BST

Bacterial Source Tracking

Texas Water Resources Institute
TR-427
June 2012

2012 Bacterial Source Tracking
State of the Science Conference

Conference Proceedings

Texas Water Resources Institute
Technical Report No. 427
June 2012

2012 Bacterial Source Tracking - State of the Science Conference

Conference Proceedings

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Foreward

The organizers of the **2012 Bacterial Source Tracking - State of the Science Conference** want to express their thanks to the organizations and individuals involved for their preparation and dedication to coordinate a successful conference. We would also like to thank our invited speakers for their support of and contributions to the conference.

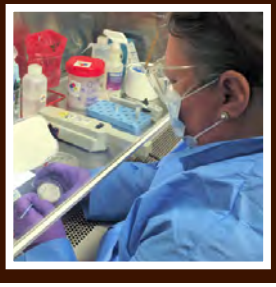
A special thank you to the conference chair, Dr. George Di Giovanni, for his countless hours and efforts to coordinate and conduct a successful conference. The science of bacterial source tracking continues to evolve and the conference provided a valuable opportunity to share developments in bacterial source tracking technology and present case studies from Texas and beyond.

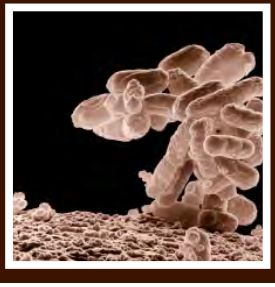
The conference was hosted by the Texas Water Resources Institute, Texas State Soil and Water Conservation Board, The University of Texas School of Public Health-El Paso Regional Campus and Texas AgriLife Research. The organizers would like to thank the Texas State Soil and Water Conservation Board for funding and support provided through a State General Revenue Nonpoint Source grant from the Board.

Visit the conference website for follow up information including presentations, videos, speaker biographies and poster abstracts: *texasbst.tamu.edu/2012-conference/*.

Table of Contents

Table of Contents continued ...





2012 Conference Planning Committee

Dr. George Di Giovanni *Conference Chair*	*University of Texas School of Public Health El Paso Regional Campus*
Dr. Elizabeth Casarez	*University of Texas School of Public Health El Paso Regional Campus*
Dr. Terry Gentry	*Texas A&M University*
Dr. Valerie Harwood	*University of South Florida*
Dr. R. Karthikeyan	*Texas A&M University*
Emily Martin	*Texas A&M University*
Dr. Joanna Mott	*James Madison University*
Dr. R. Srinivasan	*Texas A&M University*
Courtney Smith	*Texas Water Resources Institute*
Brian VanDelist	*Texas Water Resources Institute*
Dr. Kevin Wagner	*Texas Water Resources Institute*
Loren Warrick	*Texas State Soil & Water Conservation Board*
Aaron Wendt	*Texas State Soil & Water Conservation Board*

Invited Speakers

Sally Gutierrez	*US EPA National Risk Management Research Laboratory*
Dr. Chuck Hagedorn	*Virginia Tech*
Katherine McElhany	*Texas A&M University*
Dr. Mike Sadowsky	*University of Minnesota*
Dr. Orin Shanks	*US EPA National Risk Management Research Laboratory*
Dr. Don Stoeckel	*Battelle Memorial Institute*

Section 1: Introduction

Project Background

Nonpoint sources (NPS) of pollution, including agricultural activities, can greatly impact water quality. One key component in effectively implementing a NPS pollution abatement program is the identification and assessment of sources of fecal pollution. Proper evaluation of these sources is needed to target best management practices (BMPs) and develop bacterial total maximum daily loads (TMDLs) or watershed protection plans (WPPs). According to the *2010 Texas Integrated Report for Clean Water Act Sections 305(b) and 303(d): Executive Summary*, 318 water bodies do not meet applicable water quality standards for bacteria and are in need of TMDL development, standards review, and/or additional data collection.

Fecal coliform bacteria have been used extensively as an indicator of fecal pollution and the potential presence of other pathogenic microorganisms in water. It has been established that the fecal coliform bacterium *E. coli* is more closely associated with fecal pollution than other fecal coliform bacteria, which may normally reside and multiply in the environment. *E. coli* is a common inhabitant of animal and human intestines and recent studies have shown that isolates from humans and various host animals (e.g. cattle, chickens, and pigs) may differ genetically and phenotypically. Use of genetic and biochemical tests may allow the original host animal to be identified and is referred to as bacterial source tracking (BST).

The premise behind BST is that genetic and phenotypic tests can identify bacterial strains that are host-specific so that the original host animal and source of the fecal contamination can be identified. Often *E. coli* or *Enterococcus* spp. are used as the bacteria targets in source tracking, as this provides a direct link with water quality standards which are usually based on one of these two indicators.

The state of BST science, methodologies, application and confidence has evolved greatly in the past few years. A host of new information is currently available, yet not readily distributed or known to state and federal agency personnel. This lack of information transfer has spurred the need for a statewide informational workshop geared toward bringing those in attendance up to speed on recent advances in BST technologies, methodologies, applications and results.

Conference Introduction

The **2012 Bacterial Source Tracking - State of the Science Conference** was held February 28-29 at the T Bar M Resort and Conference Center in New Braunfels, Texas. Academia involved in BST analysis; state, federal, and regional agency personnel; elected officials; and other interested persons were targeted through various media outlets:

- Water Programs Listserv, Oklahoma State University
- NPSINFO Listserv, U.S. Environmental Protection Agency
- American Society for Microbiology Listserv
- Houston-Galveston Area Council Listserv
- Soil Science Society of America Listserv, Division S-3 - Soil Biology and Biochemistry
- *Conservation News*, Texas State Soil and Water Conservation Board e-newsletter
- *Conservation Matters,* Texas Water Resources Institute e-newsletter
- *News from the Texas TMDL Program,* Texas Commission on Environmental Quality e-newsletter
- *AgriLife Today,* Texas A&M University System website and newswire

Prior to the conference, the Texas State Soil and Water Conservation Board (TSSWCB) queried state and federal agencies about what their wants and needs in regards to the state of BST science. Staff from the U.S. Environmental Protection Agency (EPA), Texas Parks and Wildlife Department (TPWD), Texas Commission on Environmental Quality (TCEQ) and others were asked to identify questions and issues that should be included in conference presentations and discussion.

The conference agenda was designed around agency responses and conference objectives included:

- The Texas 303(d) List of Impaired Waters, which continues to be dominated by impairments due to indicator bacteria affecting recreational use and oyster waters use.
- The use of BST as a tool to aid stakeholders and agencies in assessing fecal pollution, developing TMDLs and WPPs, and solving water pollution issues.
- The state of BST science, methodologies, application and confidence, which has evolved greatly over the past few years. Where have we advanced the science and where do questions continue to linger?
- There has not been a concerted effort to deliver this host of new BST information currently available; therefore there is a need for this 'State of the Science' conference.

Conference speakers not only included experts from Texas, but also included speakers from the U.S. EPA Office of Research and Development, Virginia Tech, The University of Minnesota, Battelle Memorial Institute, University of South Florida, and James Madison University. See speaker biographies in Appendix C.

To provide useful information to attendees prior to the conference, organizers compiled a list of websites, presentations, documents, and publications of additional information about BST. The materials included general information on BST and detection techniques; overviews; advantages and disadvantages; applications and case studies. See the "BST Primer Materials" document that was e-mailed to registered participants in Appendix B.

Summary

Nearly 120 participants from 13 states participated in the conference to hear discussions on BST and current practices, scientific advances and improvements in application. Section 2 and 3 include conference presentations and the complete participant list can be viewed in Appendix A.

A call for posters was announced for an informal and conversational poster session. Poster abstract submissions (Section 4) were reviewed by the planning committee and seven were accepted and presented at the conference displaying a variety of BST research projects.

Presentations Summary

It was not until the 1800's that people started caring about fecal contamination, as described by Dr. Don Stoeckel (Battelle Memorial Institute). Dr. Stoeckel provided an overview of the history and the future of source tracking as well as how fecal contamination issues have been addressed over time. He also explained library-dependent and library-independent methods of source tracking.

In her presentation entitled, *The ABCs of BST*, Dr. Valerie Harwood (University of South Florida), gave her definition of microbial source tracking (MST):

The use of microbial species or types that are strongly associated with the gastrointestinal tract and

feces of specific hosts (human or animal hosts) to determine whether waste from said hosts has contaminated a water body.

Dr. Harwood discussed the challenges for developing and using library-dependent MST methods; the basis of library-independent methods; and strategies for developing MST markers including specificity, sensitivity, and limit of detection. She explained that databases with thousands of patterns are necessary to capture bacterial diversity in feces and in aquatic environments and can be very expensive to create and update.

Conference discussion shifted a bit, and Katherine McElhany (Texas A&M University), discussed food safety and how it relates to source tracking. She explained the importance of source tracking and food safety and how the two fields are intertwined. McElhany also explained that molecular methods for food testing have advanced significantly and in many cases, beyond environmental methods.

To discuss the relevance of source tracking methods and federal regulations, Sally Gutierrez from EPA's National Risk Management Research Laboratory provided an overview in the context of EPA's policies, programs and regulations, and opportunities for improvement.

Shifting from federal perspectives to state perspectives, Aaron Wendt (TSSWCB), gave a broad-scale perspective to frame remaining conference presentations in regards to general comments and observations about BST in Texas. He briefly explained Texas water quality and the need to asses bacteria TMDLs in the state:

- Texas 303(d) List of Impaired Waters dominated by elevated bacteria related to recreational use and oyster waters use
- Several watershed planning processes (TMDLs or WPPs) on-going with discontented stakeholder groups
- Variety of BST methods/approaches by a number of laboratories had been used in different watershed planning processes

In 2006, TSSWCB and TCEQ collaborated to establish a seven-member task force to:

- Examine approaches other states use to develop bacteria TMDLs
- Recommend cost-effective and time-efficient methods and approaches for developing TMDLs and Implementation Plans
- Evaluate the variety of models and BST methods available for developing TMDLs and I-Plans, and recommending under what conditions certain methods are more appropriate
- Develop a roadmap for further scientific research needed to reduce uncertainty about how bacteria behave under different water conditions in Texas

Task force research and materials can be found online (http://twri.tamu.edu/bacteriatmdl/).

Methodologies Summary

Dr. Stoeckel led in to methodology presentations by providing valuable comments on study design: know and understand the source identifier; challenge the assumptions; ensure quality of data; and validate interpretations. He also stressed the importance of defining a source tracking objective. To meet the objective: research must be quantitative; include an internal control of extraction; and researchers must understand markers. More about his study, "Evaluation of two spike-and-recovery controls for assessment of extraction efficiency in microbial source tracking studies," can be viewed in Section 2.

The conferences' common theme that fecal bacteria represent the most often exceeded water quality standard, was stressed again by Dr. Mike Sadowsky (University of Minnesota). His presentation covered the tools available to look at microorganisms in their entirety in their environment.

Dr. Sadowsky explained that organisms in the environment differ by relatively small amounts of DNA, therefore source tracking tools and methods are being used to evaluate and determine diversity and sources of fecal bacteria. Example methods he provided include: genotypic molecular methods (ribotyping, rep-PCR, species-specific hybridization markers, etc.) and phenotypic molecular methods (phage typing, antibiotic resistance, etc.).

Dr. Sadowsky explained that these types of tools provide the background to ask important questions like what are the sources and sinks of fecal bacteria in the environment, and help to understand their ecology in watersheds. His research laboratory's case study: "Temperate soils as an alternate source of *E. coli* waterways," can be viewed further in Section 2.

Shifting from organisms in the environmental to poultry litter, Dr. Valerie (Jody) Harwood (University of South Florida), explained that poultry production has increased in the United States over the last decade and Texas was ranked sixth for broiler production (3.6 billion pounds). Poultry litter samples processed by Dr. Harwood's laboratory contained: *E. coli,* Enterococci, *Campylobacter jejuni, Salmonella enterica,* and pathogenic *E. coli* strains. A small farm with four poultry houses produces 340 tons of poultry litter annually.

Dr. Harwood explained that the bacteria in poultry litter applied to land contains phosphate, nitrogen, and heavy metals spread along with bacteria, which can all affect water quality. Her dilemma–and case study–"How to specifically detect poultry litter contamination" can be viewed further in Section 2.

Case Studies Summary

Moving away from the methodologies and the latest information on the current status of source tracking, Dr. Chuck Hagedorn (Virginia Tech), discussed what happens after source tracking is used in the field. Dr. Hagedorn gave a summary of current case studies across the nation and lessons learned.

He expanded on three (of many) case studies included in the book: *Microbial Source Tracking: Methods, Applications, and Case Studies*. More on each case study can be found in his presentation in Section 2.

- **Ch. 20. Beaches and Coastal Environments**: two case studies at marine beaches (California and Florida); both beaches were impacted by non-point sources; a variety of biological, chemical and physical methods have been used for source identification. Sources of bacteria remain unknown.
- **Ch. 19. Case Studies of Urban and Suburban Watersheds**: Described the Weight-of-Evidence Approach (WOE) that allows source tracking methods to be highly focused, but used only on an as-needed basis. There were six sub-basins in Hillsborough River Watershed (Florida) used for examples for WOE approach; ten watersheds (Florida) used as case studies. Some sources are obvious, but many are not—and it takes a lot of field time and sampling (labor intensive) to trace sources to specific points of origin.
- **Ch. 18. Agricultural and Rural Watersheds:** two Case Studies—an alpine karst groundwater-spring system in Austria and a surface water system in Texas (Lake Granbury and Buck Creek). Lake Granbury and Buck Creek were both found to be impacted primarily by wildlife and livestock.

The Texas *E. coli* BST Library, a "living archive" of more than 25,000 frozen *E. coli* isolates from water and known source samples, overview was given by Dr. Elizabeth Casarez (University of Texas - Houston School of Public Health).

Dr. Casarez explained that this database of more than 10,000 genetic fingerprints has been in development over the past eight years to serve as a tool to aid TMDL and WPP development for BMPs.

She explained that *E. coli* may or may not be the best target for determining fecal pollution sources; however: levels of *E. coli* have regulatory significance; established monitoring and standard methods; there is still an uncertain relationship between library-independent targets and *E. coli* sources.

Dr. Terry Gentry (Texas A&M University) provided an overview of source tracking projects in Texas focused on library-independent work including characterization of watersheds; evaluation and development of a feral hog marker; and evaluation of grazing management practices. An ongoing project in Texas that Dr. Gentry expanded on was source tracking or Little Brazos River tributaries. He described library-independent and library-dependent approaches and analysis for this study area. For the library-independent analysis, the hog marker was detected most frequently.

Texas has a population of nearly two million feral hogs with approximately $52 million in damages each year and Joy Archuleta-Truesdale, a student at the University of Texas - Houston School of Public Health, El Paso Regional Campus, expanded on the development of a feral hog marker.

Dr. Joanna Mott (James Madison University) presented on her work: library-independent source tracking for South Texas coastal waters (marine water and fresh water). Her presentation focused on three human-specific markers: human associated *bacteroides* spp.; *Methanobrevibacter smithii;* and human polyomaviruses. Dr. Mott's studies aimed to answer the question: can human-specific molecular markers be used as a source tracking method for coastal waters?

Further information on the Corpus Christi Bay study (Cole Park and Ropes Park beaches) and the Oso Creek (south Texas) study, including lessons learned and future directions, can be viewed in Section 2. All of the human-specific markers tested could be detected in fresh and marine waters of the Coastal Bend area of Texas.

BST & Modeling Summary

To discuss source tracking and modeling Dr. R. Srinivasan (Texas A&M University) provided a review of various water quality models and their current capabilities and limitations. Dr. Srinivasan classified models into three categories: Spatially explicit statistical models; mass balance models; and mechanistic/hydrologic/water quality models. His presentation (Section 2) provided a bacteria modeling matrix discussing a few models in each category and their functions. This matrix can also be viewed in further detail in the *Bacteria Total Maximum Daily Load Task Force Final Report* (http://twri.tamu.edu/reports/2009/tr341.pdf).

Dr. Srinivasan highlighted important considerations for bacteria modeling:

- The model used will only be as good as the data used to develop it
- Models should be used as part of the TMDL framework (not as an only tool for decision-making)
- Models should continually evolve as the knowledge-base develops
- Bacteria regrowth and decay are not well represented
- Detailed models allow for spatial and temporal analysis
- Sensitivity and uncertainty in data, parameters and models

To discuss and provide more information on a bacteria load assessment tool, Dr. R. Karthikeyan (Texas A&M University) presented on the Spatially Explicit Load Enrichment Calculation Tool (SELECT). SELECT characterizes potential *E. coli* sources and estimates daily potential *E. coli* loads. Dr. Karthikeyan discussed input data for this tool and provided example watersheds in which SELECT was used.

Conclusion

Dr. George Di Giovanni, professor at the University of Texas School of Public Health and conference chair said that the science of bacterial source tracking continues to evolve and the conference provided a valuable opportunity to share developments in BST technology and present case studies from Texas and beyond.

Evaluations were collected from each participant and of the evaluations received, 68 percent of participants were 'very satisfied' with the conference and 61 percent were 'very satisfied' with the conference materials provided. Some participants stated that the conference provided a good balance of theory and application.

More case studies and case study follow up; research findings; and regulatory perspectives were just a few of the presentation topics that participants would like to see at a future conference. In addition, some participants would like to see this conference repeated depending on the changes in science or regulations. The conference speakers were rated very highly and repeatedly praised, along with the case study presentations.

Conference organizers would like to again thank TSSWCB for funding and support provided through a State General Revenue Nonpoint Source grant from the Board.

Presentations and poster abstracts can be viewed in the following sections. Presentation videos can be viewed on the conference website along with a conference participant list (http://texasbst.tamu.edu/2012-conference/).

Section 2: Presentations

Tuesday, February 28

Conference Objectives

Aaron Wendt
Texas State Soil and Water Conservation Board

Bacterial Source Tracking
State of the Science Conference
February 28-29, 2012
New Braunfels, TX

Brief BST Timeline

- Early 2000s – TCEQ, TSSWCB, and partners begin building a Texas statewide known source library and utilizing BST in watershed planning efforts
- 2006-2007 – Texas Task Force on Bacteria TMDLs
- July 2008 – Status of BST in Texas Meeting b/w TSSWCB, TCEQ, and partners
- Late 2000s – use of BST to address water quality issues continues to evolve in Texas

2012 Conference

- TSSWCB provided a State General Revenue Nonpoint Source Grant to TWRI to plan this BST State of the Science Conference
- Planning Committee queried state and federal agencies on what they wanted/needed to know about BST in order to continue advancing its use in water quality planning efforts

Objectives

- Texas 303(d) List of Impaired Waters continues to be dominated by impairments due to indicator bacteria affecting recreational use and oyster waters use
- BST is a tool to aid stakeholders and agencies in assessing fecal pollution, developing TMDLs and WPPs, and solving water pollution issues
- state of BST science, methodologies, application and confidence has evolved greatly over the past few years
- host of new information is currently available, yet not readily distributed or known to state and federal agencies in Texas
- conference will discuss BST and its application regarding current practices, scientific advances and improvements in application
- targeted audience is state, federal, and regional agency personnel; elected officials; academia; and others interested in the applicability of BST

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Past, Present, and Future of Source Tracking

Don Stoeckel, PhD

stoeckeld@battelle.org

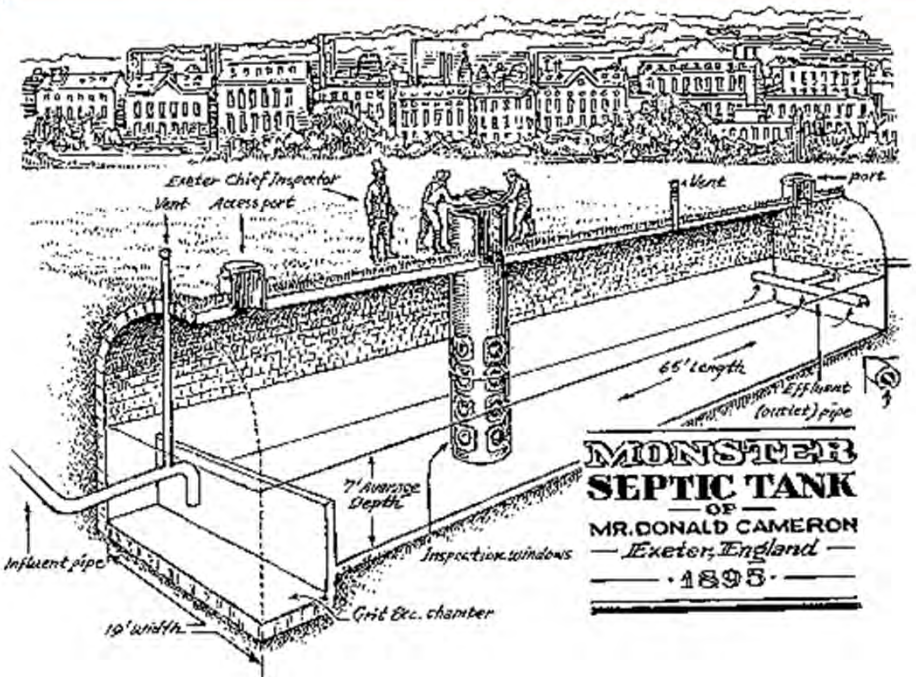
Battelle Memorial Institute

Columbus, Ohio

Overview

- Describe the problem of fecal contamination
- Solutions, almost solutions, and the state of the science
- Where are we and where are we going

FECAL CONTAMINATION



From http://www.cet.nau.edu/Projects/WDP/resources/History/History.htm

Control Fecal-oral Pathogens

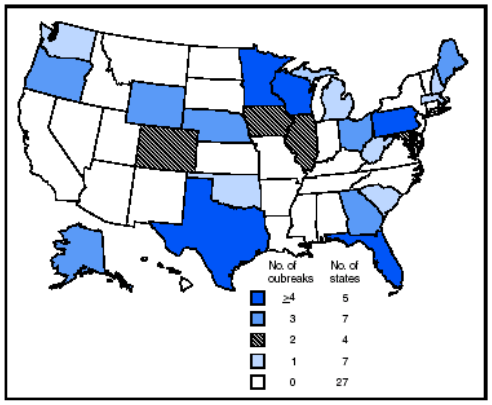
- Typically, the primary habitat is the gastrointestinal tract and the secondary habitat is the environment.
- Secondary habitat for human fecal-oral pathogens frequently includes water.
- The pathogen must survive the secondary habitat to reinfect its primary host.

Public Health Statistics

- Centers for Disease Control and Prevention
  - Morbidity and Mortality Weekly Report
  - Pathogen-specific fact sheets
- Food and Drug Administration
  - Bad Bug Book
- U.S. Geological Survey
  - National Water Information System, serving water-quality statistics

Waterborne Illness

FIGURE 1. Number of waterborne-disease outbreaks (n = 65) associated with recreational water, by state — United States, 2001–2002*



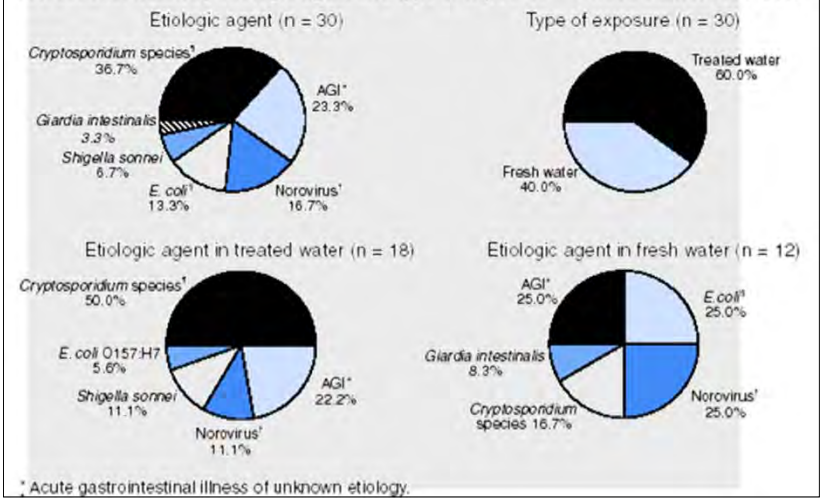
*Numbers are dependent on reporting and surveillance activities in individual states and do not necessarily indicate that more outbreaks occur in a given state.

www.cdc.gov/mmwr/preview/mmwrhtml/ss5308a1

7

Causative agents

FIGURE 3. Waterborne-disease outbreaks of gastroenteritis associated with recreational water, by etiologic agent and type of exposure — United States, 2001–2002



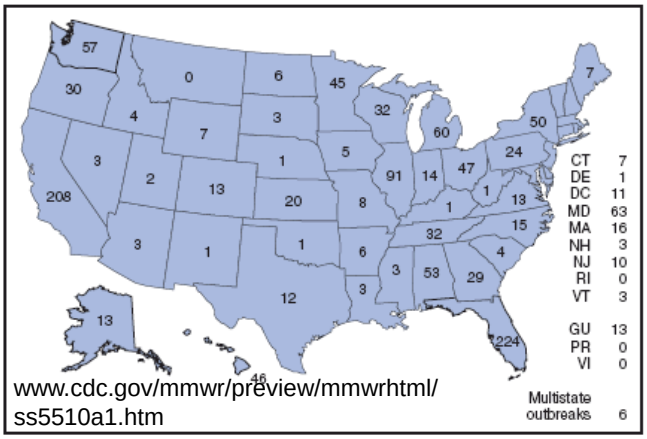
* Acute gastrointestinal illness of unknown etiology.

www.cdc.gov/mmwr/preview/mmwrhtml/ss5308a1

8

Foodborne Illness

FIGURE 6. Number of reported foodborne-disease outbreaks, by state — United States,* 2002



www.cdc.gov/mmwr/preview/mmwrhtml/ss5510a1.htm

*Includes Guam, Puerto Rico, and the U.S. Virgin Islands.

Causative agents

TABLE 1. Number of reported foodborne-disease outbreaks, cases, and deaths, by etiology — United States, 1998-2002

Etiology	Outbreaks No.	Outbreaks (%)	Cases No.	Cases (%)	Deaths No.	Deaths (%)
Confirmed etiology	2,167	(32.6)	68,981	(53.7)	76	(86.4)
Unknown etiology	4,480	(67.4)	59,389	(46.2)	12	(13.6)
Total 1998–2002	6,647	(100.0)	128,370	(100.0)	88	(100.0)

Agent	Outbreaks	Cases	Deaths
Bacterial	1,184	37,887	70
Chemical	221	1,140	1
Parasitic	23	630	0
Viral	709	28,274	5

MMWR
Surveillance for Foodborne-Disease Outbreaks --- United States, 1998--2002
http://www.cdc.gov/mmwr/preview/mmwrhtml/ss5510a1.htm

Etiology

Bacterial
- *Bacillus cereus*
- *Brucella*
- *Campylobacter*
- *Clostridium botulinum*
- *Clostridium perfringens*
- *Escherichia coli*
- *Listeria monocytogenes*
- *Salmonella*
- *Shigella*
- *Staphylococcus aureus*
- *Streptococcus*
- *Vibrio cholerae*
- *Vibrio parahemolyticus*
- *Vibrio, other*
- *Yersinia enterocolitica*

Parasitic
- *Anisakis*
- *Cryptosporidium parvum*
- *Cyclospora cayetanensis*
- *Giardia intestinalis*
- *Trichinella spiralis*

Viral
- Astrovirus
- Hepatitis A
- Norovirus
- Rotavirus

Water as a Vehicle for Foodborne Illness

TABLE 13. Number of reported foodborne-disease outbreaks, cases, and deaths, by vehicle of transmission — United States, 2002

Vehicle of transmission	Outbreaks		Cases		Deaths	
	No.	(%)	No.	(%)	No.	(%)
Beef	44	(3.3)	831	(3.3)	3	(21.4)
Dairy	16	(1.2)	704	(2.8)	0	(0.0)
Eggs	14	(1.1)	317	(1.3)	0	(0.0)
Game	3	(0.2)	33	(0.1)	0	(0.0)
Pork	26	(2.0)	360	(1.4)	0	(0.0)
Poultry	75	(5.6)	1325	(5.3)	8	(57.1)
Vegetables	44	(3.3)	1596	(6.4)	0	(0.0)
Fruits and nuts	9	(0.7)	169	(0.7)	0	(0.0)
Grains	14	(1.1)	177	(0.7)	0	(0.0)
Oils and sugars	1	(0.1)	4	(0.0)	0	(0.0)
Finfish	66	(5.0)	280	(1.1)	0	(0.0)
Shellfish	27	(2.0)	200	(0.8)	0	(0.0)
Unclassifiable vehicle	52	(3.9)	1049	(4.2)	0	(0.0)
Complex vehicle	436	(32.8)	9369	(37.5)	1	(7.1)
Known vehicle	827	(62.2)	16414	(65.7)	12	(85.7)
Unknown vehicle	503	(37.8)	8552	(34.3)	2	(14.3)
Total 2002	1330	(100.0)	24966	(100.0)	14	(100.0)

http://www.cdc.gov/mmwr/preview/mmwrhtml/ss5510a1.htm

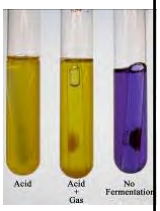
11

REGULATORY RESPONSE

12

Brief review of water quality monitoring

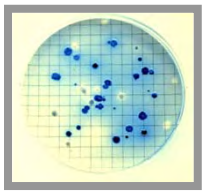
- 1885, T. von Escherich describes *Bacillus coli* in the feces of infants
- 1893, the Wurtz method of enumerating *B. coli*-like colonies on litmus lactose agar is used for sanitary bacteriology
- 1893, Durham introduces a method to detect gas production (the Durham tube)
- 1901, Horrocks introduces the term "coliform" to describe *B. coli*-like bacteria



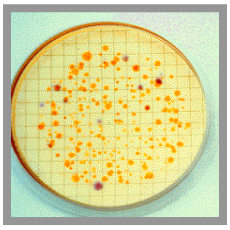
13

Media for coliforms

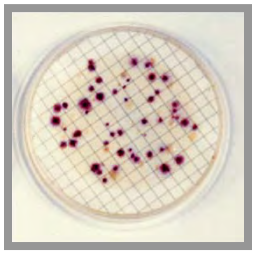
Fecal coliforms on mFC agar



Escherichia coli on mTEC agar



Escherichia coli on modified mTEC agar



Total coliforms (and *E. coli*) on Colilert in Quantitray format

14

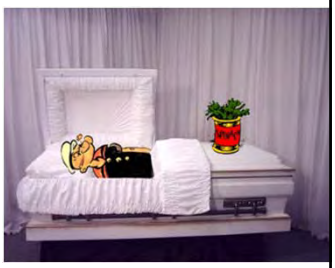
Water Regulations

- Drinking water
  - USEPA total coliform rule (total coliforms and *E. coli*)
  - USEPA ground water rule (total coliforms, *E. coli*, enterococci, and coliphages)
- Recreational water
  - USEPA BEACH act (enterococci and *E. coli*)
- Shellfish-harvesting water
  - FDA CFSAN National Shellfish Sanitation Program (total coliforms or fecal coliforms)

15

Regulations are Reactive Example -- Irrigation water

- Spinach outbreak in 2006.
- Adoption of recreational water quality standards in California Leafy Greens document.
  - What are the costs?
  - What are the health benefits?
  - What are the remedies?



13gb.com/media.php?media_id=2023

16

Fecal-indicator microorganisms

U.S. historic, regulatory:
- Total coliform
- Fecal coliform
- Fecal streptococci

Alternate, nonregulatory:
- *Clostridium perfringens*
- Staphylococci
- *Aeromonas hydrophila*
- *Bacteroides spp.*

Direct, pathogens:
- *Cryptosporidium*
- *Giardia*
- Enteric viruses

U.S. current, regulatory:
- Total coliforms (drinking water, ground water, shellfish-harvesting water)
- Fecal coliforms (shellfish-harvesting water, recreational waters
- *Escherichia coli* (fresh water)
- Enterococci (fresh water or marine water)
- Coliphage (ground water)

17

Relations to public health

Fecal-indicator bacteria indicate the presence of fecal-oral pathogens

- BUT—they are unrelated to the presence of other pathogens (swimmers ear, skin rashes, etc.) and other issues such as toxic cyanobacteria
- BUT– the relations are not consistent
  - Pathogens are detected in recreational waters with fecal-indicator bacteria concentrations lower than regulatory limits
  - Pathogens are absent in recreational waters with fecal-indicator bacteria concentrations higher than regulatory limits

18

MICROBIAL SOURCE TRACKING

Fundamental concept of source tracking

- Some intestinal bacteria of animal groups are expected differ because of:
  - Basic habitat
    - Body temperature, food supply, digestive system
  - Natural selection
    - Direct competition, pathogenicity factors, prior exposure to agents like antibiotics

The process

- Choose source-specific targets that are in the feces of local source groups
- Characterize “reference material” (also known as manure and sewage) from local sources
- Test water for fecal contamination
- Associate contamination with sources

Early methods

- Fecal coliform-Fecal streptococci ratio
  - FC/FS about 4 in human fecal material
  - FC/FS 1 or lower in other warm-blooded animals
  - Only valid for recent contamination (differential die-off)
- Cultivation of host-associated microbes
  - Sorbitol-fermenting bifidobacteria
  - *Rhodococcoccus coprophilus*
- Chemical methods
  - Detection of human-origin biochemicals and metabolites

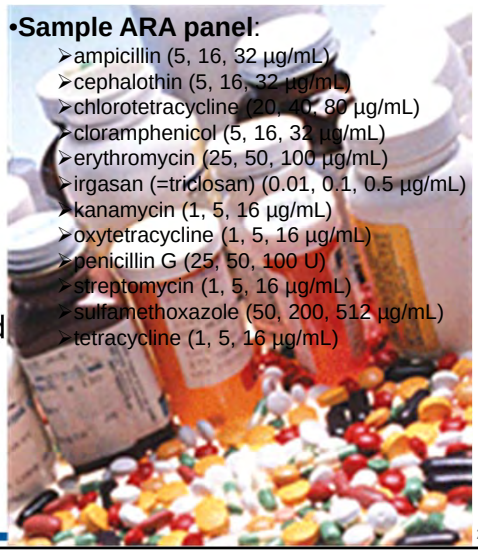
Pedigree of currently-applied methods

- Library-dependent
  - Cultivate target microorganism from known-source feces
  - Generate a descriptive pattern of data (profile) of the isolates
    - Library of profiles from human-origin isolates
    - Alternate libraries of profiles from other fecal sources
  - Cultivate target microorganisms from environment
    - Recreational water, shellfish-harvesting water, drinking water (less common)
  - Generate descriptive profiles of environmental isolates
  - Query libraries for exact or statistical matches

23

Library markers used for MST

- Phenotypic patterns
  - Antibiotic resistance
  - Carbon Utilization
  - Fatty acid profiles
- Genotypic patterns
  - REP-PCR
  - Macrorestriction followed by PFGE
  - Ribotyping
  - MLST and other sequencing patterns

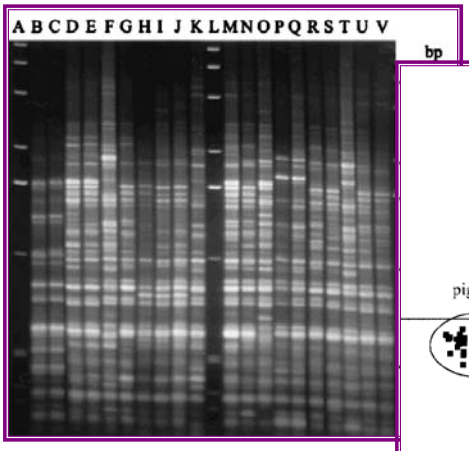


- **Sample ARA panel**:
  - ampicillin (5, 16, 32 µg/mL)
  - cephalothin (5, 16, 32 µg/mL)
  - chlorotetracycline (20, 40, 80 µg/mL)
  - cloramphenicol (5, 16, 32 µg/mL)
  - erythromycin (25, 50, 100 µg/mL)
  - irgasan (=triclosan) (0.01, 0.1, 0.5 µg/mL)
  - kanamycin (1, 5, 16 µg/mL)
  - oxytetracycline (1, 5, 16 µg/mL)
  - penicillin G (25, 50, 100 U)
  - streptomycin (1, 5, 16 µg/mL)
  - sulfamethoxazole (50, 200, 512 µg/mL)
  - tetracycline (1, 5, 16 µg/mL)

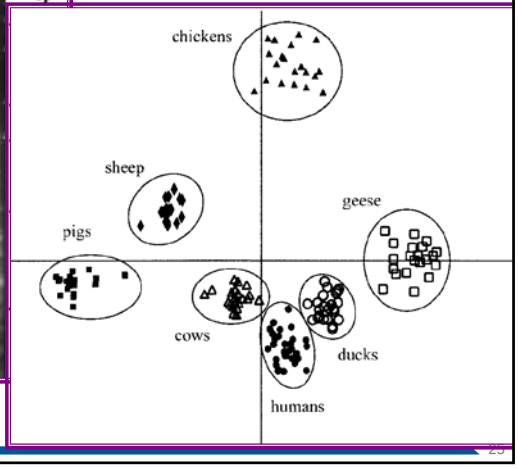
24

Statistical analysis methods take this ...

from Dombek and others, 2000 Appl. Environ. Microbiol.



to this

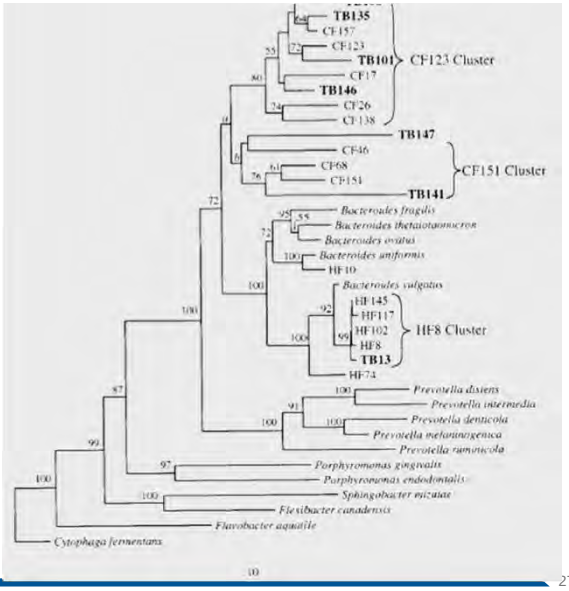


Pedigree of currently-applied methods

- Library-independent
  - Extract DNA from known-source feces
  - Generate a descriptive pattern of data (profile) of the "metagenome" and search for host-associated markers
    - Species level (phylogeny, DNA sequences in the 16S rDNA
    - Functional level (genes enhancing the host-microbe interaction)
  - Extract DNA from environment
    - Recreational water, shellfish-harvesting water, drinking water (less common)
  - Test environment for presence of the host-associated marker

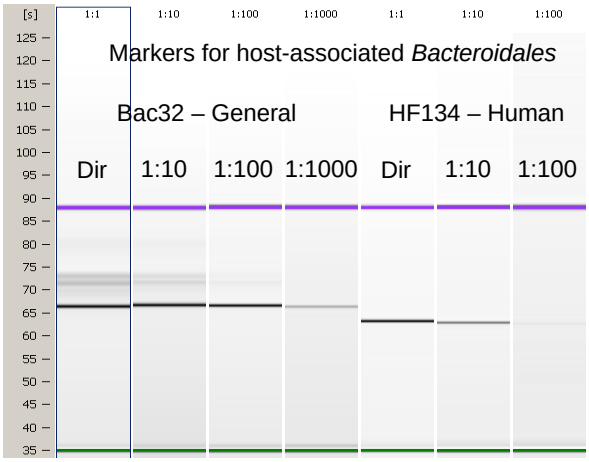
DNA markers used for MST

- Species-level genetic markers



From Bernhard and Field, 2000. "A PCR Assay To Discriminate Human and Ruminant Feces on the Basis of Host Differences in *Bacteroides-Prevotella* Genes Encoding 16S rRNA." Appl. Environ. Microbiol. 66:4571

Library-independent markers



EVOLUTION OF MST

Performance of common MST tools

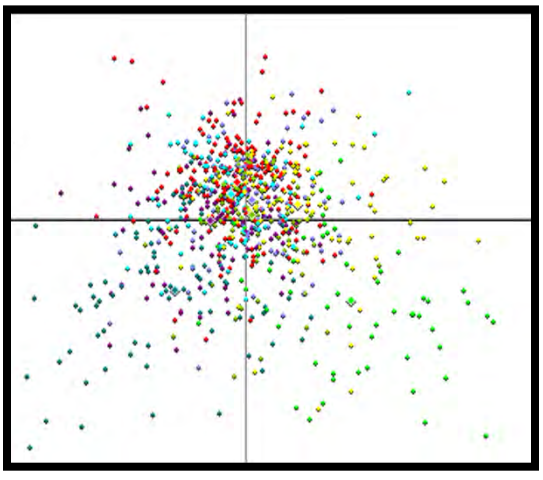
- Measurements of performance
  - Accuracy
    - the ability to correctly identify the source of a fecal-indicator isolate or a contaminated sample.
    - balance between Sensitivity and Specificity
  - Sensitivity
    - the rate of true-positive results
    - the proportion of samples that ARE from a source that CAN be (correctly) classified to that source
  - Specificity
    - one minus the rate of false-positive results
    - the proportion of samples NOT from a source that is (correctly) NOT classified to that source

Challenges with current use of libraries

- Specificity and Sensitivity (in the statistical sense)
  - Many profiles are promiscuous, found in more than one fecal source type
  - Even with statistical methods, clear separation is not always possible

Separation of profiles

- REP-PCR profiles
- 8 host species,
- @100 isolates per



Stoeckel, et al., 2004. ES&T

Dk green=horse Lt green=human

Head-to-head evaluations of some early MST tools

- Southern California Coastal Water Research Project J. Water and Health issue in 2003
- USGS methods-comparison studies ES&T in 2004
- European methods-comparison studies (TOFPSW) – Multiple individual and group publications 2004-2007

33

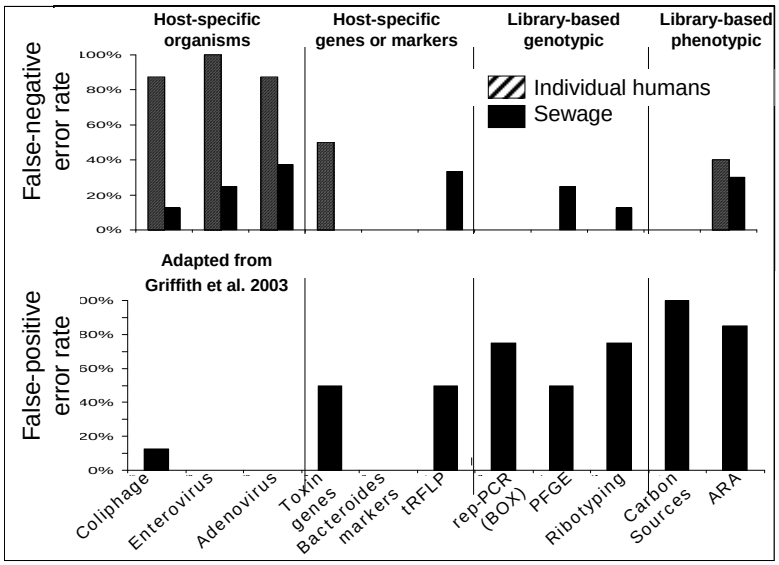
The SCCWRP study

- Southern California Coastal Waters Research Project, with additional funding by other California agencies and USEPA
- Library-dependent methods tended to have lower specificity
  - all sources found in all samples
- Library-independent methods tended to have lower sensitivity but excellent specificity
  - Major source of contamination not detected in some cases

J. Water Health Vol 1, issue 2; variously cited in Griffith, 2003

34

SCCWRP study results for human-source feces



Evolution of Library-Dependent Methods

- Many researchers avoid use as stand-alone methods
  - Tiered approaches use library methods as one level of analysis
  - Toolbox approaches use library methods as one line of evidence
- Migration to standardized protocols
  - Regional or national level, larger libraries
  - Data migration and sharing among researchers

Challenges with current use of markers

- Specificity and Sensitivity (in the statistical sense)
  - False positive, false negative
- Sensitivity (in the analytical sense)
  - Limit of detection
- Interpretation paradigm
  - Management of cultivated fecal-indicator bacteria concentrations using DNA-based markers from other bacteria

37

MST markers do not have absolute host specificity

- Statistical sensitivity and specificity of markers
- Presence-absence data (Stoeckel and Harwood

TABLE 2. Performance statistics for tests in which MST methods were tested with reference samples to determine the ability or failure to detect the sole source of fecal contamination

Test[a]	Target	Host category	Sample type	Sensitivity (*n*)[b]	Specificity (*n*)[c]	Reference(s)
Isolate-by-isolate classification						
ARA	*E. coli*	Human	Blind samples	1.00 (7)	0.80 (5)	41
MAR, CUP, ribotyping, PFGE, rep-PCR also included in table						
Marker detection						
Bacteroides thetaiotaomicron PCR	B.thetaF/B.thetaR	Human	Individual feces	0.92 (25)	0.98 (241)	11
Bacteroides thetaiotaomicron PCR	B.thetaF/B.thetaR	Human	Wastewater	1.00 (20)	NR (NR)[d]	11
Bacteroides thetaiotaomicron PCR	Primers, two internal probes described	Human	Individual feces	0.78 (9)	0.76 (71)	57
Bacteroidales PCR (two trials)	HF183F, HF134F/Bac708R	Human	Blind samples	0.70, 1.00 (10, 14)	1.00, 1.00 (6, 7)	26
Bacteroidales PCR	HF183F/Bac708R	Human	Individual feces	0.20–0.85 (7–25)	0.85–1.00 (46–73)	6
Bacteroidales PCR	HF183F/Bac708R	Human	Wastewater	1.00 (41)	1.00 (75)	6, 9, 11, 91
Bacteroidales qPCR	HF183F/reverse primer described	Human	Individual feces	0.86 (7)	1.00 (19)	91
Bacteroidales qPCR	HF183F/reverse primer described	Human	Wastewater	1.00 (4)	NR (NR)	91
Bacteroidales PCR (two trials)	CF128F, CF193F/Bac708R	Ruminants and	Blind samples	1.00 (7, 9)	0.89, 0.92 (9, 12)	26
Various other markers also included in table						

38

End-point PCR detection limits are lower than standards for fecal-indicator bacteria

- McLellan (Bower et al. 2005 AEM 71:8305)
  - detection of HF183 in diluted sewage by PCR
  - 0.2 to 82 CFU/100 mL E. coli (n=14)
- Internal research (unpublished data)
  - Detection of qHF183 in sewage by qPCR
  - Detection limit 4 copies/5 uL reaction
  - 78 to 4,800 CFU/100 mL *E. coli* (geometric mean 480, n=30)

39

Evolution of Library-Independent Methods

- Toolbox approaches using complementary lines of evidence
- Statistical analysis of data and relation to fecal indicators
- Quantification
- Development and validation of additional markers

40

FUTURE DIRECTIONS

Source Tracking is a Valuable Tool

- Build upon experience
  - Enhance the demonstrated capabilities of BST tools
  - Avoid pitfalls related to sensitivity, specificity
- Use an appropriate tool to meet the indicated need
  - Different stakeholders have different needs
    - Recreational water may continue to be linked to fecal indicators
    - Irrigation water might utilize other measures
    - Ecological health research, control of antibiotics, endocrine disruptors, and other emerging contaminants, might use markers independent from cultivated fecal indicators
  - Interpretations can be enhanced by application of appropriate statistical and modeling methods

Thank you

Battelle
The Business of Innovation

The ABCs of Microbial Source Tracking

Valerie J. Harwood

University of South Florida

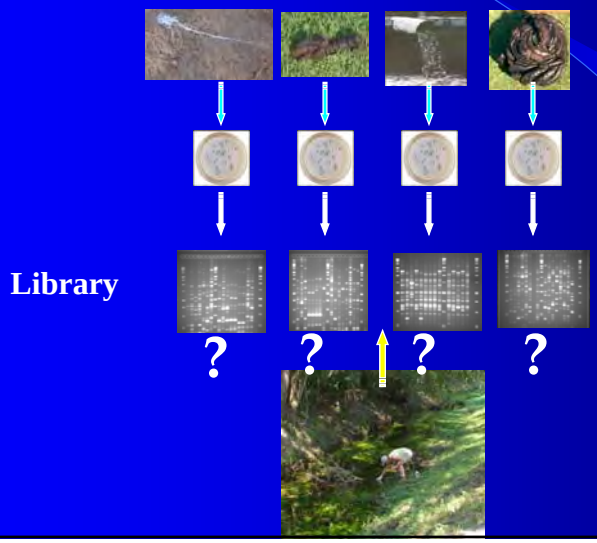
USF
UNIVERSITY OF SOUTH FLORIDA

2012 Bacterial Source Tracking State of the Science Conference, New Braunfels, TX. Feb 28-29

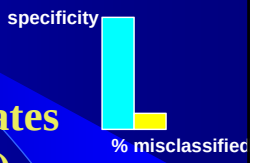
Harwood's Definition of MST

- The use of microbial species or types that are strongly associated with the gastrointestinal tract and feces of specific hosts (human or animal hosts) to determine whether waste from said hosts has contaminated a water body.

Library-Dependent MST Approach



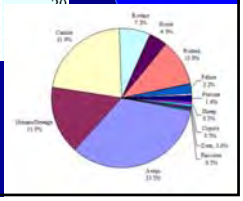
Before the Library Is Deployed… Its Performance Must Be Assessed



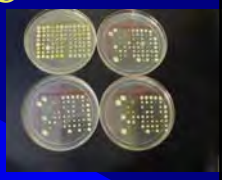
- Specificity – denotes frequency of misclassification of negative control isolates (high specificity = low false-positive rate)
- Sensitivity – denotes frequency of correct classification of positive control isolates (high sensitivity = low false-negative rate)

Antibiotic Resistance Analysis Data (Library)

112197	dairy cow	H6	AMP	AMX	CEP	CTC	ERY	OTC	STR	TET	VAN
30598	birds	A1	15	5	0	0	15	0	0	0	30
30598	birds	A2	15	5	0	0	50	0	0	0	30
112597	dairy cow	A1	0	5	15	20	15	0	0	0	30
112597	dairy cow	A2	0	5	20	40	30	0	0	0	30
112597	dairy cow	A3	0	5	15	40	30	80	0	50	30
112597	dairy cow	A4	0	5	15	80	15	80	80	50	30
112597	dairy cow	A5	10	5	25	40	50	80	0	50	30
112597	dairy cow	A6	0	0	15	20	15	0	0	0	30
112597	dairy cow	B1	0	5	15	40	15	0	0	0	30
112597	dairy cow	B2	0	5	20	40	50	0	0	0	30
112597	dairy cow	B3	10	5	25	40	50	0	0	0	30
112597	dairy cow	B4	10	10	20	40	50	0	20	0	
112597	dairy cow	B5	0	5	15	40	30	0	0	0	
112597	dairy cow	B6	0	5	15	40	30	0	0	0	



Library-Dependent MST
Antibiotic Resistance Analysis



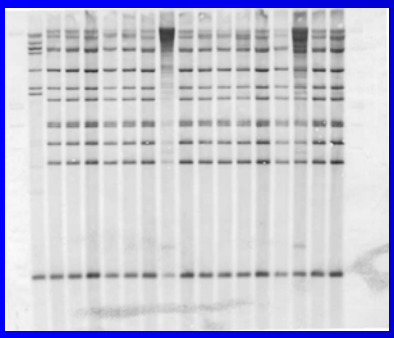
Harwood et al. 2000. Classification of the antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical Florida waters. Appl. Environ Microbiol. 66: 3698.

TABLE 4. Identification of isolate source from samples collected at failing septic system

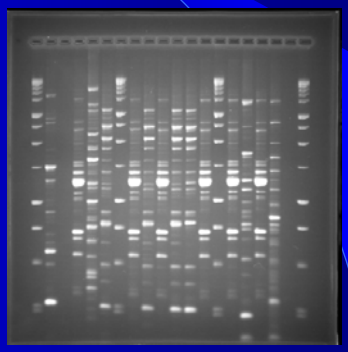
Indicator	Source	No. (%) of database isolates assigned to each source category							
		Human	Bird	Chicken	Cow	Dog	Pig	Raccoon	Total
Fecal streptococcus	Curb	10 (66.7)	0 (0)	4 (26.6)	0 (0)	0 (0)	0 (0)	1 (6.7)	15 (100)
	Ditch	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)
	Pasture	26 (76.5)	1 (2.9)	0 (0)	0 (0)	5 (14.7)	0 (0)	2 (5.9)	34 (100)
	Total	38 (74.5)	1 (2.0)	4 (7.8)	0 (0)	5 (9.8)	0 (0)	3 (5.9)	51 (100)
Fecal coliform	Curb	40 (88.9)	3 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	2 (4.4)	45 (100)
	Ditch	36 (87.8)	4 (9.8)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.4)	41 (100)
	Pasture	5 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (100)
	Total	81 (89.0)	7 (7.7)	0 (0)	0 (0)	0 (0)	0 (0)	3 (3.3)	91 (100)

Library-Dependent MST
From Phenotype (ARA) to Genotype

Ribotyping

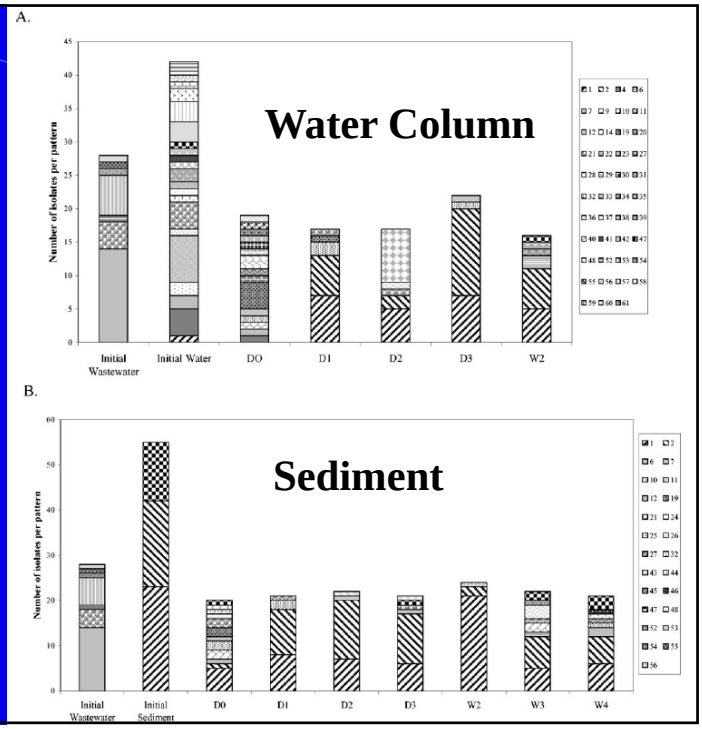


BOX-PCR



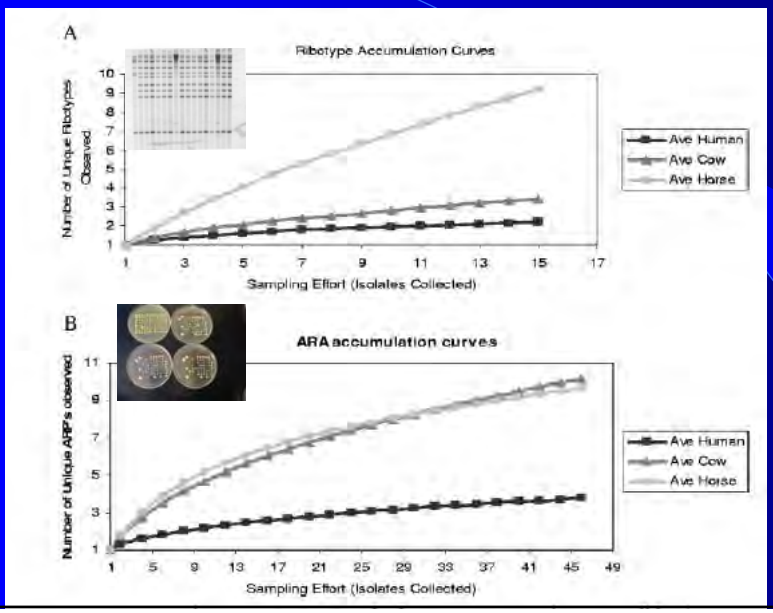
Not All *E. coli* Are Created Equal (Differential Survival)

Hood et al., 2005. Appl. Environ. Microbiol. 71: 3041-3048.



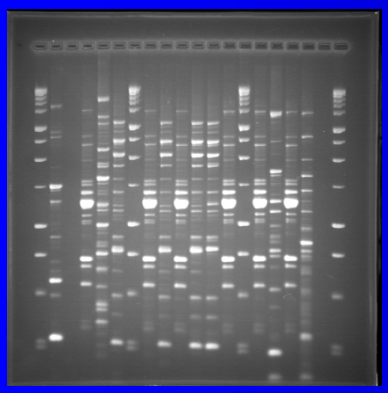
E. coli Diversity in Feces

Anderson et al 2006. Appl. Environ Microbiol. 72: 6914-6922.



E. coli and *Enterococcus* Diversity in Stormwater

Brownell, et al 2007. Water Research. 41:3747-3757



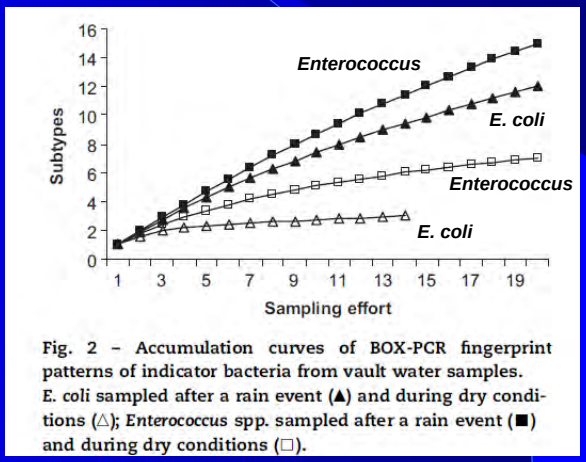
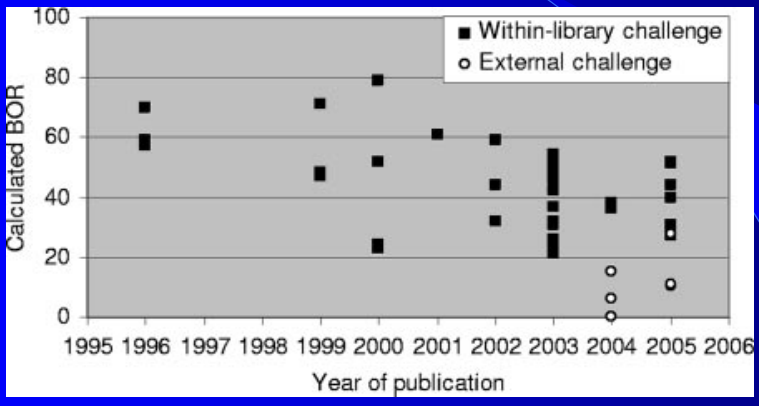


Fig. 2 – Accumulation curves of BOX-PCR fingerprint patterns of indicator bacteria from vault water samples. *E. coli* sampled after a rain event (▲) and during dry conditions (△); *Enterococcus* spp. sampled after a rain event (■) and during dry conditions (□).

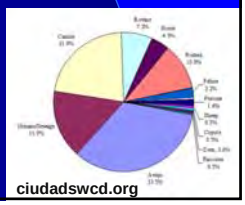
Classification Accuracy (Benefit Over Random)



Stoeckel and Harwood, 2007. Performance, Design, and Analysis in Microbial Source Tracking Studies. Appl. Env. Micro 73:2405

Challenges for Developing and Using Library-Dependent MST Methods

- Data bases with many thousands of patterns are necessary to capture bacterial diversity in feces and in aquatic environments.
- These data bases are expensive to create.
- They must be updated (expensive)
- The larger the database, the more we tend to see non-host-specific (promiscuous) patterns….
- Making the data very hard to interpret.



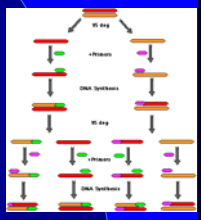
What Is the Basis of Library-Independent MST Methods?

- Some microorganisms are confined to the gastrointestinal tract of a particular host group….
- If we can find a "signature" to identify these source-specific microbes, we can use that signature to trace pollution to its source.
- Frequently, the "signature" is a DNA sequence (part of a gene).

Strategy for Developing MST Markers

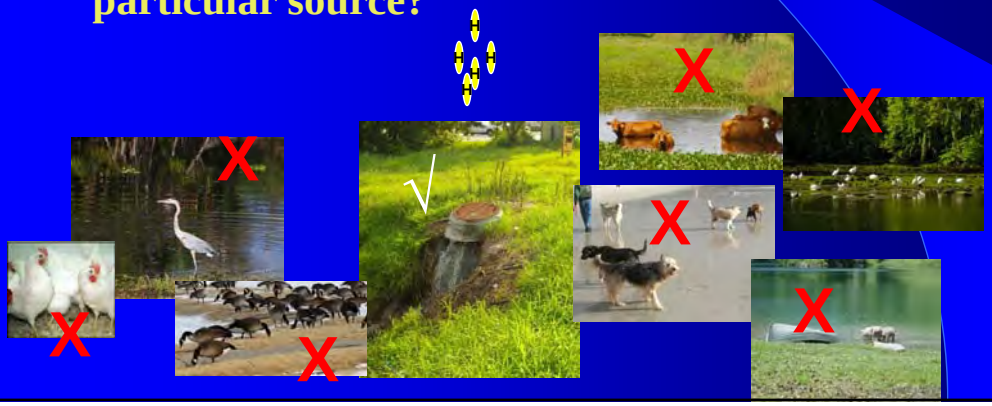
1. Identify microorganism that is confined to a particular host.
2. Identify gene that will discriminate this organism from all others.
3. Develop PCR method to selectively amplify the gene.
4. Test the PCR method for sensitivity, specificity, and other performance characteristics.

•Human polyomavirus



Sounds Simple – How Hard Could MST Be?

A. Specificity – if we detect a given "signature" (marker), how sure are we that it came from a particular source?



Sounds Simple - How Hard Could It Be?

B. Sensitivity

- **If contamination from a given source is present, how sure are we that our marker will be detected?**

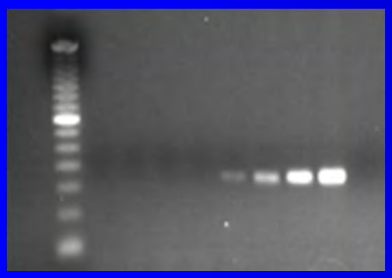
Sounds Simple - How Hard Could It Be?

C. Limit of Detection

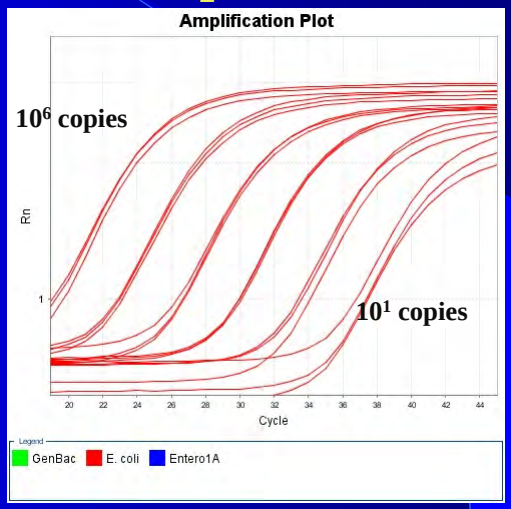
- **Quantitative assessment of sensitivity, i.e. how little can we reliably detect?**
- **Or…how much can contamination be diluted and still be detected?**

Conventional vs. Quantitative PCR

Conventional PCR



qPCR

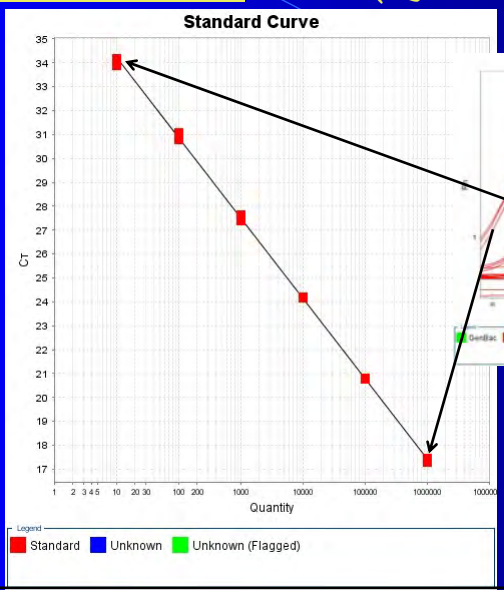


MST Success Story: Ben T. Davis Beach (+/- PCR)

How Hard Could it Be?
Quantitative PCR (QPCR)

$r^2 = 0.9995$
Efficiency = 98.6

Standard deviation (C_T)
=0.063 for 10^6 copies
=0.325 for 10^1 copies

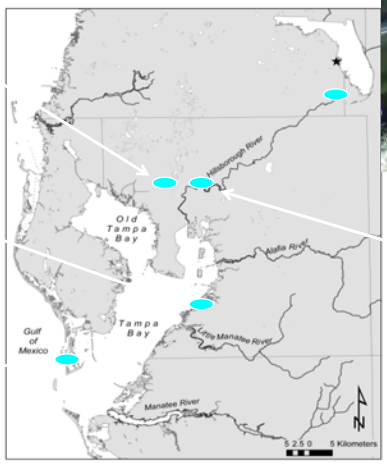


QPCR Study in a Range of Waters


Lake Carroll


Bahia Beach (Tampa Bay)


Fort DeSoto




Green Swamp

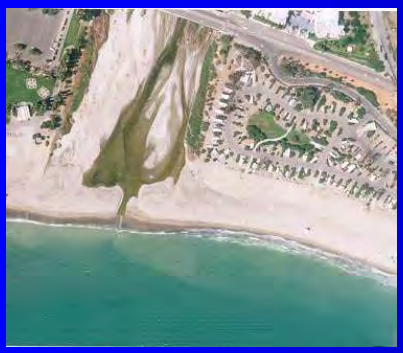

Riverfront Park
Hillsborough River

PCR Inhibition in Ambient Waters Detected by Internal Amplification Control

Sample Site	C_T Value	
	Sampling Date 1	Sampling Date 2
Distilled water	35-38	
Bahia Beach	35.1	35.4
Fort DeSoto	36.4	35.6
Green Swamp	40.1*	37.8
Lake Carroll	39.0*	37.9
Hillsborough River	42.4**	Undetermined**

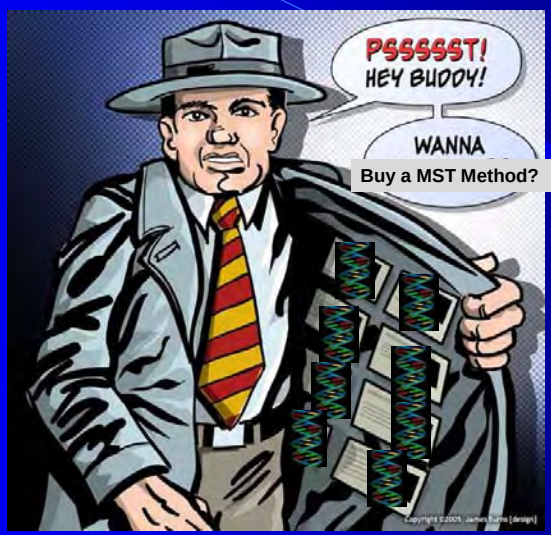
Inhibition best relieved by template dilution

Doheny & Avalon Beach



Correlation of FIB, Human Markers and Adenoviruses

	Doheny Beach						
	Total Coliforms	Fecal Coliforms	Enterococci	HPyVs	H-Bac	M. smithii	Adenovirus
Fecal Coliforms	r = 0.8780						
Enterococci	r = 0.8480	r = 0.8620					
HPyVs	NS	NS	NS				
H-Bac	R^2 = 0.1370	R^2 = 0.1900	R^2 = 0.1920	NS			
M. smithii	R^2 = 0.4660	NS	R^2 = 1.000	NS	NS		
Adenovirus	NS	NS	NS	R^2 = 0.0870	R^2 = 0.1078	NS	
	Avalon Beach						
Fecal Coliforms	r = 0.8926						
Enterococci	r = 0.6277	r = 0.7282					
HPyVs	NS	NS	NS				
H-Bac	R^2 = 0.061	R^2 = 0.074	NS	NS			
M. smithii	NS	NS	NS	NS	NS		




Questions?
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Microbial Source Tracking Applications in Food Safety

Katherine McElhany

Molecular Microbiology-Food Safety &
Environmental Microbiology Program

Texas A&M University

Outline

- Food & Environment
- Applications for Source Tracking in Food
- Methods
- Regulation
- Viruses in Source Tracking
- Final Thoughts and Summary

Food & Environment

- BST has largely focused on identifying sources of fecal bacteria in the environment
- Source tracking in food more mature
- Two fields are very much linked
- Food field developed from environmental work
  - Molecular tools initially developed in environmental microbiology
  - Food microbiologists and the industry expanded upon these tools

Foodborne Pathogens

	Organism	Est. Illnesses/Yr
Bacterial	*Salmonella* spp.	>1 million
	Clostridium perfringens	>900,000
	Campylobacter spp.	>800,000
	STEC *E. coli*	>150,000
	Shigella spp.	>100,000
	Listeria monocytogenes	>1,500
Viral	Norovirus	>5 million
	Hepatitis A	>1,500

(Scallan et. al., 2011)

Input Factors

Animals
- Direct contamination
- Manure

Water
- Irrigation water
- Wash water

People
- Handling (farm, packing house)
- Preparation (in home, restaurant, etc.)

Environmental Influence

	Organism	Reservoir	Transmission		
			Humans	Water	Animals
Bacterial	*Salmonella* spp.	Cattle & Poultry	X	X	X
Bacterial	*Clostridium perfringens*	Ubiquitous in environment	X	X	X
Bacterial	*Campylobacter* spp.	Poultry, Pigs, Cattle, Wild Birds	X	X	X
Bacterial	*Shigella* spp.	Humans	X	X	
Bacterial	STEC *E. coli*	Ruminants	X	X	X
Bacterial	*L. monocytogenes*	Soil & Water	X	X	X
Viral	Norovirus	Humans	X	X	
Viral	Hepatitis A	Humans	X	X	

Applications for ST in Food

- Outbreak Response & Traceback
- Product Quality & Control (emerging)
- Research

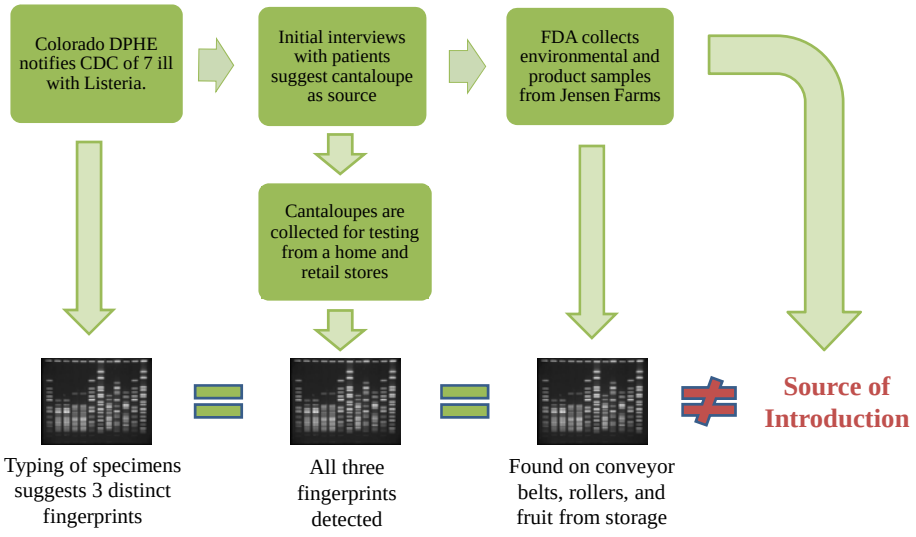
Foodborne Outbreaks & Illnesses

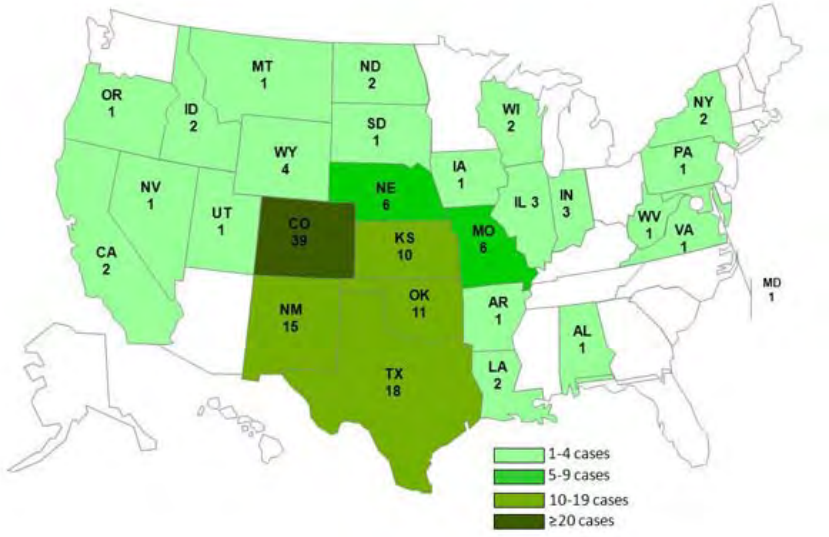
- The CDC collects data on foodborne disease outbreaks from all states and territories through the Foodborne Disease Outbreak Surveillance System
- A foodborne disease outbreak is defined as the occurrence of two or more cases of a similar illness resulting from the ingestion of a common food

Outbreak Response & Traceback

- Identify food
  - Allow recall
  - Prevent further illness
- Identify scope of outbreak
  - Link patients across multiple states or countries
- Trace back to source
  - Allow extended recall, if necessary
  - Identify contributing risk factors
  - Liability issues

2011 Listeria Outbreak





(CDC, 2011)

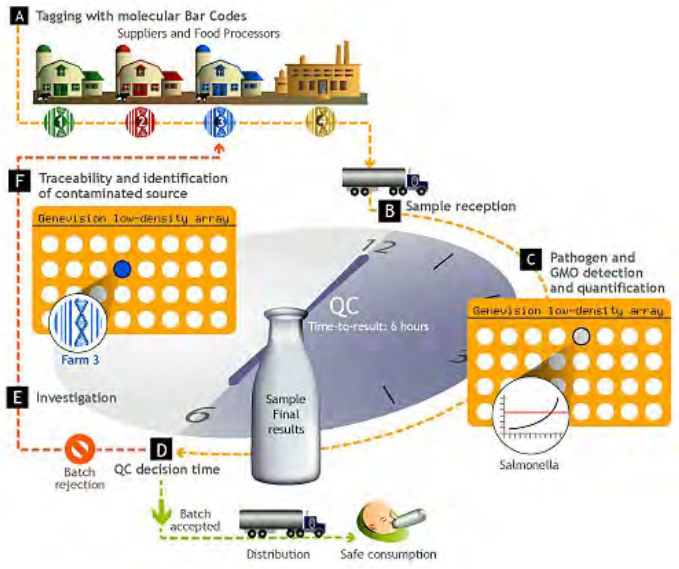
Product Quality & Control

- Food safety is not a competitive advantage—food quality is!
- Uses of molecular tools for traceback starting to be used for purposes other than food safety
  - Quality indicators
  - Fraud/counterfeit detection
  - Food origin
- Traditionally done by manual or digital trace back, but molecular methods now emerging
  - Tagging with custom-designed molecular barcodes

Tagging with Molecular Bar Codes

- Information that can be encoded
  - Name of product
  - Name of company
  - Processing plant
  - Date of production
  - Batch number
  - Customized information

- Added to packaging or product
- The information encoded is secure



Courtesy Warnex, 2004

Research

- Understanding transmission
- Evaluating risk
- Tracking evolution of organisms and environmental influence
  - Acquired virulence genes
  - Adaptation
  - Antibiotic resistance

Molecular Methods

- Developed initially for environmental sampling (water and soils)
- Methods for food testing have now advanced significantly
- In many cases, beyond environmental methods

Food Industry & Reg. Agencies

- Source tracking has become very refined process in food industry
- Not just research purview
- Commercial diagnostic companies investing resources to commercialize kits for sampling, sample processing and detection

Advanced Techniques

- Easy-to-use accelerated fingerprinting methods (i.e. DiversiLab) becoming commonplace
- MLST and other methods used in combination with PFGE and DiversiLab
- TAMU graduate students learning methods as part of Molecular Methods course

Why?

Regulation

- Regulations have been the main driving force for developing source tracking in the food industry
- Influenced:
  - Testing
  - Standardization & Coordination
  - Liability

Testing

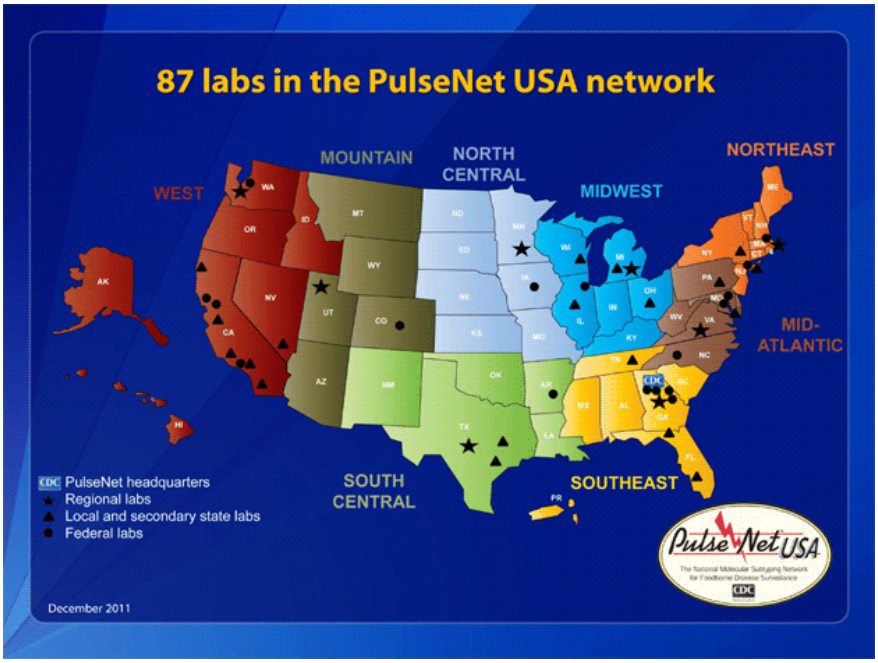
- Regulations mandating testing
  - Frequency
  - Methods
- Federal regulations have forced companies to test for “adulterants”
  - *E.coli* O157:H7
  - *Listeria monocytogenes*
  - non-O157:H7 *E.coli*

Standardization & Coordination

- Regulations requiring reporting of foodborne illness have encouraged:
- Standardization of Methods
  - PFGE technology developed by CDC now used all over the world
  - PulseNet Europe, PulseNet Asia, PulseNet Latin America
- Coordination of agencies
  - Necessary for trace back in outbreaks involving multiple states
  - PulseNet

PulseNet

- National network of public health and food regulatory agencies coordinated by CDC.
  - State Health Departments
  - Local Health Departments
  - USDA/FSIS
  - FDA
- Participants perform PFGE analysis of foodborne disease-causing bacteria from specimens
- PFGE patterns are uploaded automatically to a CDC database and are available for rapid comparison



Liability

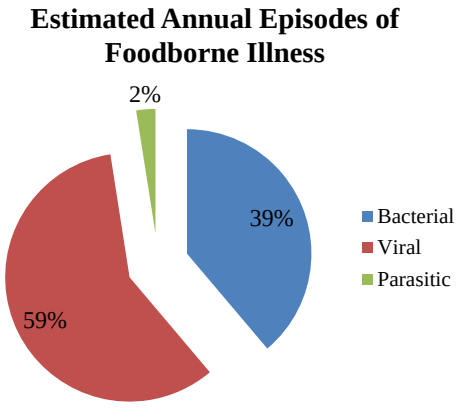
- The *Food Safety Modernization Act* and other regulations have increased liability issues for companies
- Encouraged fingerprinting and traceback
- This and consumer pressures have encouraged the food industry to develop own standards
  - Global Food Safety Initiative (GFSI)

Challenges

- "Fitting a loaf of bread into a microcentrifuge tube"
  - How much to test?
  - Inhibitors in molecular work
  - Detection limits
- No quantitative detection—food companies and testing laboratories often test only for presence/absence
- Most molecular testing done in 3rd party labs
  - Equipment needs
  - Personnel needs

Viruses

- Viruses are often overlooked in food and environmental studies, even though they are the most common agents of foodborne illness

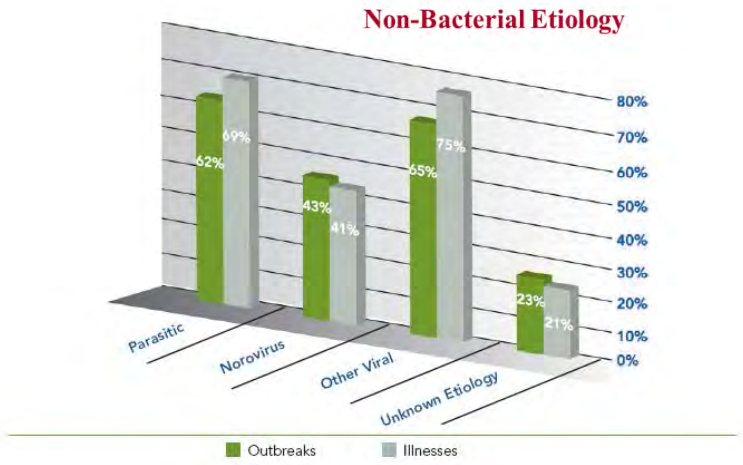


(Scallan et. al., 2011)

Viral Sources in Foods

- We know very little where and how viruses enter our foods
  - At the source?
  - During processing and packing?
  - At retail?
- Source tracking used to address such questions
- Underused because of challenges:
  - More difficult to culture
  - More difficult to recover

% of Foodborne Outbreaks and Illnesses Associated with Produce

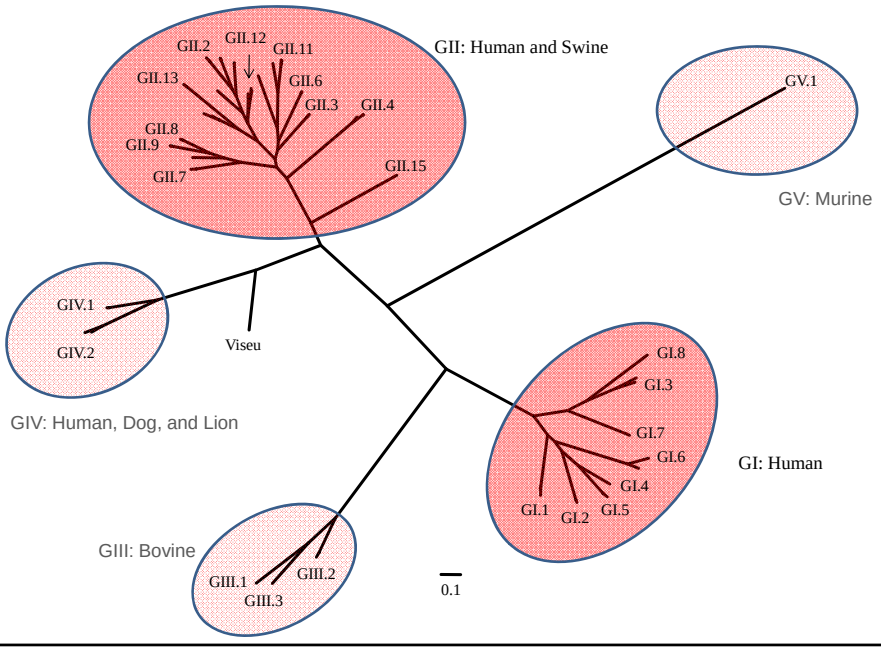


(Scharff, 2010)

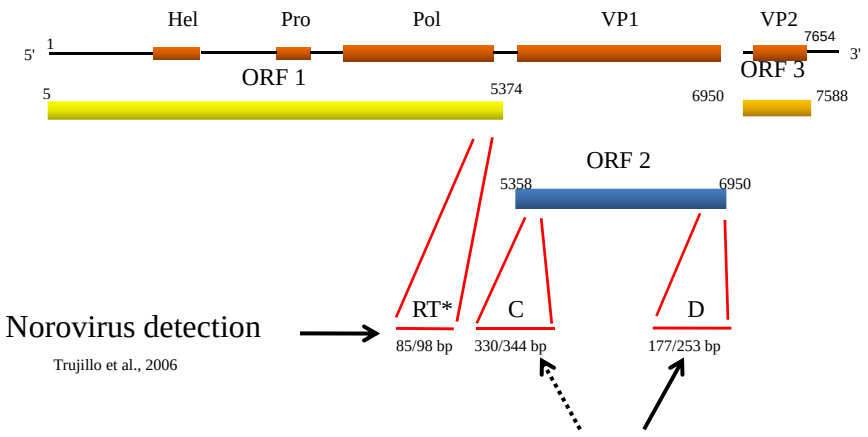
Noroviruses

- Noroviruses are the principal cause (>85%) of outbreaks of viral gastroenteritis
  - Significant cause of morbidity, but self-limiting
  - Transmission routes
    - Food (~20-30%)
    - Water (~<1%)
    - Person to person (70-80%)
- Norovirus genotyping also provides information about the possible etiology
  - Genogroup I and II strains are responsible for majority of disease in humans
  - Genotype II.4 is responsible for the majority of outbreaks

Genetic Classification of Noroviruses



Genomic Typing Regions



* RT = TaqMan realtime RT-PCR

Viral Indicators in Salad

- Coliphages are viruses that infect *E. coli*—commonly used as indicator organisms
- Male-specific RNA coliphage (FRNA) genogroups show some source specificity.
- F+RNA Male-Specific Coliphages
  - Genogroup I: Animal-associated
  - Genogroup II: Human-associated
  - Genogroup III: Human-associated
  - Genogroup IV: Animal-associated

(Friedman et. al., 2009)

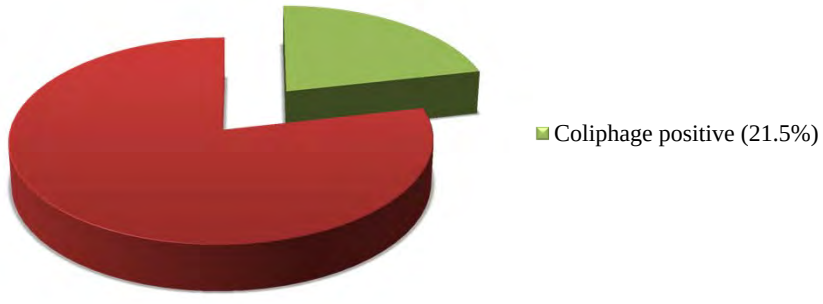
Viral Indicators in Salad

- Study sought to determine sources of fecal contamination in restaurant salads
  - Samples collected from local restaurants
  - Male-specific coliphages extracted from salads using Method 1602
  - Phages were genotyped using established RT-PCR assay
- Example of method adaptation:
  - Methods established for water and environment
  - Adapted for food product

(EPA, 2001; Friedman et. al, 2009)

Viral Indicators in Salad

Of 200 Restaurant (House and Specialty Salad) Samples:



Prince & Pillai, 2010 – unpublished data

Viral Indicators in Salad

- Of 43 Samples genotyped:
  - 1 positive for Genogroup I (suggesting animal)
  - 5 positive for Genogroup III (suggesting human)
- These results indicate main contributor to fecal contamination in sampled salads was human (suggests processing or food handling)

Prince & Pillai, 2010 – unpublished data

What are significant organisms?

- Determining sources of fecal contamination
  - Water
  - Soil
  - Food
- Targeted organisms generally pathogens or fecal indicators
- How are significant organisms chosen?

Organisms Detected in Food

Bacterial genera detected in 16S rRNA-based tag pyrosequencing of ground beef:

Anaerobiospirillum spp.	*Escherichia* spp.	*Prevotella* spp.
Bacteroides spp.	*Lactobacillus* spp.	*Propionibacterium* spp.
Brocothrix spp.	*Lactococcus* spp.	*Proteus* spp.
Buttiauxella spp.	*Micrococcus* spp.	*Pseudomonas* spp.
Chryseobacterium spp.	*Niastella* spp.	*Ruminococcus* spp.
Clostridium spp.	*Nocardioides* spp.	*Staphylococcus* spp.
Desulfovibrio spp.	*Peptoniphilus* spp.	*Succinivibrio* spp.
Enterococcus spp.	*Photobacterium* spp.	*Sutterella* spp.

McElhany & Pillai, 2009 – unpublished data

Organisms Detected in Food

Organisms detected in ground beef that are known human gut microbiota:

Anaerobiospirillum spp.	*Escherichia* spp.	*Prevotella* spp.
Bacteroides spp.	*Lactobacillus* spp.	*Propionibacterium* spp.
Brocothrix spp.	*Lactococcus* spp.	*Proteus* spp.
Buttiauxella spp.	*Micrococcus* spp.	*Pseudomonas* spp.
Chryseobacterium spp.	*Niastella* spp.	*Ruminococcus* spp.
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Desulfovibrio spp.	*Peptoniphilus* spp.	*Succinivibrio* spp.
Enterococcus spp.	*Photobacterium* spp.	*Sutterella* spp.

McElhany & Pillai, 2009– unpublished data

Summary

- For food, source tracking driven by regulations
  - Advanced testing methods
  - Standardized methods
  - Coordinating agencies and databases
- Viruses are often overlooked in source tracking
- More research needed to validate choice of organisms used in source tracking

BST: Relevance to EPA Policies, Programs and Regulations

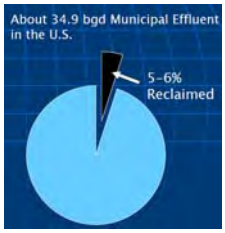
2012 BST Conference
State-of-the-Science
New Braunfels, Texas

Sally C. Gutierrez, EPA/ORD
Cincinnati, Ohio
4/4/2012

U.S. Environmental Protection Agency

Sustainability Context

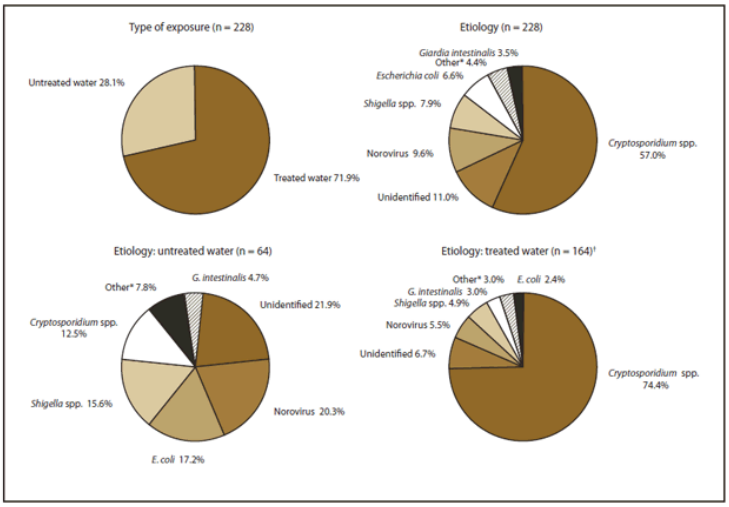
Sustainability Advancement Opportunity



Statutory Frameworks

- Federal Clean Water Act
  - Water quality standards
  - Total Maximum Daily Loads
  - Biosolids management
  - Water reuse
- Federal Safe Drinking Water Act
  - Source water protection
  - Control of contaminants

U.S. Recreational Waterborne Disease Outbreaks (AGI) 1999-2008



MMWR, Sept. 23, 2011 /60(ss12);38-68

Water Quality Standards

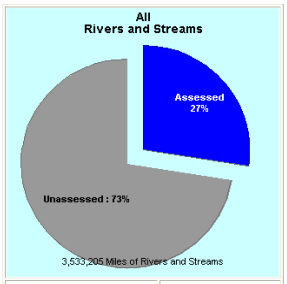
BST Relevance

6

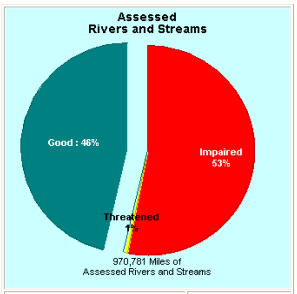
CWA Water Quality Standards Elements

- Waterbody use designation
- Criteria (numeric or narrative)
- Antidegradation

U.S. Rivers and Streams Assessment Status and Quality



Assessed Status	Miles
Assessed	970,781
Unassessed	2,562,424
Total Miles	**3,533,205**



Attainment Status	Miles
Good	449,617
Threatened	6,369
Impaired	514,795
Total Miles Assessed	**970,781**

NRDC v. EPA (2006)

- NRDC sued EPA on its lack of progress to comply with BEACH Act requirements
- Issues
  - Timetable for proposing new standards
  - Setting standards that fully protect public health
  - Establishing test methods to allow prompt decision making about beach closings and advisories

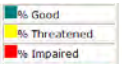
Total Maximum Daily Loads

BST Relevance

10

U.S. Rivers and Streams
Designated Use Condition

Designated Use Group	Miles Assessed	Percent Good	Percent Threatened	Percent Impaired
Fish, Shellfish, And Wildlife Protection And Propagation	762,113	55.0	.5	44.5
Recreation	390,603	55.2	1.4	43.3
Agricultural	351,971	93.7	.6	5.8
Aquatic Life Harvesting	261,832	34.0	.3	65.7
Industrial	210,190	93.3	.0	6.7
Public Water Supply	187,707	73.9	.8	25.3
Other	99,646	84.5	.3	15.3
Aesthetic Value	26,064	89.0	.0	11.0
Exceptional Recreational Or Ecological Significance	14,663	11.3	4.4	84.4



U.S. Rivers and Streams
Designated Impaired for Pathogens Cause

Cause of Impairment	Miles Threatened or Impaired
Escherichia Coli (E. Coli)	70,849
Fecal Coliform	66,874
Pathogens	10,916
Enterococcus Bacteria	9,029
Bacteria	7,347
Total Coliform	3,571
Fecal Bacteria	108
Indicator Bacteria	64
Bacterial Slimes	30

Biosolids Management

BST Relevance

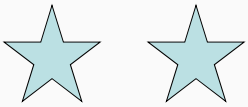
13

Biosolids

- EPA has approved the following microbial methods for use in biosolids: EPA Method 1680 and 1681 for fecal coliforms and EPA Method 1682 for Salmonella.
- All approved methods use culture techniques

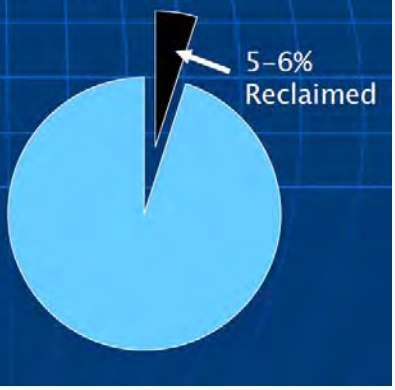
Water Re-use

BST Relevance



15

- About 5-6% of municipal wastewater effluent in the U.S. is reclaimed and beneficially reused
- Israel reuses more than 70%
- Singapore reuses 30%, up from 15% in recent years
- Australia, now at 8%, has a national goal of 30% by 2015



CWA Analytical Methods

- Approved EPA methods mandatory for CWA compliance activities
- Approved Methods codified in 40 CFR 136

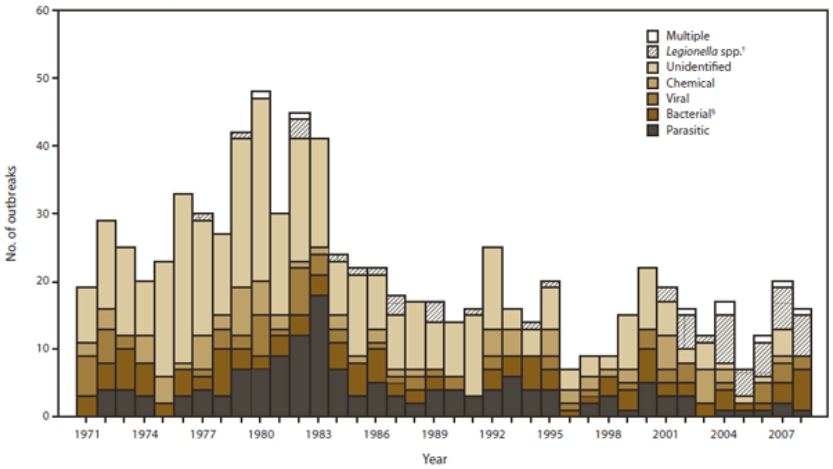
CWA Approved Methods 40 CFR 136

- 1603 Escherichia coli (E. coli) in Water by Membrane Filtration Using Modified membrane-Thermotolerant Escherichia coli Agar (Modified mTEC)
- 1604 Total Coliforms and Escherichia coli in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium) (PDF)
- 1605 Aeromonas in Finished Water by Membrane Filtration using Ampicillin-Dextrin Agar with Vancomycin (ADA-V) (PDF)
- 1622 Cryptosporidium in Water by Filtration/ Immunomagnetic Separation/Immunofluorescence Assay Microscopy
- 1623 Cryptosporidium and Giardia in Water by Filtration/IMS/FA

Other CWA Methods *not* Currently Approved for use at 40 CFR 136

- *Escherichia coli* (*E. coli*) in Water by Membrane Filtration
  - 1103.1 Using membrane-Thermotolerant *Escherichia coli* Agar (mTEC)
  - 1603 Modified membrane-Thermotolerant *Escherichia coli* Agar (Modified mTEC)
- Enterococci in Water
  - 1106.1 Using membrane-Enterococcus-Esculin Iron Agar (mE-EIA)
  - 1600 By Membrane Filter Test Method for Enterococci in Water
- Male-specific (F+) and Somatic Coliphage in Water
  - 1601 By Two-step Enrichment Procedure (PDF) (40 pp, 259K)
  - 1602 By Single Agar Layer (SAL) Procedure (PDF) (38 pp, 207K)
- 1604 Total Coliforms and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous DetectionTechnique (MI Medium) (PDF) (18 pp, 384K)
- 1605 Aeromonas in Finished Water by Membrane Filtration using Ampicillin-Dextrin Agar withVancomycin (ADA-V) (PDF) (36 pp, 141K)
- 1622 *Cryptosporidium in Water by Filtration/ Immunomagnetic Separation/Immunofluorescence Assay Microscopy*
- 1623 *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA

U. S. Drinking Waterborne Disease Outbreaks



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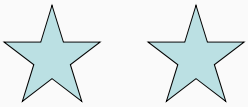
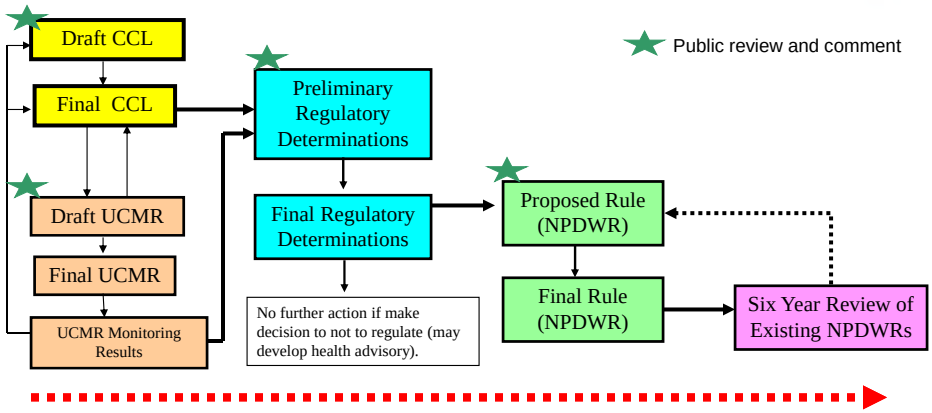
Drinking Water Safety

BST Relevance



21

SDWA Regulatory Process



At each stage, need increased specificity and confidence in the type of supporting data used (e.g. health, occurrence, treatment).

22

National Public Drinking Water Regulations
Microorganisms

Contaminant	MCLG[1] (mg/L)[2]	MCL or TT[1] (mg/L)[2]
Cryptosporidium	zero	TT [3]
Giardia lamblia	zero	TT[3]
HPC	n/a	TT[3]
Legionella	zero	TT[3]
Total Coliforms (including fecal coliform and *E. Coli*)	zero	5.0%[4]
Turbidity	n/a	TT[3]
Viruses (enteric)	zero	TT[3]

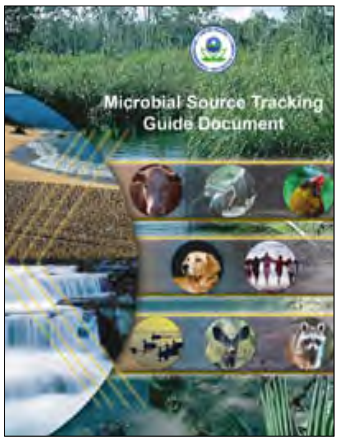
Homeland Security/Emergency Response

BST Relevance

24

Water Security/Emergency Response

EPA MST Resource Documents

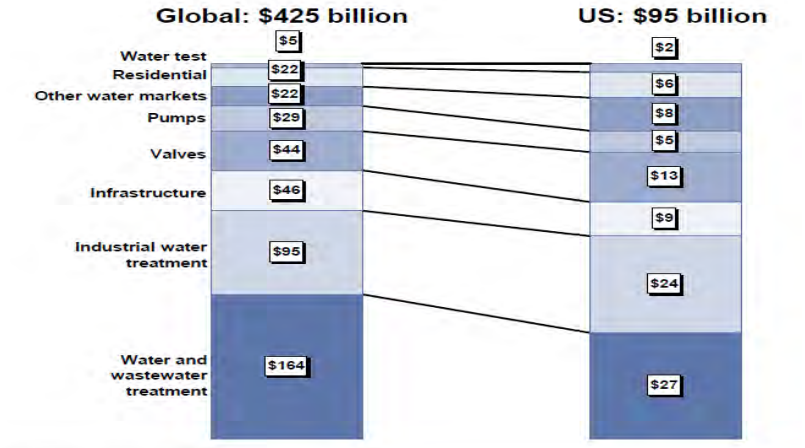



Using Microbial Source Tracking to Support
TMDL Development and Implementation

"A New Hope"

Technology Innovation Clusters and New Technology Development

Water Market Segments - 2008



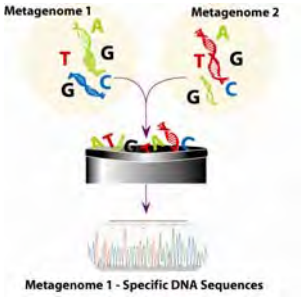
Source: Goldman Sachs Research estimates.

Advanced Molecular Tools for Protecting Recreational and Drinking Water Sources

Patent No.: US 7,572,584 B2
Issue Date: August 11,2009
Title: Species-Specific Primer Sets and Identification of Species-Specific DNA Sequences using Genome Fragment Enrichment

- Newly developed **Microbial Source Tracking** methods for distinguishing human, cattle, and chicken sources of fecal contamination

- **Genome Fragment Enrichment** DNA sorting technology to identify unique and divergent sequences between two DNA preparations



SBIR Pathogen Technology Development Investment

Acknowledgements

- **Pamela Barr, Director, Standards and Risk Management Division, Office of Ground Water and Drinking Water**
- **Dr. Orin Shanks, Dr. Jorge Santo-Domingo, USEPA NRMRL**

31

Recommendations from the Texas Task Force on Bacteria TMDLs

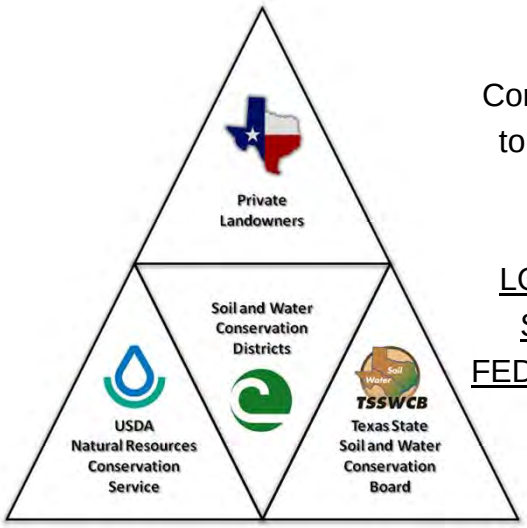
Aaron Wendt
Texas State Soil and Water Conservation Board

Bacterial Source Tracking
State of the Science Conference
February 28-29, 2012
New Braunfels, TX

Water Quality in Texas

- Texas State Soil and Water Conservation Board (TSSWCB)
  - Agricultural and Silvicultural Nonpoint Source
- Texas Commission on Environmental Quality (TCEQ)
  - Point Source Permitting (WWTF, CAFO, MS4)
  - All other forms of Nonpoint Source

Texas Conservation Partnership



Providing
Conservation Assistance
to Private Landowners
for 70+ Years

LOCAL = 216 SWCDs
STATE = TSSWCB
FEDERAL = USDA-NRCS

Why Texas Needed a Task Force

- Texas 303(d) List of Impaired Waters dominated by elevated bacteria related to recreational use and oyster waters use
- Several watershed planning processes (TMDLs or WPPs) on-going with discontented stakeholder groups
- Variety of BST methods/approaches by a number of laboratories had been used in different watershed planning processes

TSSWCB and TCEQ Establish Task Force

- September 27, 2006
- examine approaches other states use to develop bacteria TMDLs
- recommend cost-effective and time-efficient methods and approaches for developing TMDLs and Implementation Plans
- evaluate the variety of models and BST methods available for developing TMDLs and I-Plans, and recommending under what conditions certain methods are more appropriate
- develop a roadmap for further scientific research needed to reduce uncertainty about how bacteria behave under different water conditions in Texas

Task Force Members

- Allan Jones (chair) – Texas Water Resources Institute
- George DiGiovanni – Texas Agricultural Experiment Station
- Larry Hauck – Texas Institute for Applied Environmental Research
- Joanna Mott – Texas A&M University–Corpus Christi
- Hanadi Rifai – University of Houston
- Raghavan Srinivasan – Texas A&M University
- George Ward – University of Texas at Austin

Task Force Report

- http://twri.tamu.edu/what-we-do/finished/bacteria-tmdl/
  - Task Force website with all background information, membership lists, meeting summaries, report drafts, comments rec'd
- June 4, 2007
- TR-341 published by Texas Water Resources Institute
- http://twri.tamu.edu/publications/reports/2009/tr-341/

Task Force Report

- recommended the use of a Three-Tier Approach for bacteria TMDL and Implementation Plan development that is designed to be
  - cost-effective
  - time-efficient
  - scientifically credible
  - accountable to watershed stakeholders
- tiers move through increasingly aggressive levels of data collection and analysis (including BST) in order to achieve stakeholder consensus on needed load reductions and strategies to achieve those reductions

TSSWCB and TCEQ Adopt Recommendations

- June 29, 2007
- adopted the principles and general process recommended by the Task Force
- directed staff to
  - incorporate the principles of the recommendations into an updated joint-agency TMDL guidance document
  - move diligently to expedite the development of bacteria TMDLs that were paused during the work of the Task Force
  - establish a multi-agency bacteria work group to continue examining the scientific research and development needs identified by the Task Force

What did Task Force say about BST?

- examined use of ERIC-PCR, Ribotyping, PFGE, KB-ARA, CSU, Bacteroidales PCR
- recommended using library-independent methods like Bacteroidales PCR for preliminary qualitative analyses (Tier 2)
- recommended using library-dependent methods if more quantitative data are needed (Tier 3)

Appendix 4. Table 2. Bacteria TMDLs Under Development in Texas.

Project	HSPF	Load Duration	Other Models	Bacteria Source Tracking Method
Upper San Antonio River	●			ERIC-PCR and RiboPrinting
Leon River	●			ERIC-PCR and RiboPrinting
Peach Creek	●			ERIC-PCR and RiboPrinting
Adams and Cow Bayous	●		RMA2/ACE	No BST
White Oak and Buffalo Bayous	●			ARA and CSU
Lower San Antonio River		●		ERIC-PCR and RiboPrinting
Atascosa River		●		No BST
Elm and Sandies Creeks		●		No BST
Upper Trinity River		●		Ribotyping (Institute for Environmental Health, Inc., Seattle, WA)
Guadalupe River above Canyon Lake		●		Ribotyping (Source Molecular Corporation, Inc., Miami, FL)
Upper Oyster Creek		●		Ribotyping (Institute for Environmental Health, Inc., Seattle, WA)
Copano Bay and Mission and Aransas Rivers			ArcHydro\Monte Carlo Simulation	ARP and PFGE
Oso Bay and Oso Creek			ArcHydro\SWAT	No BST
Gilleland Creek		●		No BST
Clear Creek		●		
Metropolitan Houston (Brays, Greens, Halls and other Bayous)	●			ARA and CSU
WPP – Lake Granbury				
WPP – Buck Creek			TBD	*E. faecium*, ERIC-PCR, RP
WPP – Bastrop Bayou				
WPP – Plum Creek		●	SELECT, SPARROW, SWAT	No BST

Table 2. Relative comparison of several bacterial source tracking techniques

Technique	Acronym	Target organism(s)	Basis of characterization	Previously Used or in Progress in Texas	Used in other states	Accuracy of source identification	Size of library needed for water isolate IDs	Capital cost	Cost per sample (reagents and consumables only)	Ease of use	Hands on processing time for 32*** isolates	Time required to complete processing 32 isolates
Enterobacterial repetitive intergenic consensus sequence polymerase chain reaction	ERIC-PCR	*Escherichia coli* (*E. coli*) and *Enterococcus* spp.	DNA fingerprint	Yes (Di Giovanni)	Yes	Moderate	Moderate	$20,000 ($15,000 BioNumerics software, $5,000 equipment)	$8	Moderate	3 h	24 h**
Automated ribotyping (RiboPrinting)†	RP	*E. coli* and *Enterococcus* spp.	DNA fingerprint	Yes (Di Giovanni)	Yes	Moderate	Moderate	$115,000 ($100K RiboPrinter, $15K BioNumerics software)	$40	Easy	1 h	24 h
Pulsed field gel electrophoresis	PFGE	*E. coli* and *Enterococcus* spp.	DNA fingerprint	Yes (Pillai and Lehman)	Yes	High	Large	$30,000	$40	Difficult	10 h	5 days
Kirby-Bauer antibiotic resistance analysis‡	KB-ARA	*E. coli* and *Enterococcus* spp.	Phenotypic fingerprint	Yes (Mott)	Yes	Moderate*	Moderate	$35,000	$15	Easy	3 h	24 h**
Carbon source utilization	CSU	*E. coli* and *Enterococcus* spp.	Phenotypic fingerprint	Yes (Mott)	Yes	Moderate	Moderate	$15,000	$10	Easy	4 h	24 h**
Bacteriodales polymerase chain reaction	*Bacterio-dales* PCR	*Bacteriodales* species	Genetic marker presence or absence (not quantitative)	Yes (Di Giovanni)	Yes	Moderate to high for only human, ruminant, horse, and pig sources	Not applicable	$5,000	$8	Easy to moderate	3 h	8 h**
Enterococcus faecium surface protein polymerase chain reaction or colony hyb.	*E. faecium* esp marker	*E. faecium*	Genetic marker presence or absence (not quantitative)	Yes (Di Giovanni)	Yes	High for only human	Not applicable	$8,000	$8 to $12	Easy to moderate	3 to 6 h	8 to 24 h**
ERIC and RP 2-method composite	ERIC-RP	*E. coli*	DNA fingerprints	Yes (Di Giovanni)	No	Moderate to high	Moderate	$120,000	$48	Moderate	4 h	24 h
ERIC and KB-ARA 2-method composite	ERIC-ARA	*E. coli*	DNA and phenotypic fingerprints	Yes (Di Giovanni)	No	Moderate to high	Moderate	$55,000	$23	Moderate	6 h	24 h
KB-ARA and CSU 2-method composite	ARA-CSU	*E. coli* and *Enterococcus* spp.	Phenotypic fingerprints	Yes (Mott)	Yes	Moderate to high	Moderate	$30,000	$23	Easy to moderate	7 h	24 h

†A manual ribotyping version is also used by some investigators (i.e. Dr. M. Samadpour with IEH Laboratories and Consulting Group in Seattle), but no detailed information is available for comparison.
‡A variation of this technique using replica plating and +/- scoring of growth on media with different concentrations of antibiotics, called ARA, has been used extensively in Virginia for TMDLs.
*This technique is better for distinguishing broader groups of pollution sources. For example, "wildlife" and "livestock" as opposed to "avian wildlife", "non-avian wildlife," "cattle," etc.
**With sufficient personnel, up to approximately 150 isolates can be analyzed in 24 h.
***Thirty two isolates selected for comparison because it is the maximum throughput per day of the RiboPrinter, which is the only automated system described.

What did Task Force say about BST?

- Need to clearly define agency/stakeholder expectations for BST and capabilities of BST
- Impact of indicator bacteria survival and regrowth in the aquatic environment, sediment, and soils on BST
- Appropriate level of discrimination of BST results – individual species, human or animal, or some level between
- BST typically identifies only source, not entry pathways of fecal pollution – importance of sampling regime
- In nearly all cases, no single BST method should be solely relied upon
- Laboratory infrastructure for BST work in Texas needs to be expanded for both library dependent as well as library independent methods

What did TF say about Library-Dependent BST?

- Recommend composite library-dependent BST using 1 of 3 combination methods:
  - ERIC-PCR and RiboPrinting (ERIC-RP)
  - ERIC-PCR and KB-ARA (ERIC-ARA)
  - CSU and KB-ARA (CSU-ARA)
- Library development is one of the most costly components of BST
  - most economical to build upon the libraries already established in Texas
  - recommended to use BST methods that will strengthen and expand the current Texas library and follow previously approved SOPs

What did TF say about Library-Independent BST?

- Library-independent methods are cost-effective, rapid and potentially more specific and accurate than library dependent methods
- Concerns regarding geographical stability of markers
- Concerns about the difficulty of interpreting results in relation to water quality standards (i.e., Bacteroidales vs. E. coli)
- Recommend library-independent PCR genetic test for Bacteroidales markers
  - human
  - ruminants
  - horse
  - swine

What did Task Force say about Tier 2 BST?

  - conducted in conjunction with the targeted monitoring
  - determine if livestock, humans and/or non-domestic animals are contributing bacteria
- Library Independent
  - samples analyzed using PCR genetic test for the Bacteroidales markers for human, ruminants, horse and swine
- Library Dependent (limited)
  - E. coli isolates from water samples analyzed using the Tier 3 methods
  - Compared to previously developed Texas Known Source Library
  - determine the need for development of a local source library
  - confirm that the sources of E. coli and Bacteroidales are comparable

What did Task Force say about Tier 3 BST?

- Library Dependent
  - Use 1 of 3 combination methods
  - ERIC-RP, ERIC-ARA or CSU-ARA
- If Tier 2 BST does not provide 80% identification using existing statewide library, then statewide library needs to be augmented with local known sources
  - Add isolates from known fecal samples (~3 isolates/sample)
- Conduct BST on ambient water samples using the selected combination method
  - identified to cattle, other livestock, avian and non-avian non-domestic animals, domestic sewage, pet sources, unknown
  - Sources should be expressed as percentages of total isolates with appropriate confidence intervals

What did Task Force say about BST R&D?

- Improve linkages of BST and computer modeling. Models can be validated with BST or vice versa.
- Determine reasonable expectation for the level of source identification by BST
- Refinement of library-independent BST methods and species-specific markers
- Investigate geographic and temporal stability of BST known source libraries
- Define appropriate ambient water sampling protocol to provide desired statistical confidence with BST

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Battelle
The Business of Innovation

Source Tracking Accuracy and Study Design

Don Stoeckel, PhD
stoeckeld@battelle.org
Battelle Memorial Institute
Columbus, Ohio

Battelle
The Business of Innovation

Collaborators

- U.S. Geological Survey
- The Ohio State University
- Ohio River Valley Water Sanitation Commission
- Colorado Department of Public Health and the Environment
- US Environmental Protection Agency

Definition of Objective

Measure *E. coli* and source tracking markers in streams and rivers in order to detect areas where high *E. coli* are contributed by human sources

- Internal spike-and-recovery controls for DNA extraction
- Confirmation of MST marker specificity and sensitivity
  - Seasonal variability
  - Understand the relationship between *Bacteroidales* and *E. coli* density
- Confirmation of equivalent persistence for *E. coli* and MST markers
- Positive-control tests in environmental waters
- Techniques to limit false-positive interpretations

INTERNAL SPIKE-AND-RECOVERY CONTROLS

Spike-and-recovery controls at the sample level (filtration, extraction, and qPCR)

- Introduction of spike-and-recovery controls into samples to control for losses during processing

WATER RESEARCH 43 (2009) 4820–4827

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/watres

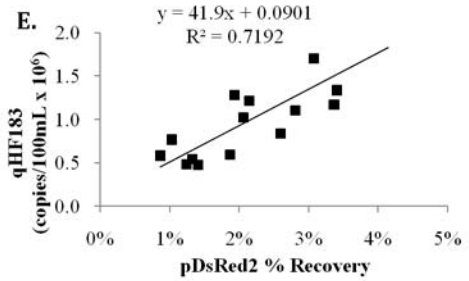
Evaluation of two spike-and-recovery controls for assessment of extraction efficiency in microbial source tracking studies

Donald M. Stoeckel[a], *Erin A. Stelzer*[a], *Linda K. Dick*[b,*]

[a]US Geological Survey, Ohio Water Microbiology Laboratory, Columbus, OH 43229, USA
[b]School of Environment and Natural Resources, The Ohio State University, Columbus, OH 43210, USA

Controls used

- Cells of plasmid-containing *E. coli*
  - Normalized to cell count by average plasmid number
- Cells of *P. sterwartii*
  - Corn pathogen, gram-negative, enteric
  - Normalized to cell count (chromosomal marker, single copy)



SENSITIVITY AND SPECIFICITY

Collection of reference material

Test human and nonhuman sources

Study of fecal contamination sources to the Ohio River (USGS, Ohio State and ORSANCO)

- Collect sewage from five treatment plants along the Ohio River
- Collect feces from other animals at five locations along the Ohio River
- Repeat collections quarterly
- Analyze for
  - Fecal-indicator bacteria
  - MST markers

Results

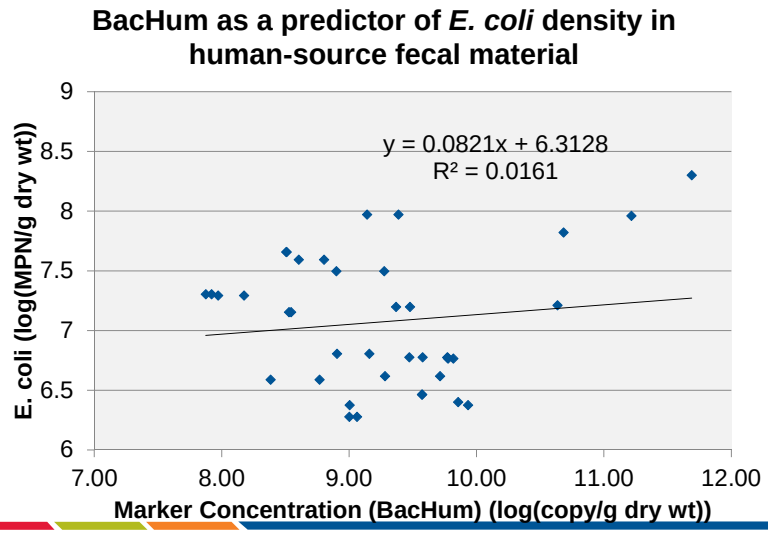
Characteristics of fecal material in the Ohio River Valley:

- Concentration of *E. coli* in MPN/g dry weight
- Concentration of general (AllBac) and human-associated (qHF183 and BacHum) MST markers in copies/g dry wt

Category	Location	*E. coli*				AllBac				qHF183				BacHum			
		Spr	Sum	Fall	Win	Spr	Sum	Fall	Win	Spr	Sum	Fall	Win	Spr	Sum	Fall	Win
Human	Bridgeport	6.7	--	7.7	6.6	11.7	--	11.0	11.2	10.2	--	9.2	8.9	9.8	--	8.8	9.2
Human	New Martins	6.9	8.0	7.5	--	11.7	11.8	10.1	--	10.7	9.8	8.5	--	10.2	9.7	8.2	--
Human	Parkersburg	6.8	--	7.5	7.4	11.7	--	10.7	11.2	10.7	--	9.5	9.1	10.2	--	9.1	9.1
Human	Steubenville	6.8	7.2	7.1	6.7	11.3	11.1	10.3	10.9	10.5	9.0	9.0	9.4	10.0	8.9	8.7	9.3
Human	Wheeling	6.3	7.3	7.8	7.7	11.0	10.5	10.6	10.9	10.0	8.3	9.0	8.9	9.5	8.3	8.6	8.8
Bird	Duck	--	--	6.8	**8.2**	--	11.1	10.3	***7.7***	--	ND	ND	ND	--	ND	ND	ND
Bird	Goose	--	**8.3**	7.5	--	--	10.8	10.6	10.3	--	ND	ND	ND	--	ND	ND	ND
Pets	Dog	--	**8.5**	**8.5**	**8.3**	--	11.5	10.2	11.4	--	6.4	5.8	6.4	--	6.6	6.4	7.4
Rodents	Raccoon	--	**9.6**	**8.0**	--	--	***9.7***	10.2	--	--	ND	ND	--	--	ND	ND	--
Ruminants	Cow	--	6.7	***5.8***	***5.3***	--	11.8	10.9	11.5	--	ND	ND	ND	--	ND	5.7	ND
Ruminants	Deer	--	**8.6**	--	6.0	--	11.2	--	10.3	--	ND	--	ND	--	ND	--	ND

9

Note... there is no consistent ratio between *E. coli* and marker in the individual sample



10

STABILITY OF SIGNALS

Microcosms study

Test to see how aging affects MST markers and fecal-indicator bacteria in sewage

USGS and Ohio State

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zjs / DOCHEAD=ARTICLE zjss / DOCTOPIC=PUBLIC HEALTH MICROBIOLOGY
FROM COVER SHEET: Editor: Schaffner Article No.: T Section: Public Health

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2010, p. 000 Vol. 76, No. 10
0099-2240/10/$12.00 doi:10.1128/AEM.02636-09

Relative Decay of *Bacteroidales* Microbial Source Tracking Markers and Cultivated *Escherichia coli* in Freshwater Microcosms[▽]

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School of Environment and Natural Resources, The Ohio State University, Columbus, Ohio 43210,[1] and U.S. Geological Survey, Ohio Water Science Center, Columbus, Ohio 43229[2]

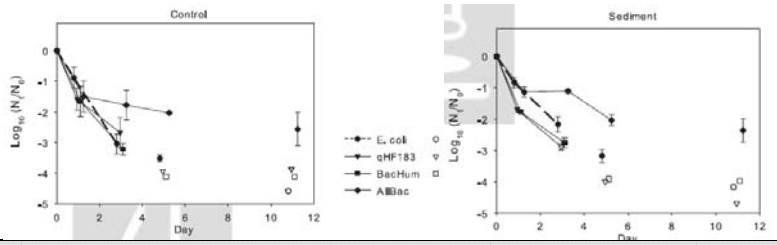
Received 29 October 2009/Accepted 20 March 2010

Results from Task

- Create microcosms under five conditions
- Sample at 4 time steps, to 11 days
- Evaluate *E. coli* and MST markers
- Consistency of relations
  - Human-associated marker decayed slightly more rapidly than did E. coli
    - If human-associated marker is still detected, then human-source E. coli of the same age are still present.
  - AllBac general marker decayed more slowly than did E. coli
  - Relative decay rates of human-associated marker and E. coli remained the same with different applied stressors

Summary of results

- Microcosm study (t_{99} values, days)



Target	Control (25˚C)	Light (25˚C)	Sediment (25˚C)	Reduced Temperature (15˚C)	No Competition (25˚C)
	Mean T99 (standard deviation) (significance p<0.05)				
E. coli	2.05 (0.31) (a)	2.28 (0.13) (b)	2.75 (0.37) (b)	3.03 (1.03) (a)	6.17 (0.69) (b)
qHF183	1.86 (0.81) (a)	1.63 (0.29) (a)	1.34 (0.13) (a)	2.60 (0.59) (a)	3.16 (0.25) (a)
BacHum	1.30 (0.47) (a)	1.44 (0.19) (a)	1.60 (0.13) (a)	2.36 (0.53) (a)	3.45 (0.12) (a)
AllBac	2.49 (1.76) (a)	2.37 (0.08) (b)	4.34 (0.49) (c)	2.97 (1.05) (a)	2.54 (0.41) (a)

15

SEMI-QUANTITATIVE INTERPRETATION OF DATA

16

Why quantify?

- Known failure of sensitivity, specificity, and evenness of marker distribution across host populations, geography, and perhaps time.
  - Stoeckel and Harwood review of markers from 2007

TABLE 2. Performance statistics for tests in which MST methods were tested with reference samples to determine the ability or failure to detect the sole source of fecal contamination

Test[a]	Target	Host category	Sample type	Sensitivity (*n*)[b]	Specificity (*n*)[c]	Reference(s)
Isolate-by-isolate classification						
ARA	*E. coli*	Human	Blind samples	1.00 (7)	0.80 (5)	41
MAR, CUP, ribotyping, PFGE, rep-PCR also included in table						
Marker detection						
Bacteroides thetaiotaomicron PCR	B.thetaF/B.thetaR	Human	Individual feces	0.92 (25)	0.98 (241)	11
Bacteroides thetaiotaomicron PCR	B.thetaF/B.thetaR	Human	Wastewater	1.00 (20)	NR (NR)[d]	11
Bacteroides thetaiotaomicron PCR	Primers, two internal probes described	Human	Individual feces	0.78 (9)	0.76 (71)	57
Bacteroidales PCR (two trials)	HF183F, HF134F/Bac708R	Human	Blind samples	0.70, 1.00 (10, 14)	1.00, 1.00 (6, 7)	26
Bacteroidales PCR	HF183F/Bac708R	Human	Individual feces	0.20–0.85 (7–25)	0.85–1.00 (46–73)	6
Bacteroidales PCR	HF183F/Bac708R	Human	Wastewater	1.00 (41)	1.00 (75)	6, 9, 11, 91
Bacteroidales qPCR	HF183F/reverse primer described	Human	Individual feces	0.86 (7)	1.00 (19)	91
Bacteroidales qPCR	HF183F/reverse primer described	Human	Wastewater	1.00 (4)	NR (NR)	91
Bacteroidales PCR (two trials)	CF128F, CF193F/Bac708R	Ruminants and	Blind samples	1.00 (7, 9)	0.89, 0.92 (9, 12)	26

Various other markers also included in table

Markers can be detected when water quality is "acceptable"

- McLellan (Bower et al. 2005 AEM 71:8305)
  - detection of HF183 in diluted sewage by PCR
  - **0.2 to 82 CFU/100 mL *E. coli*** (n=14)
- This research (unpublished data)
  - Detection of qHF183 in sewage by qPCR
  - Detection limit 4 copies/5 uL reaction
  - **78 to 4,800 CFU/100 mL *E. coli*** (geometric mean 480, n=30)

qPCR is complicated, why not use presence/absence distributions?

- Probabilistic distribution of qPCR detections
- For a generic Human-associated marker
  - Marker has 100% sensitivity
  - Marker specificity is incomplete; present in 8% of Dogs (92% specificity)
- The marker is detected in a watershed
- The probability that the marker detection truly represents human source contamination is a function of
  - the contributing populations of dogs and humans
  - the prevalence of marker in those populations
  - **If we know the contributing population sizes, we don't need to do microbial source tracking**

19

Variability in fecal material – be conservative

$$E.coli_{feces} / \text{Marker}_{feces} \approx E.coli_{water} / \text{Marker}_{water}$$

- Rearrange the equation to solve for *E. coli*$_{\text{water}}$ indicated by Marker$_{\text{water}}$

$$E.coli_{water} \approx \text{Marker}_{water} * E.coli_{feces} / \text{Marker}_{feces}$$

- Calculate the 10$^{\text{th}}$ and 90$^{\text{th}}$ confidence intervals in fecal material
- Substitute to maximize the quotient

$$E.coli_{water} \leq \text{Marker}_{water} * E.coli_{feces,0.9} / \text{Marker}_{feces,0.1}$$

20

VALIDATION

Laboratory Evaluation -- USGS

- Prepared samples were analyzed "blind"
- High degree of accuracy in presence/absence
  - BoBac was detected in sample 1 because it is carried at low concentration in cat fecal material

		QC Blind 1	QC Blind 2	QC Blind 3	QC Blind 4
Source		Cat and human	Cattle	Horse	Human
Observed	***E. coli***	***>24,000***	***24,000***	***830***	***930***
Marker detected	qHF183	**Detected**	Not detected	Not detected	**Detected**
	BacHum	**Detected**	Not detected	Not detected	**Detected**
	BoBac	**Detected**	**Detected**	Not detected	Not detected

Detailed semi-quantitative data

		QC Blind 1	QC Blind 2	QC Blind 3	QC Blind 4
Observed	***E. coli***	***>24,000***	***24,000***	***830***	***930***
Estimated added to test mixture	Human	810	0	0	500
	Ruminants	0	42,000	0	0
	Pets	620,000	0	0	0
	Other	0	0	710	0
Calculated upper limit	Human	**62,000**	ND	ND	7,900
	Ruminant	**67,000**	**350,000**	ND	ND
	Pets	*1,300,000*	*200,000*	*4,700*	*3,500*

Data from Stoeckel, Stelzer, Mau, and Stogner *Water Research* 2011

ND, marker not detected, value presented is the threshold above which marker should have been detected

Pets values in *italics* because no pet-associated markers were tested. The value is based on pets carrying human- and ruminant-associated markers

$$E.coli_{water,source} \leq \text{Marker}_{water,adj} * E.coli_{feces,0.9} \Big/ \text{Marker}_{feces,adj,0.1}$$

23

Small-Scale Field Validation

- Preliminary data from contaminated river
- Reach includes two rural communities with limited waste treatment before discharge

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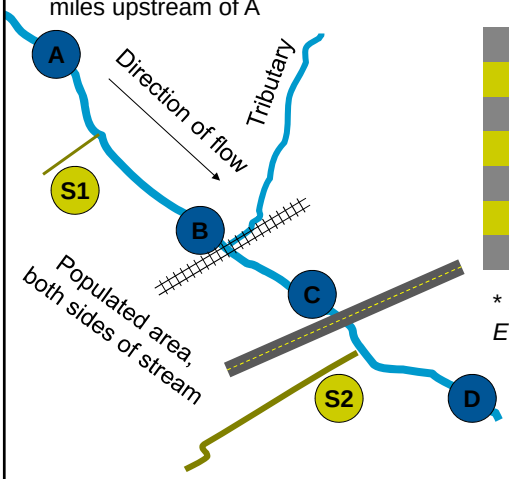
Study area

Unpopulated for several miles upstream of A



Site	*E. coli* density* (MPN/100 mL) Pass 1	Pass 2	Geomean
A	37	5	14
S1	11,000		
B	260	34	95
Trib	170		
C	240	100	160
S2	>240,000		
D	1,200	740	950

* single-sample criterion used locally is 240 *E. coli*/100 mL

25

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Source 1 (S1)

Light flow from an 8-inch pipe, outfall of a permitted package plant

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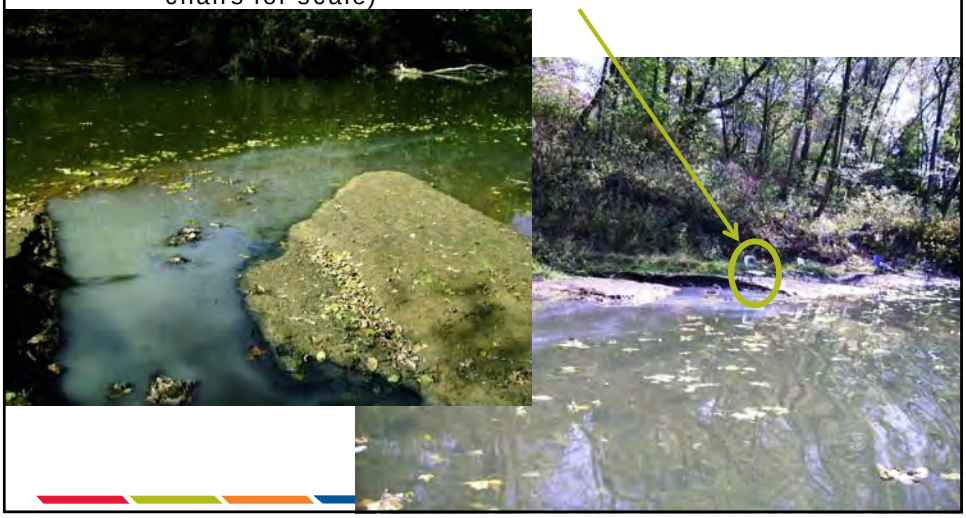
Source 2 (S2)

Unpermitted discharge, steady flow, 3- to 4-feet broad and about 1-foot deep (note chairs for scale)



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Small-Scale Evaluation

		Site A	*Source 1*	Site B	Site C	*Source 2*	Site D
Measured	***E. coli***	**37**	***11,000***	**260**	**240**	***240,000***	**1,200**
Potential (pass 1)	Potential Human*	370	*5,100*	110	700	*3,600,000*	4,900
Measured	***E. coli***	**5**		**34**	**100**		**740**
Potential (pass 2)	Potential Human	120		170	290		8,300

*note: Alternate sources, including ruminants, pets, and birds, could not be excluded as potential sources because no host-associated markers were measured for these sources

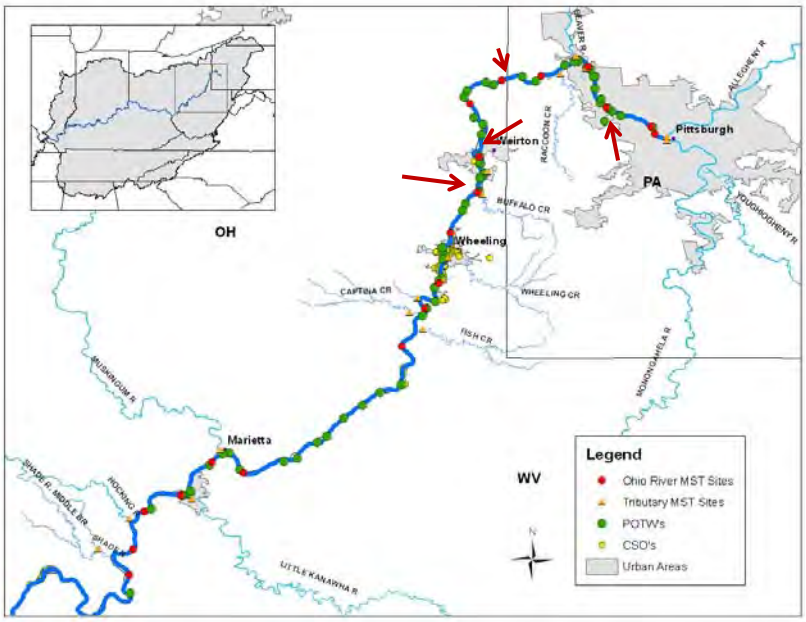
28

Large-scale Field Evaluation

Ohio River Survey		Sample set – Human source *E. coli*			
Sample	**Location**	**Exp(Bayes)**	**Exp(mean)**	**Exp(pctiles)**	**Measured**
Ohio RM457 RDB	**Ohio side, upstream**	57	25	67	**15**
Ohio RM457 LDB	**KY side, upstream**	31	13	36	**5**
Ohio RM459 RDB	**Ohio side, up Miami**	86	38	105	**29**
Miami mouth	**Mouth, Miami (CSO)**	12,445	5,641	16,311	**2,809**
Ohio RM464 RDB	**Ohio side, down Miami**	1,057	476	1,316	**178**
Ohio RM470 RDB	**Ohio side, at City**	703	319	924	**173**
Ohio RM470 LDB	**KY side, at City**	136	61	177	**12**

29

Area Map



30

Specific areas

- Especially large increases in human-associated marker, indicating inputs of human fecal contamination, were apparent at four locations
  - LDB 3.3 just downstream from the ALCOSAN POTW. *E. coli* increased from 1,600 to 4,600 MPN/100 mL.
  - RDB 22.9 just downstream from Elkhorn Run and Moon Run POTW and Conway POTW. *E. coli* increased from 930 to 5,500 MPN/100 mL.
  - ***LDB 66.4 downstream from Weirton POTW, across the river from Steubenville. E. coli increased from 270 to 2,600 MPN/100 mL.***
  - RDB 74.9 downstream from Wellsburg POTW and off the shoreline of Brilliant, Ohio. *E. coli* increased from 570 to 630 MPN/100 mL.

31

Comments on Study Design

- Know the source identifier
  - Sensitivity and specificity
- Challenge the assumptions
  - Relation between marker and cultivated fecal indicator
  - Relative persistence in the environment
- Ensure quality of data
  - Internal controls
  - Good laboratory practice and procedures
- Validate interpretations
  - Laboratory and field controlled tests
  - Use of external information -- serendipity

Persistence of Fecal Indicator Bacteria in the Environment: from Indicators to Pathogens and Metagenomes

Michael J. Sadowsky

University of Minnesota
Department of Soil, Water and Climate; and
BioTechnology Institute

Fecal Bacteria

- Represent the Most Often Exceeded Water Quality Standard

Environmental Cleansing of Fecal Bacteria

- Occurs easily if the fecal load is small (privies and small farm systems).
- Does not occur well at all if loads are large (big spills).
- Die off of fecal bacteria (due to U.V. light and nutrient starvation) does occur.

Fecal Bacteria are Clever

Given enough numbers and selection pressure (death), alternate hosts and reservoirs become a strategy for bacterial survival.

Hope for the Present and Future

- Molecular technologies have the necessary sensitivity and accuracy to differentiate among ecotypically-distinct bacteria.
- Microbial Source Tracking (MST) – a new? science is born. Others will talk about this.

MST Methods can be used to assess who is there, and how long it persists

Methods Currently Being Evaluated to Determine Diversity and Sources of Fecal Bacteria

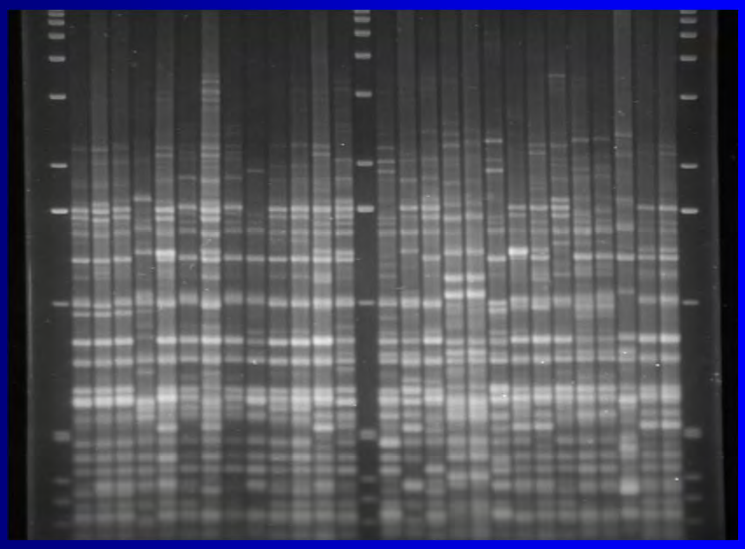
- Genotypic Molecular Methods
  - Ribotyping
  - AFLP
  - RFLP
  - 16S rDNA
  - **rep-PCR**
  - UidA gene sequencing
  - Species-specific PCR
  - Pulsed-field gel electrophoresis
  - Species-specific hybridization markers
- Phenotypic Methods
  - Antibiotic resistance
  - Carbohydrate utilization
  - Phage typing
  - Biolog analyses – N and C

Can DNA Fingerprinting and Other Methods be Used to Identify Diversity and Ecology of of Fecal Contamination in Watersheds?

rep-PCR DNA Fingerprinting

- Exploits naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in all bacterial genomes,
- Allows snapshot of genome without sequencing.
- Many families of repetitive sequences:
  - REP
  - ERIC
  - BOX: BOXA1R primer used our studies
  - Many others

rep-PCR Gel Image



These and New Tools Allow us to Probe the Environment for New Sources and Sinks of Fecal Bacteria and underatnd their Ecology in Watersheds

There are many sources of *E. coli* and pathogens in the environment!

Despite what you learned in microbiology class:

E. coli is not limited to the intestinal tract of warm blooded animals!

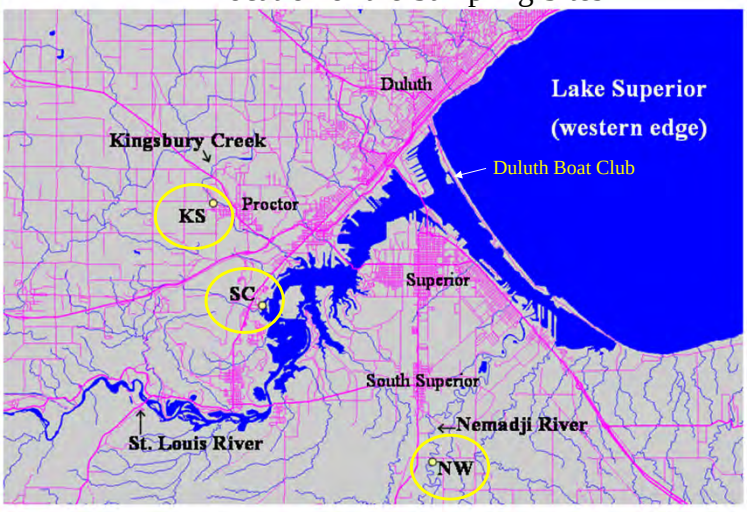
Temperate Soils as a Source of *E. coli*

Collaborative studies with Winfried Ksoll & Randy Hicks (UMD) and Richard Whitman & Murulee Byappanahali (USGS)

Stems from Initial Studies by Fujioka and others that tropical soils in Hawaii and Guam are sources of *E. coli*.

Case study – Temperate Soils as an Alternate Source of *E. coli* Waterways

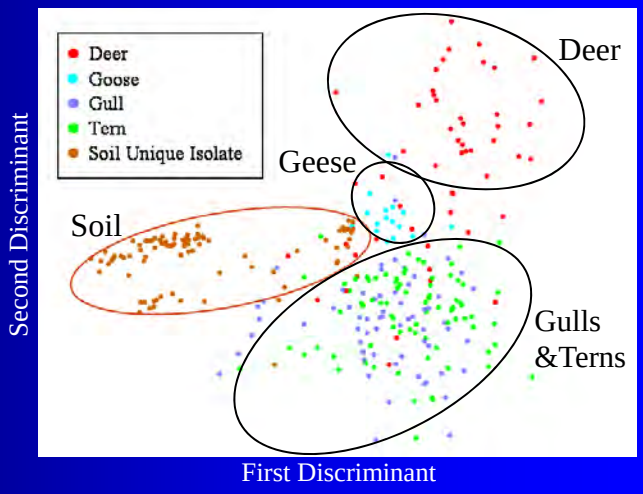
Location of the Sampling Sites



MANOVA

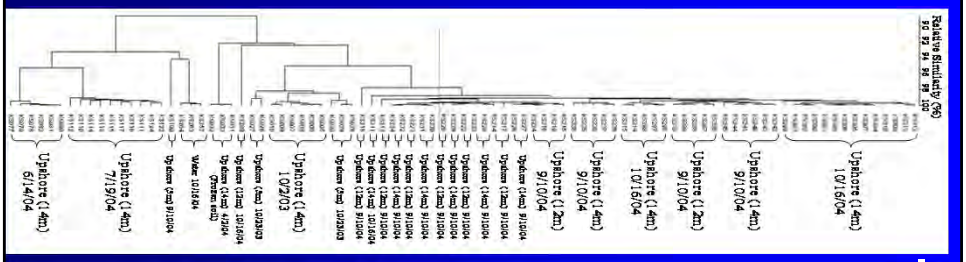
E. coli isolated from soil were relatively unique compared with *E. coli* from other animal hosts

19% make their way to water and beaches



The first and second discriminants account for 80% of the variation.

HFERP Analysis of Soil *E. coli*



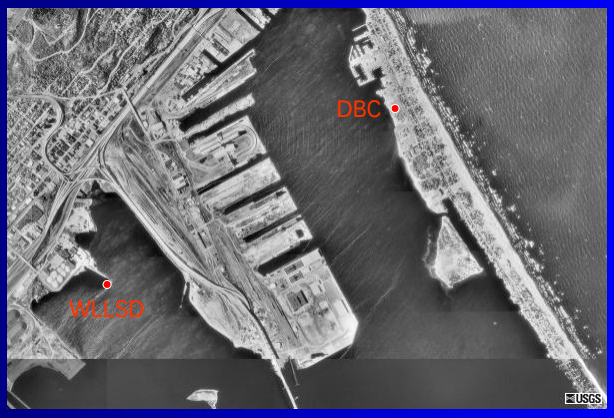
Genetically Identical Soil *E. coli*

We refer to these as Naturalized *E. coli*

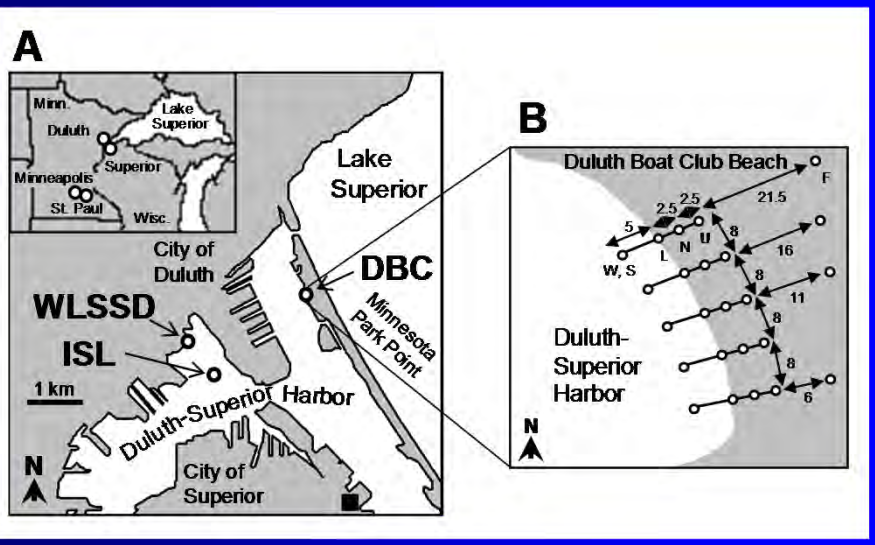
Sand and Sediment as Sources of *E. coli*

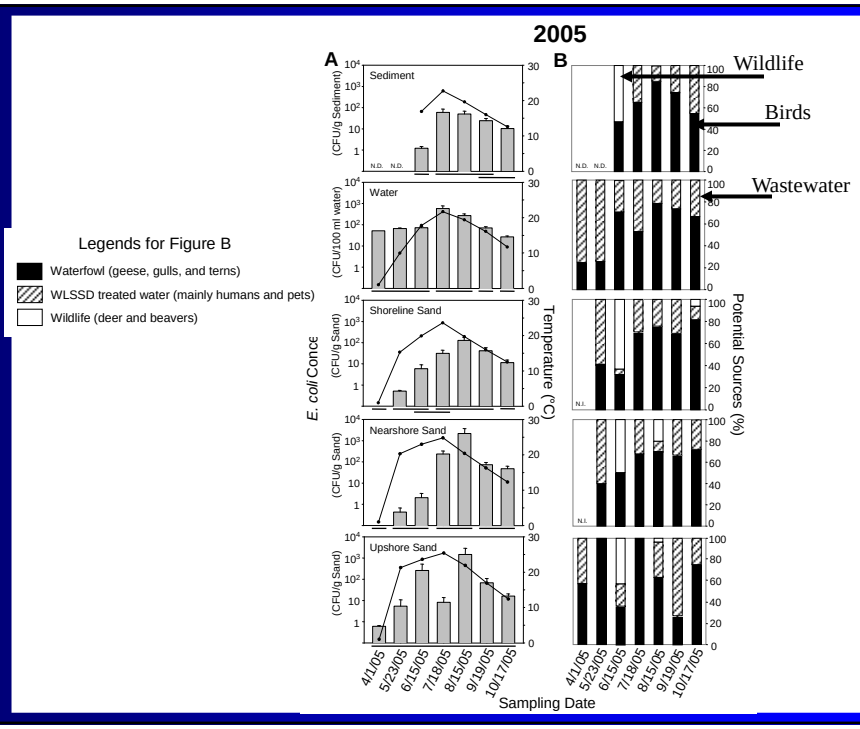
Collaborative studies with Winfried Ksoll & Randy Hicks (UMD)

Duluth Harbor- Western Lake Superior Sanitary District and Duluth Boat Club

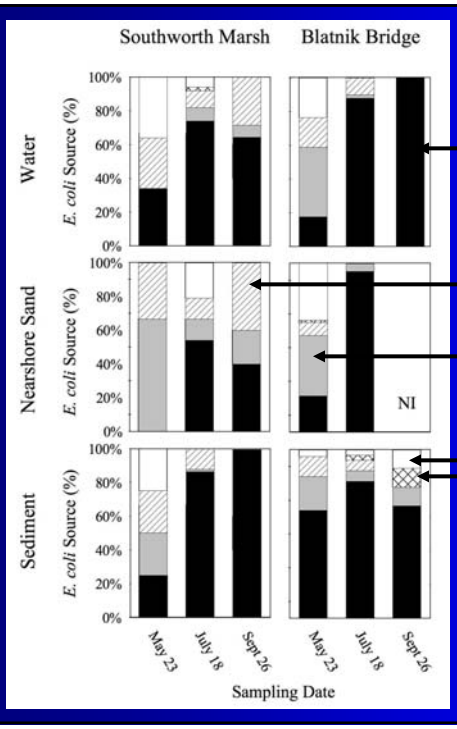


Study Site


A
Minn.
Duluth
Lake Superior
Superior
Minneapolis
St. Paul
Wisc.
Lake Superior
City of Duluth
DBC
WLSSD
ISL
1 km
Minnesota Park Point
Duluth-Superior Harbor
City of Superior
N
B
Duluth Boat Club Beach
F
21.5
2.5
2.5
5
N
U
L
W, S
8
16
8
11
8
8
6
Duluth-Superior Harbor
N


2005
A
B
Sediment
Water
Shoreline Sand
Nearshore Sand
Upshore Sand
(CFU/g Sediment)
(CFU/100 ml water)
(CFU/g Sand)
E. coli Conce
Temperature (°C)
Potential Sources (%)
Wildlife
Birds
Wastewater
N.D.
N.I.
4/1/05
5/23/05
6/15/05
7/18/05
8/15/05
9/19/05
10/17/05
Sampling Date
Legends for Figure B
Waterfowl (geese, gulls, and terns)
WLSSD treated water (mainly humans and pets)
Wildlife (deer and beavers)

So, where are these bacteria coming from?



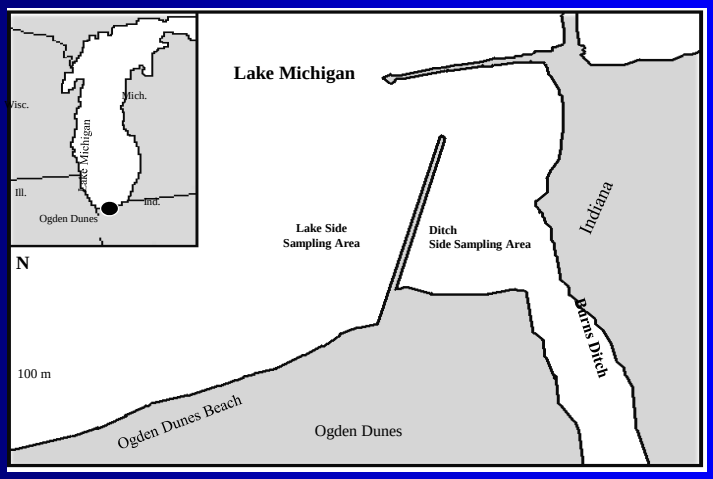
Alternate Sources of *E. coli* in the Great Lakes and Oceans

Cladophora (Algae) as a Source of *E. coli* and Pathogens

Collaborative studies with Richard Whitman and Murulee Byappanahali (USGS)

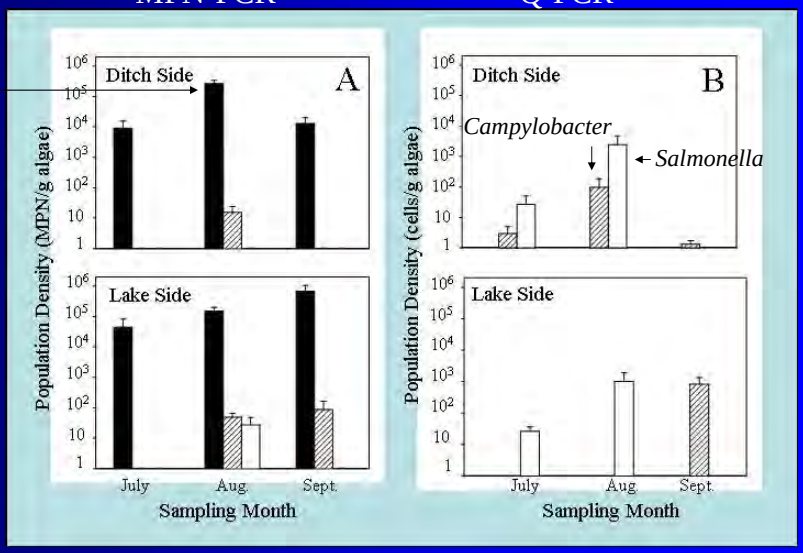
Byappanahalli *et. al.*, 2003

Sampling Sites in Lake Michigan



MPN-PCR Q-PCR

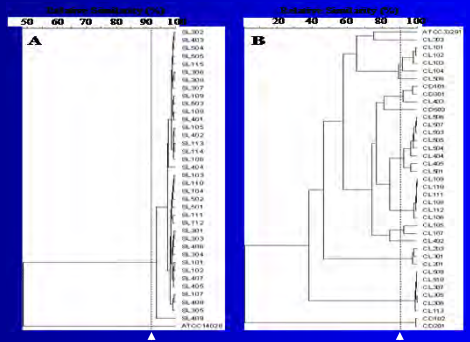
E. coli



Seasonal shifts in the population densities of *E. coli* and potential pathogens in lake- and ditchside *Cladophora* samples.

Salmonella

Monogenotypic



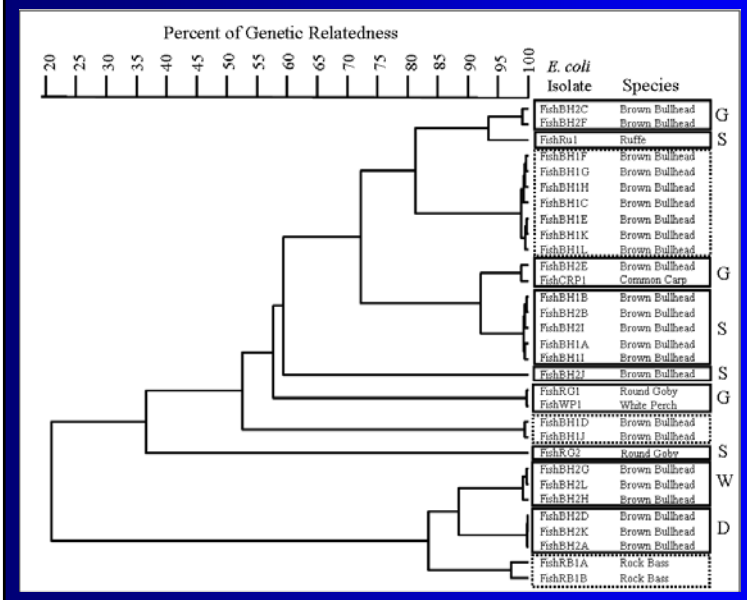
Campylobacter

Pathogens Associate with *Cladophora*!

Are There Other Sources of Environmental *E. coli* That We do Not Know About?

Do cold blooded animals like fish harbor *E. coli*?

Fish as Sources of *E. coli*



Key

D = Ducks

S = Sediment

G = Geese

W = Wastewater

Growth, Survival, and Genetic Structure of *E. coli* Populations at the Seven Mile Creek Watershed

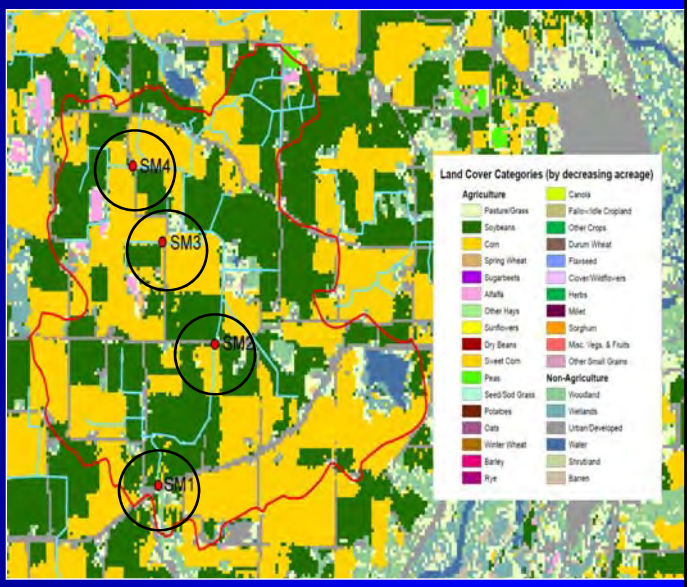
Fecal Bacteria Persist in the Environment

Seven Mile Creek(SMC) Watershed

Study Sites: SM1-SM4

Sampling period:

2008: July-October
2009: April-October
2010: April- October



➢ Analysis of Dendrograms:

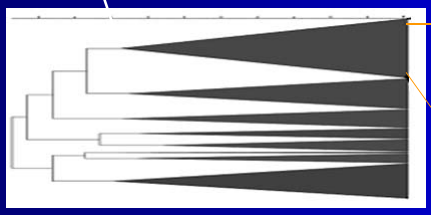
- Similarity ranged from 1.98 to 100% and the Shannon diversity index was calculated as 5.45 suggesting that the *E. coli* population in SMC was quite diverse.

- A total of 606 different strains were detected .

  - 356 strains were represented by a single isolate suggesting that many of the *E. coli* present in SMC water and sediment may occur intermittently as a result of new inputs.

  - The remaining 250 strains were represented by isolates between 2 and 112. Some of these strains were found in samples from all the three years and across different sampling sites and types suggesting that they may be growing in the water and sediments.

93.8%



Sediment:
SM1 –Jul 08 (3), Aug 09 (1)
SM 2 –Sep 08 (5), Jul 08 (17), Jul 09 (9), Aug 09 (5)
SM 3 –Jul 08 (3)
SM 4 - Jul 08 (2), Jul 10 (22), Aug 10 (6)

Water:
SM 2 - Aug 08 (2), Jun 09 (2), Jul 09 (3), Jul 10 (1)
SM 3 - W-Aug 08 (4),
SM 4 – W-Jul 10 (19), Aug 10 (1), Sep 10 (7)

Enlarged image of a cluster containing 112 clonal isolates

Fundamentally Two Different Types of MST Approaches

a. Library – Dependent
b. Library – Independent

Limitations to Library-Independent Approaches

1. Limited number of Host Source-Specific PCR Primers and Cross Reactions

2. Inherent problems with qPCR

3. Inhibitors

Plate Count and qPCR Data Severely Limits What you can See

Can Metagenomics Save the Day?

Collaborations with Prof. Hur and Tatsuya Unno

What is a Metagenomics?

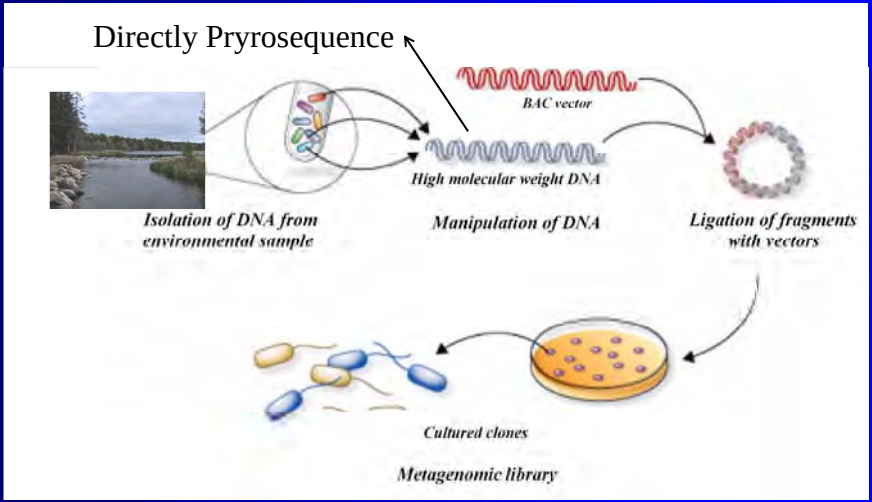
- The study of the totality of genetic material (genomes or their fragments) recovered directly from environmental samples.

- Many types of Metagenomic Analyses
  a. Diversity (16S rDNA)
  b. Microbial Community Analyses
  c. Functional Gene Discovery Analyses

Why use Metagenomic Analyses

•The majority of microorganisms in environmental and animal samples (estimated to be less than 1%) remain uncultured or non-culturable.

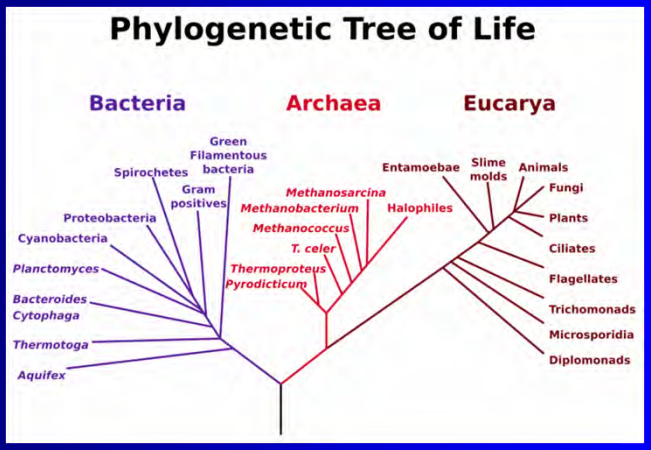
General Metagenomic Approaches



Pyro - DNA Sequencing

- High Throughput, Large Scale, and Inexpensive DNA Sequencing Technology
- Initially used 454 Platform – produced 700,000 - 500 bp reads per run
- Now using Illumina platform – produces 140,000,000 – 100 bp reads.

Microbes and The Tree of Life



Our studies are only targeting 1 domain of life – the bacteria

Methods

Sample preparation

- Fecal DNA
  - Human and livestock animals (cows, pigs, chickens, and ducks)
  - Pooled by each source (30 feces per animal species)
- Freshwater DNA
  - Surface water - 500 ml to 4L
  - DNA extraction
  - MoBio DNA extraction kit
  - Barcoding

Illumina Pyrosequencing

Sample → DNA Extraction → 16SrDNA amplification

→ Sequencing each PCR product

Produces 100s of millions of DNA sequences

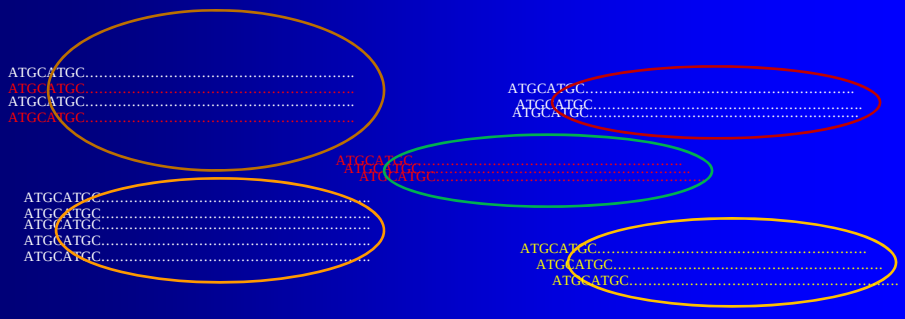
Overall Goals

Match DNA Sequences in Data Sets created from feces of known animals to those recovered in rivers samples.

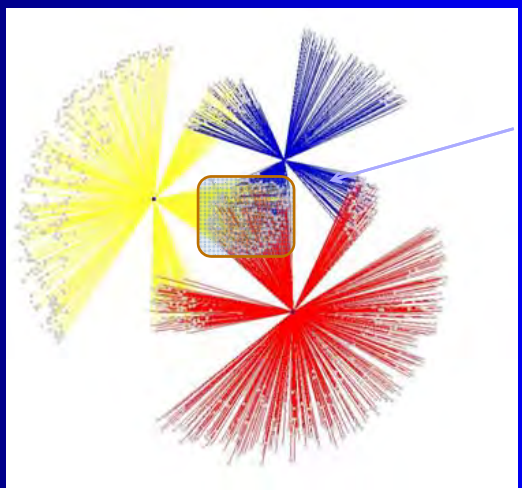
1. Shared OTUs – Taxonomy Independent
2. Shared Taxonomic Units - Genera

What are Shared OTUs?

OTUs containing fecal and environmental DNA
Independent of Taxonomy



Network image of shared OTUs



OTU based network analysis of
sample A, sample B, AND sample C

New MST method with Next Generation Sequencing technique

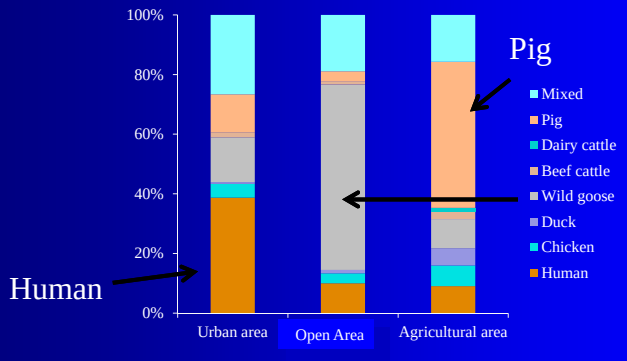
Environ. Sci. Technol. **2010**, *44*, 7777–7782

Use of Barcoded Pyrosequencing and Shared OTUs To Determine Sources of Fecal Bacteria in Watersheds

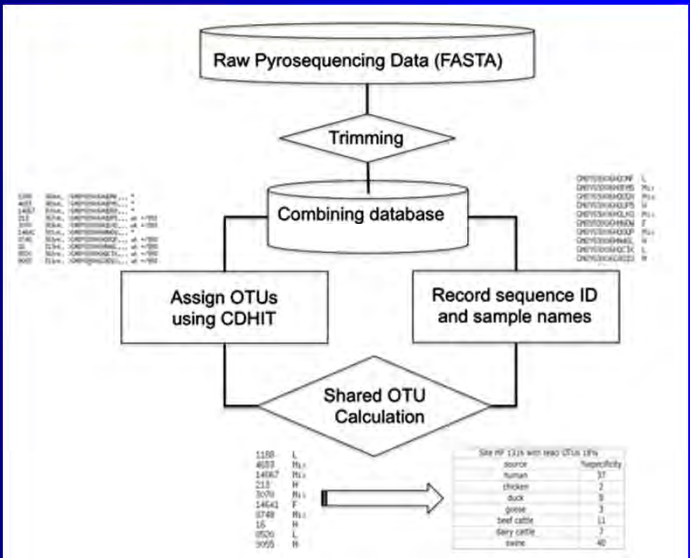
TATSUYA UNNO,[†] JEONGHWAN JANG,[†] DUKKI HAN,[†] JOON HA KIM,[†] MICHAEL J. SADOWSKY,[§] OK-SUN KIM,[∥] JONGSIK CHUN,[∥] AND HOR-GIL HUR*[,†,‡]

Department of Environmental Science and Engineering and International Environmental Research Center, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea, Department of Soil, Water, and Climate and BioTechnology Institute, University of Minnesota, St. Paul, Minnesota 55108, and School of Biological Sciences, Seoul National University, NS70, 56-1 Daehak-dong, Kwanak-gu, Seoul 151-742, Republic of Korea

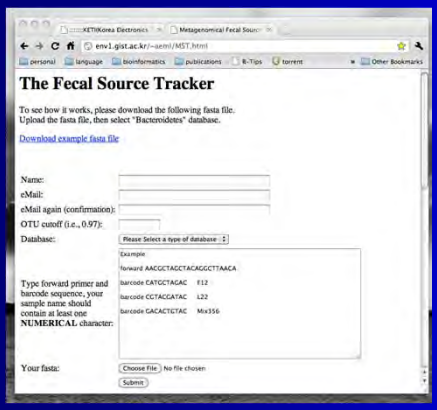
Result: Number of shared OTUs



Everything at once



Development of automated MST system



Applying batch program to automate pyrosequencing analysis

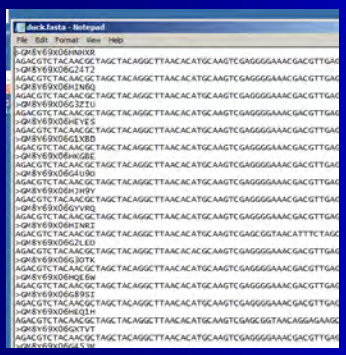
http://env1.gist.ac.kr/~aeml/MST.html

Integrated Online System for a Pyrosequencing-Based Microbial Source Tracking Method that Targets Bacteroidetes 16S rDNA.

Tatsuya Unno, Doris Di, Jang Jeonghwan, Yaeseul Suh, Michael Sadowsky and Hor-Gil Hur . Environ Sci Technol **DOI:** 10.1021/es201380c

Illumina Pyrosequencing

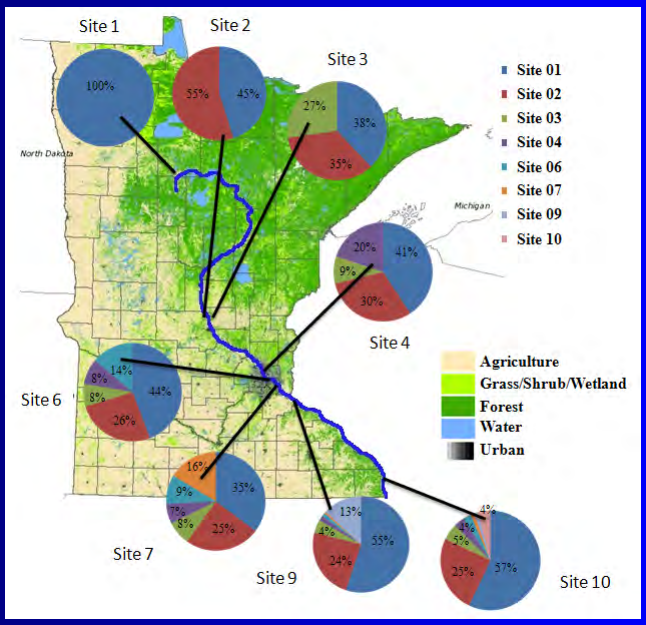
Pyrosequencing results



Software names	Purposes
Mothur	Sequence trimming Alignment Species richness analysis Cluster analysis OTU assignment Taxonomic classification

Next Generation Fecal Taxon Libraries - FTL

- Contains all the taxonomic units and OTUs in pooled fecal samples from known animal sources.
- Gives information about all potential pathogens and commensals in the fecal and environmental sample.

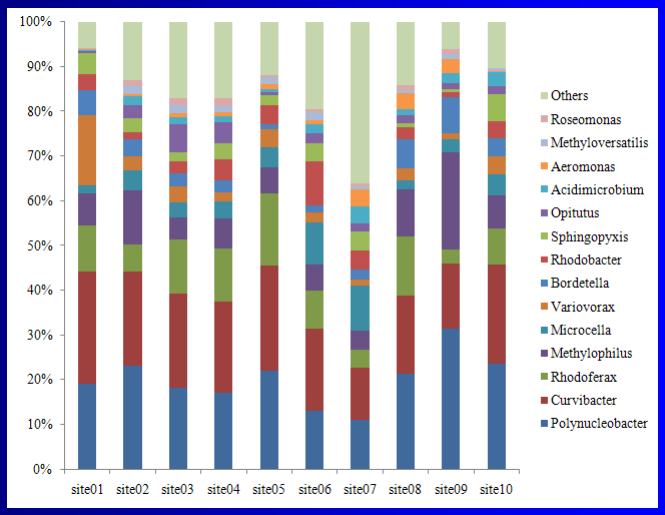


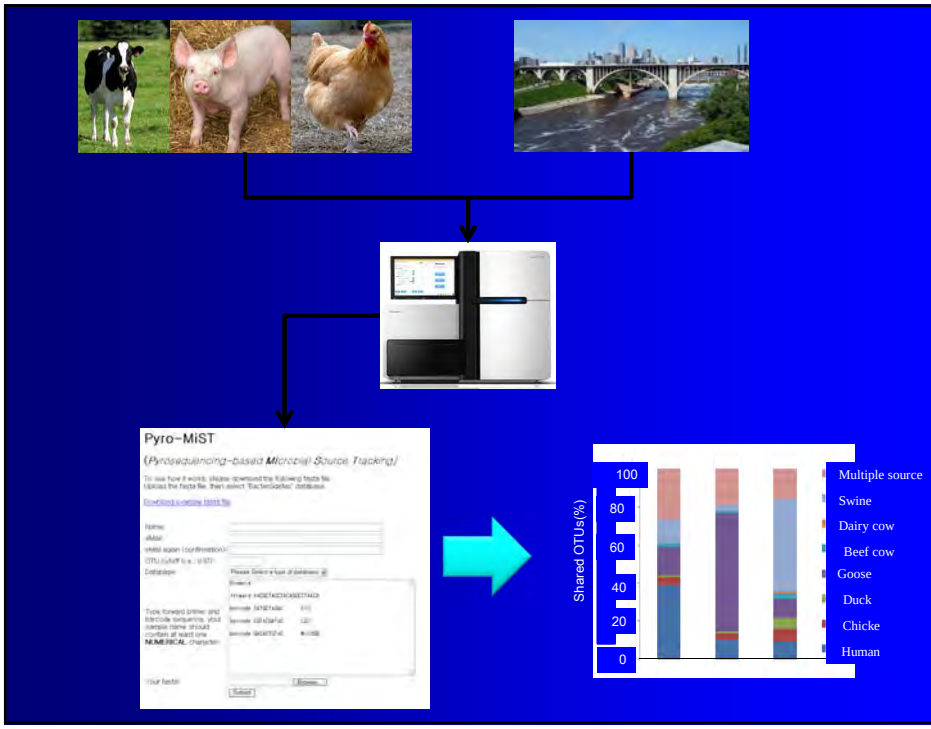
Pyrosequencing Runs

Allows Analysis of about 200 samples per Illumina Run

About $25 per sample for Complete Taxonomic Analysis

Genera of Classified Bacteria Recovered at Each Site





Visit our Website

- WWW.Ecolirep.umn.edu
- Project overview
- Methods
- Links

If your interested …

Michael J. Sadowsky, University of Minnesota
Richard L. Whitman, U.S. Geological Survey

ASM Press, 2010

About the Book

The Fecal Bacteria offers a balanced, integrated discussion of fecal bacteria and their presence and ecology in the intestinal tract of mammals, in the environment, and in the food supply. This new volume covers their use in examining and assessing water quality in order to offer protection from illnesses related to swimming in or ingesting contaminated water, in addition to discussing their use in engineering considerations of water quality, modeling, monitoring, and regulations. Fecal bacteria are additionally used as indicators of contamination of ready-to-eat foods and fresh produce. The intestinal environment, the microbial community structure of the gut microbiota, and the physiology and genomics of this broad group of microorganisms are explored in the book.

Acknowledgements

- Project Collaborators and Cooperators
  - Matt Hamilton, Tatsuya Unno, Hor-Gil Hur, LeeAnn Johnson, John Ferguson, Satoshi Ishii, Brian Badgley, many many others– UMTC
  - Randy Hicks, Wendy Hieb, and Dennis Hansen, Matt Kading -UMD
  - Minnesota Pollution Control Agency
  - Minnesota Department of Agriculture
  - Metropolitan Council Environmental Services
  - Western Lake Superior Sanitary District
  - Trappers, Hunters, and Public
- Funding
  - Legislative Commission on Minnesota Resources
  - Metropolitan Council Environmental Services
  - Sea Grant
  - US- EPA
  - MN Ag
  - USGS

Thank you for Inviting me and for the Opportunity to Speak with you.

Thanks for your attention!

The Thick and Thin of Poultry Fecal Identification

Valerie J. Harwood, Ph.D.

Department of Integrative Biology, University of South Florida

2012 Bacterial Source Tracking State of the Science Conference, New Braunfels, TX. Feb 28-29

Poultry Production in U.S.: A Steady Increase Over the Past Decade.

1990 - 2010 (USDA figures)

- **Broilers up 47% to 8.6 billion birds in 2010**
- **Highest producers are AL, AR, GA, MS, NC**
- **Texas was ranked 6th for broiler production in 2010 (3.6 billion pounds)**
- **In TX in 2008, meat and eggs valued at 2.1 billion**
- **Broilers and turkeys produced on 800 contract farms**

TEXAS POULTRY FEDERATION AND AFFILIATES

What's In That Stuff? (Poultry Feces)

- *E. coli* (~1,200 CFU/g poultry litter)
- Enterococci (~51,000/g poultry litter)
- *Campylobacter jejuni, C. coli*
- *Salmonella enterica*
- Pathogenic *E. coli* strains like 0157:H7



And There's a Lot of It!

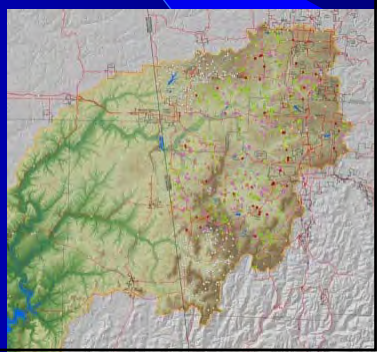
- Up to 0.5 lbs soiled litter per pound of meat produced
- = 340 tons annually from a farm with 4 houses

What Do We Do With It?

- For the most part, it is "land-applied."
- ~1.6 billion kg/year in U.S.
- Phosphate, nitrogen, heavy metals spread along with bacteria

The Lawsuit

- 2005: Oklahoma Attorney General Drew Edmonson sued 13 poultry integrators including Tyson in federal court for degrading water quality in the Illinois River Watershed by land application of poultry litter.



The Dilemma: How to Specifically Detect Poultry Litter Contamination: QPCR for *Brevibacterium* LA35

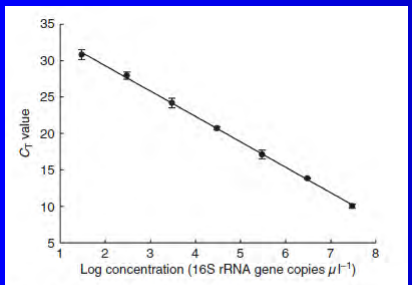


Figure 1 A representative standard curve of C_T values vs. poultry litter marker gene concentration in plasmid pLA35. The mean and standard deviation of triplicate samples is graphed vs. LA35 16S rRNA gene copies per microlitre.

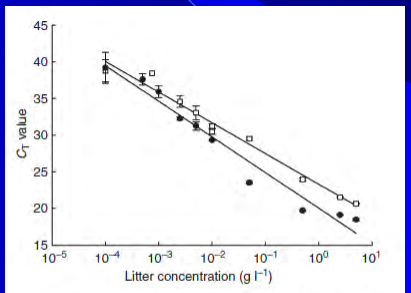


Figure 2 Used poultry litter samples were seeded into water to determine the qPCR detection limit in terms of gram of litter per litre of water. Mean and standard deviation of triplicate measurements at each concentration are shown for litter samples A (□) and B (●).

Sensitivity and Specificity of *Brevibacterium* LA35 Poultry Litter Marker

Weidhaas, Harwood et al 2010. J. Appl. Microbiol.

Table 1 Sensitivity and specificity of the *Brevibacterium* 16S rRNA sequence by qPCR and nested SYBR green PCR against animal and human faecal sources and used and unused poultry litter

Faecal source	Type of sample	Geographical location	Number tested	Number positive
Soiled	Composites (broiler)*	Oklahoma	10†	10
Poultry litter	Composites (broiler)	Georgia	7	7
Clean litter	Duplicates	Oklahoma	3	0
Chicken	Composites of scats‡ (broiler)	Georgia	2	2
	Faecal swabs (layer hens)	Florida	7	3
	Composites of faecal slurry (layer hens)	Florida	5	4
	Composites of ten scats (layer hens)	Utah	4	2
	Individual faecal samples (layer hens)	Minnesota	5	3
Turkey	Faecal swabs	Minnesota	2	1
Beef cow	Composites of ten pats§	Oklahoma	9†	0
	Composites of ten pats§	Arkansas	6†	0
	Composite of ten pats	Colorado	1	0¶
Dairy cow	Faecal slurries§	Missouri	3†	0
	Faecal slurries§	Oklahoma	3†	0
	Individual pats	Florida	10	0¶
	Individual pats	Minnesota	6	0¶
Swine	Faecal slurries§	Arkansas	2†	0
	Composite of faecal slurries	Missouri	1†	0
Duck	Composites of ten scats§	Oklahoma	3†	1
	Composites of ten scats§	Arkansas	2†	0
	Individual scats	Florida	8	0¶
Goose	Composites of ten scats§	Oklahoma	3†	1
	Composites of ten scats§	Arkansas	2†	0§
	Individual scats	Idaho	10	1
	Individual scats	Florida	10	1
Human	Septic system	Florida	1	0
		Colorado	1¶	0
		Oklahoma	3†	0§
	WWTP influent	Florida	5	0
		West Virginia	3¶	1
		Ohio	2¶	0
		Minnesota	5	1
		Oklahoma	1†	0§
		Arkansas	2†	0§
		Colorado	9¶	2
	WWTP effluent	Florida	1	0
		Minnesota	2	0
		Idaho	2	0

Relationship between Fecal Indicator Bacteria (FIB) and LA35 in Poultry Litter

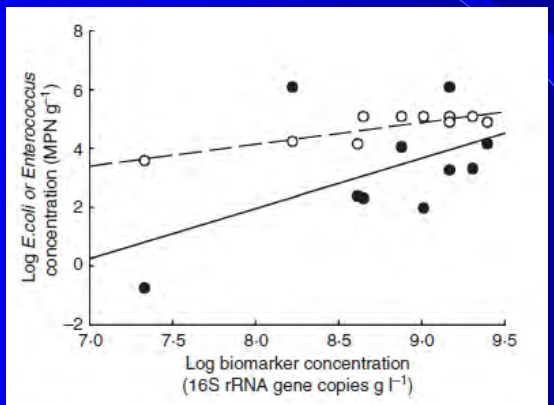


Figure 3 Correlation between *Escherichia coli* (●) or *Enterococcus* spp. (○) and LA35 marker gene concentrations in poultry litter samples. All values are $\log_{10}$ transformed.

Illinois River Watershed LA35 Detections/All Samples

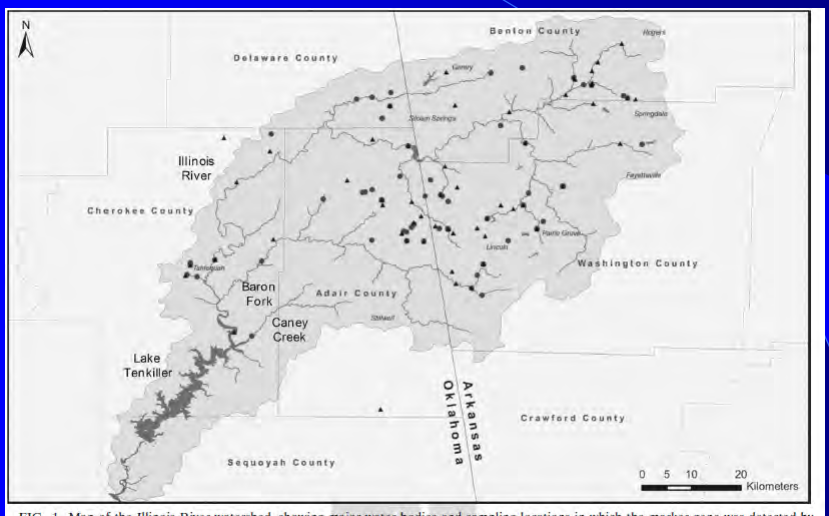


FIG. 1. Map of the Illinois River watershed, showing major water bodies and sampling locations in which the marker gene was detected by qPCR or by nested PCR (●) and those in which it was not detected by either assay (▲).

LA35 Concentrations in Environmental Samples

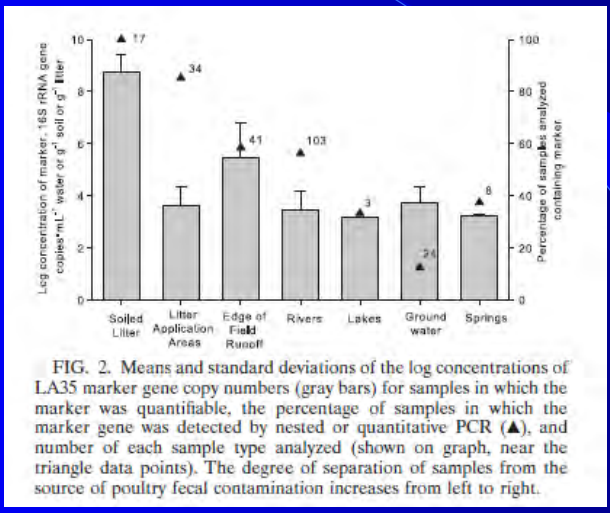
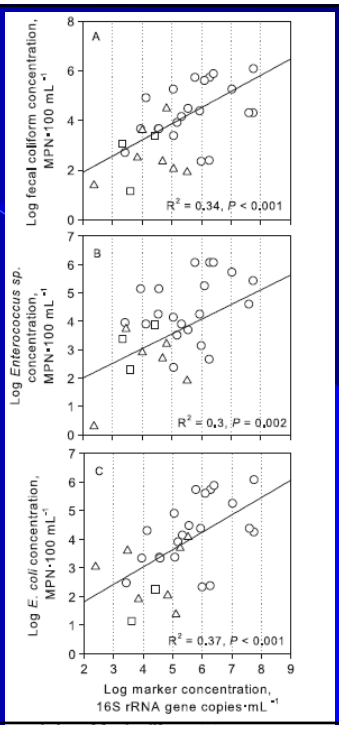


FIG. 2. Means and standard deviations of the log concentrations of LA35 marker gene copy numbers (gray bars) for samples in which the marker was quantifiable, the percentage of samples in which the marker gene was detected by nested or quantitative PCR (▲), and number of each sample type analyzed (shown on graph, near the triangle data points). The degree of separation of samples from the source of poultry fecal contamination increases from left to right.

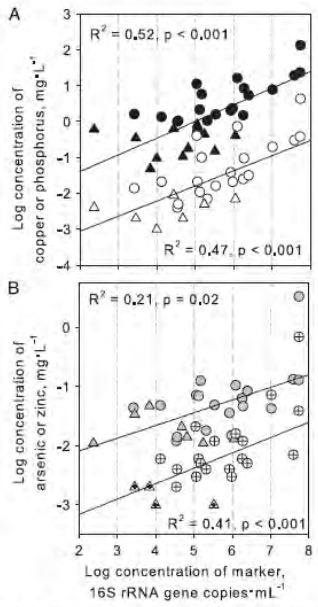
Weidhaas, Harwood et al 2011. Appl Env. Microbiol. 77:2094

Correlation of FIB with LA35 in River▲, Groundwater ■ and Edge-of-Field Runoff● Samples



LA 35 vs.
(A) Copper and Phosphorus and

(B) Arsenic and Zinc



CU

P

Zn

As

FIG. 6. Linear regression analysis showing the correlation of the LA35 marker gene with Cu and P (A) and As and Zn (B) in edge-of-field runoff (circles) and river water (triangles). Symbols: white symbols, Cu; black, P; crossed symbols, As; gray symbols, Zn.

TaqMan qPCR Assay Developed by J. Weidhaas, West VA University

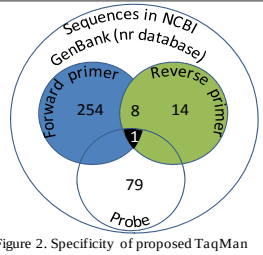


Figure 2. Specificity of proposed TaqMan primers and probe. Numbers indicate organisms with similar sequences to proposed TaqMan primers and/or probe and thus may amplify during PCR. Acceptable sequence homology between the primers and/or probe was defined as 1) less than 2 base pair (bp) mismatch between sequences in the middle of the sequence, or 2) less than 4 bp mismatch in sequence at the ends of the sequences.

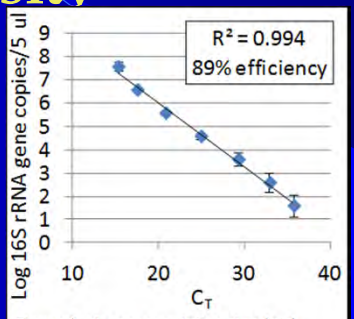


Figure 3. A representative standard curve of CT values vs. poultry litter marker gene concentration in plasmid pLA35. Mean and standard deviation of triplicate samples is shown.

Additional Information

- Poultry production figures: http://www.census.gov/compendia/statab/2012/tables/12s0878.pdf
- **Texas Poultry Federation http://www.texaspoultry.org/default.cfm**

VirginiaTech
Invent the Future

2012 Bacterial Source Tracking: *State of the Science Conference*

New Braunfels, Texas

Overview of Case Studies

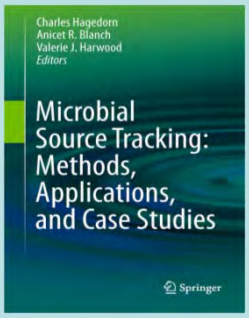
Charles Hagedorn
Professor
Crop and Soil Environmental Sciences
Virginia Tech

Overview of Case Studies

TIME

August 2011. Forget Irene: The Drought in Texas Is the Catastrophe That Could Really Hurt

Overview of Case Studies



Eight of 26 chapters dealt with case studies.

Three chosen as examples,

What can be learned from these?

Ch. 20. Beaches and Coastal Environments

Helena Solo-Gabriele, Ai Boehm, Troy M. Scott, and Chris Sinigalliano

Two Case Studies at Marine Beaches.

Huntington Beach in SoCal, Hobie Cat Beach in FL (Atlantic side).

Both impacted mainly by non-point sources.

Used a variety of biological, chemical, and physical methods for source ID.

Ch. 20. Beaches and Coastal Environments

H. Solo-Gabriele, A. Boehm, T.M. Scott, and C. Sinigalliano

Huntington Beach in SoCal
Antibiotic resistance profiling
Fecal Steroids
Human-specific *Bacteroidales* Marker
Enterococcus species distribution

Process of Elimination: Talbert Marsh, Santa Ana River, Groundwater Discharge, Wastewater Outfall, Animal Sources, Sediments and Sands

***Main Source of Bacterial Contamination Remains Unknown.**
***Some human is present, some dogs, migratory and shore birds are seasonal, no "smoking gun."**
***"The findings have taught stakeholders and researchers that contamination of urban coastal waters is a complex and difficult problem to solve."**

Ch. 20. Beaches and Coastal Environments

H. Solo-Gabriele, A. Boehm, T.M. Scott, and C. Sinigalliano

Hobie Cat Beach in FL
Less complicated than Huntington
On Virginia Key (small island)
Variety of human, animal and gull markers
Enterococcus species distribution
Concurrent measurements of pathogens

Process of Elimination: Shoreline sand,
Source added to shoreline sands, direct and indirect contributions from gulls, dogs, and humans, Wastewater Outfall

***Main Source of Bacterial Contamination Remains Unknown.**
***Dogs are an issue, but cannot explain the consistently elevated counts.**
***Migratory and shore birds are seasonal, no "smoking gun."**
***Prolonged persistence due to re-growth in sands?**

Ch. 19. Case Studies of Urban and Suburban Watersheds

Cheryl W. Propst, Valerie J. Harwood, and Gerold Morrison

Described the Weight-of-Evidence Approach (WOE) that allows MST methods to be highly focused, but used only on an as-needed basis.

WOE involves Categorization of sites by microbial water-quality assessment (MWQA).

A priority ranking, most probable source categories, and recommended management options are developed for each site.

Six sub-basins in Hillsborough River Watershed (FL) were examples for WOA approach.

Ten watersheds in FL used as case studies, one beach near Tampa as an example.

Ch. 19. Case Studies of Urban and Suburban Watersheds

Cheryl W. Propst, Valerie J. Harwood, and Gerold Morrison

Conclusions:

Local knowledge and agency "buy in" are essential for project success.

Some sources are obvious, but many are not - and it takes a lot of field time and sampling (labor intensive) to trace sources to specific points of origin.

One small cross-connection or faulty lift station, or chronic SSOs can impact a large area. High success rate in finding sources.

There are not many situations where changes were made and then subsequent sampling was performed to assess the impact of the changes.

Ch. 18. Agricultural and Rural Watersheds

Andreas K. Farnleitner, George H. Reischer, Hermann Stadler, Denny Kollanur, Regina Sommer, Wolfgang Zerobin, Gunter Bloschl, and George D. Di Giovanni

Two Case Studies: An alpine karst groundwater-spring system in Austria and a surface water system in Texas (Lake Granbury and Buck Creek).

Lets skip the Austrian Karstic Springs Study, not a lot of relevance to TX.

Ch. 18. Agricultural and Rural Watersheds

Andreas K. Farnleitner, George H. Reischer, Hermann Stadler, Denny Kollanur, Regina Sommer, Wolfgang Zerobin, Gunter Bloschl, and George D. Di Giovanni

Lake Granbury, TX.
Highly developed landscape, sanitary surveys indicated human sources would be a major component, noted older housing developments in man-made coves (prior to current septic regulations) as potential problems.

Ch. 18. Agricultural and Rural Watersheds

Andreas K. Farnleitner, George H. Reischer, Hermann Stadler, Denny Kollanur, Regina Sommer, Wolfgang Zerobin, Gunter Bloschl, and George D. Di Giovanni

Lake Granbury, TX.

Methods: Non-library - *Methanobrevibacter*, human polyomavirus, *Bacteriodales* PCR (GenBac); Library - ERIC-PCR and ribotyping.

Results: Data indicated that Lake Granbury was impacted primarily by wildlife and livestock to a lesser degree, with only a minor human allocation.

Explanation:
MST results wrong? Unlikely.
Insufficient sampling over time for a large watershed?
Human pollution staying in man-made coves (little mixing)?
Wildlife/bird populations larger than expected?
Subsurface flow (wildlife) impacting lake?
Fecal bacteria carried by fish in the lake?

Suggestion: Maybe use intensive targeted sampling to ID hot spots before using MST? This could initially mean hundreds of samples.

Ch. 18. Agricultural and Rural Watersheds

Andreas K. Farnleitner, George H. Reischer, Hermann Stadler, Denny Kollanur, Regina Sommer, Wolfgang Zerobin, Gunter Bloschl, and George D. Di Giovanni

Buck Creek, TX (Red River Basin).
Primarily a rural and agricultural landscape, row crops, grasslands, relatively undeveloped, ranches and livestock. Survey indicated wildlife and livestock.

Ch. 18. Agricultural and Rural Watersheds

Andreas K. Farnleitner, George H. Reischer, Hermann Stadler, Denny Kollanur, Regina Sommer, Wolfgang Zerobin, Gunter Bloschl, and George D. Di Giovanni

Buck Creek, TX (Red River Basin).

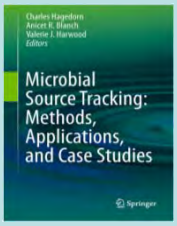
Methods: Non-library - *Methanobrevibacter*, human polyomavirus, *Bacteriodales* PCR (GenBac); Library - ERIC-PCR and ribotyping.

Results: Data indicated that Buck Lake was impacted primarily by wildlife and livestock (as expected), but also had a human allocation (unexpected). Where did this human attribution come from?

Explanation: MST results wrong (especially library)? Unlikely.
Insufficient sampling over time and seasons and storms?
Human markers and isolates carried by some wild species?

Suggestion: Watershed with low ambient levels of fecal pollution, abundant wildlife – tough to pin down human source. Need lots of samples from wildlife that may harbor human isolates/markers.

Overview of Case Studies



Two other chapters are pertinent:

Chapter 16: Minimizing Microbial Source Tracking at All Costs
Peter G. Hartel

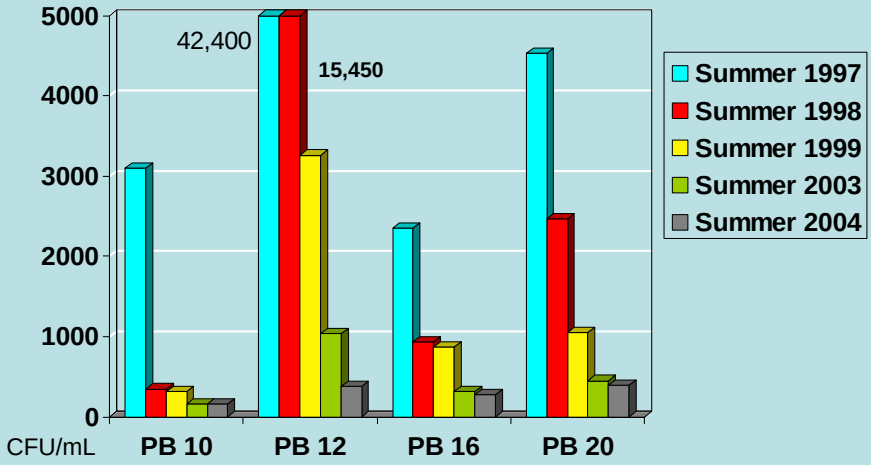
*Emphasized the importance of local knowledge.
*Used MST only when absolutely necessary. Most cases could be solved without MST.
*Trade-off, large numbers of samples needed and very labor intensive.

Chapter 21: Source Tracking in Australia and New Zealand: Case Studies
Warish Ahmed, Marek Kirs, and Brent Gilpin

*Twelve case studies, all expertly done.
*Methods evolved from library-based to library independent over time.
*Like and use the fecal stanols and sterols, and their ratios (same for EU).
*Found regional differences in markers – a warning for the rest of us.

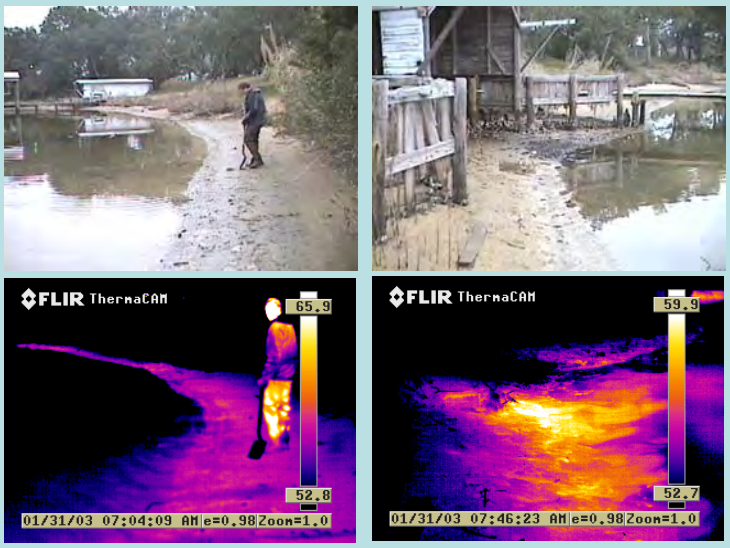
My Own Experiences – An 8 Year Study on a Rural Virginia Watershed

Reductions (%) in Fecal Coliforms at Four Page Brook Sampling Locations
Was MST Even Needed Here?



Finding a “point” source with infrared detection.

(Photographs courtesy of Dr. Joe Lepo, Univ. of West Florida)



Final Thoughts on Case Studies

By now we understand how to do:

Sanitary Surveys, Sampling (Intensive and WOI) Seasonality Events, Watershed Characterization, Prioritize Potential Sources, Develop a Cost-Effective Plan, Select the MST Tools Needed, Implementation of BMPs, Technology Transfer

What's needed?

*Still developing and testing MST tools (current SCCWRP methods study).
*When will this end? Maybe a microarray approach is needed?
*Once MST has been applied, and you have results, many studies end there.
*Too few involve going back out and locating the sources of those results; plus being able to implement BMPs on sources (if found) and then monitor to demonstrate BMP effectiveness (labor intensive, years are involved)!

Anything to add to this list?

UTHealth | School of Public Health
El Paso Regional Campus
The University of Texas Health Science Center at Houston

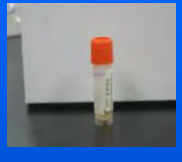
Texas *E. coli* Bacterial Source Tracking Library

Elizabeth Casarez
and George D. Di Giovanni

University of Texas School of Public Health
El Paso Regional Campus,
UT Health Science Center at Houston

Texas *E. coli* BST Library

1) AN ARCHIVE
>25,000 frozen *E. coli* isolates from water and known source samples



2) A DATABASE
>10,000 Genetic fingerprints

3) A TOOL
Current Texas *E. coli* BST Library
1393 isolates from 1232 source samples
Screened, self-validated ERIC-RP prints
Identify sources of fecal contamination
aid TMDL and WPP development for BMPs

Texas *E. coli* BST Library
Why Target *E. coli*?

Is *E. coli* the best target for determining fecal pollution sources?

Maybe not

However

- ✳ Levels of *E. coli* have regulatory significance
- ✳ Established monitoring and standard methods
- ✳ Uncertain relationship between library-independent ST targets and *E. coli* sources

There Are *E. coli* in the Water, But Where Did They Come From?

- BST - laboratory tests to determine if *E. coli* in water samples came from animal or human feces
- Most *E. coli* BST methods are Library Dependent
  - Need database of reference bacteria from known animal and human sources
- Large "local" watershed libraries currently considered most useful
  - Cost and time considerations

Early Texas BST Studies

- Texas State Soil and Water Conservation Board (TSSWCB) – Waco Study
  - N. Bosque, Leon River Watersheds – Lakes Waco and Belton
  - 3,061 *E. coli* from 765 source samples
  - 634 *E. coli* from 415 water samples
  - Collected over 12 month period
- Texas Commission on Environmental Quality (TCEQ) – San Antonio Study
  - San Antonio River, Salado and Peach Creeks, Leon River
  - 3,382 *E. coli* from 759 source samples
  - 3,348 *E. coli* from 851 water samples
  - Collected over 4 month period

Goals:
ID Contamination Sources, Standardize Protocols, Compare BST Methods

Source Sample Collection

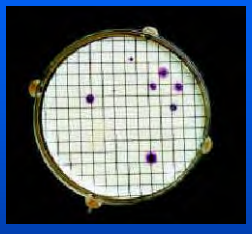
Maximize diversity (even if bad for statistics)

- "Sanitary survey" of watershed stakeholder concerns
- High numbers of source samples – approx 750 each study
- Animals from different areas
- 1 sample per animal *(sewage)
- *E. coli* isolation by water compliance methods

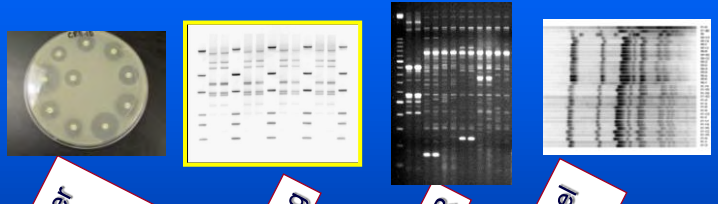
→ **5 isolates archived**
3 screened by ERIC-PCR

Isolation of *E. coli* from Source and Water Samples

- *E. coli* isolation from samples using same media for compliance water monitoring
  - USEPA Method 1603 – modified mTEC medium
  - Confirmation of β-D-glucuronidase activity of isolates using NA-MUG (same as Colilert and Quanti-Tray)
  - No broth enrichment or clinical media - avoid selecting different populations of *E. coli*

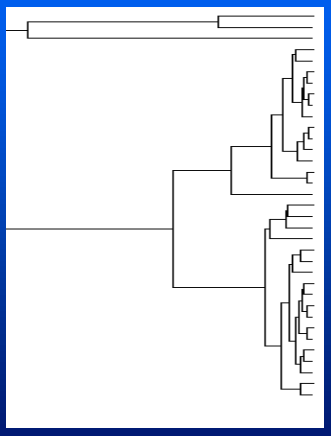


Ability of Methods to Discriminate Differences Between Bacterial Strains



Which method or combination is best?

Isolate Screening:
Send out the clones!



80% similarity or BUST!

- Genotypic screening of isolates from each sample using ERIC-PCR and Applied Maths BioNumerics Software

EXCLUDE CLONES

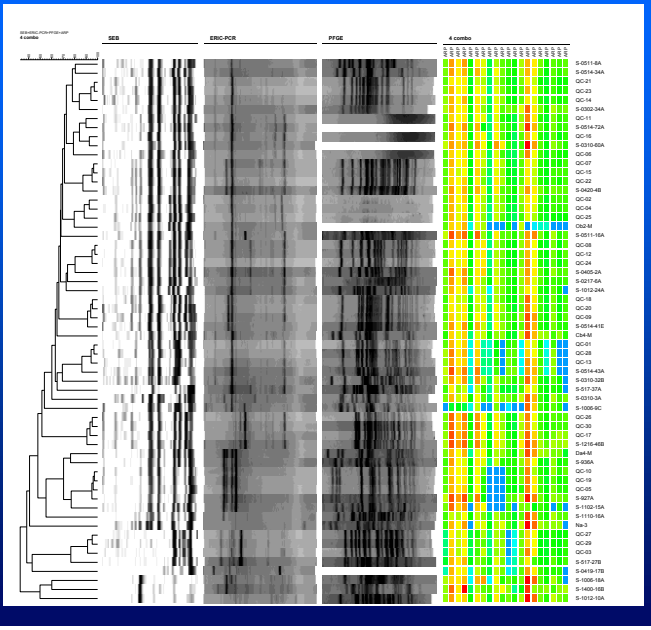
maximize diversity of isolates in library (even if bad for statistics)

- Isolates considered clones at ≥ 80% similarity
- At least one isolate from each sample included in library
- If all ERIC-PCR types already in library (≥ 80% sim), *most abundant type selected* – representative of sample

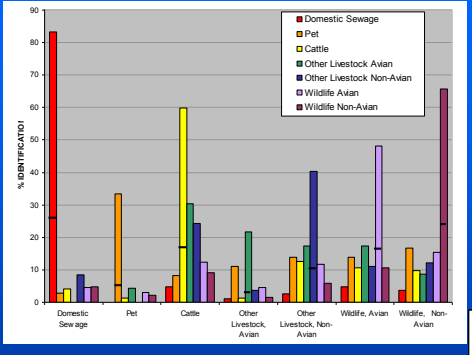
Data Analysis
Best Match Approach

- DNA fingerprints – Pearson correlation curve-based analyses
- "Best Match" approach with minimum similarity cutoff based on laboratory QC data
  - Water isolate must match library isolate ≥ minimum similarity or unidentified
  - Identification to single library isolate with highest similarity – max similarity epidemiology approach
- Match to single isolate but sorted by host class
- Library accuracy - jackknife rates of correct classification (RCC) or average RCC (ARCC) for ID attempts
  - Pick one isolate at a time--treat as unknown
  - Compare to rest of library

Data Analysis: Applied Maths BioNumerics
Composite Data Sets

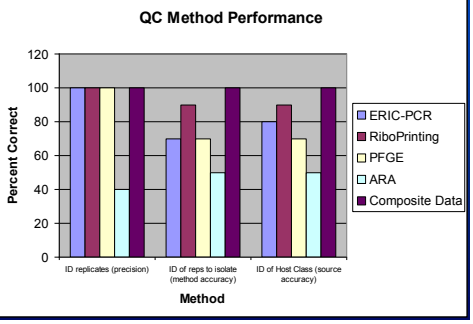


4 Method Composite Gives Best Results

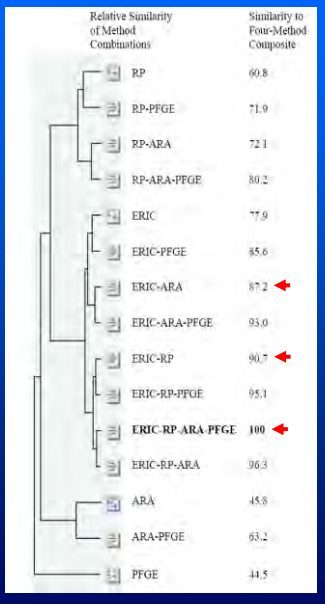


Jackknife Cross-Identification of 4-method Library (RCCs)

Blind (Really) QC Study



Congruence of Methods = 2-Method Composites Nearly as Good



- ERIC-RP
  90.7% similar to 4-method
- ERIC-ARA
  87.2% similar
- Accuracy vs. cost and ease of use

Conclusions – Waco Study

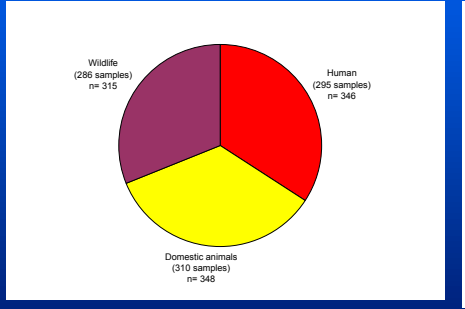
- **Cattle suspected as main source – BST identified wildlife>livestock>human**
- **PFGE had the highest RCCs of any single method, but only 20% water isolates could be identified**
- **Four-method composite data set had the highest accuracy and ability to identify water isolates**
  - **ARCC of 50% for seven-way split - 4X better than random, and 83% RCC domestic sewage, 95% animal**
  - **91% of water isolates identified**
- **Two-method composites better than any single method – *ERIC-RP***
- **Time and cost considerations for future projects**

Next Step:
Determine Usefulness to other Watersheds
E. coli Library Refinement and Challenge

- REFINEMENT
  - Remove library isolates incorrectly identified in their local watershed library using Jackknife Analysis.
    - Correct in stringent 7-way split of source classes
    - Unique patterns (left unidentified) for diversity
    - < 80% similarity ERIC-RP composite data set
  - Combine libraries from Waco and San Antonio studies
    - **SELF-VALIDATED LIBRARY ISOLATES**
- CHALLENGE: *E. coli* fecal isolates from Lake Granbury, Oyster Creek-Trinity River, and Buck Creek

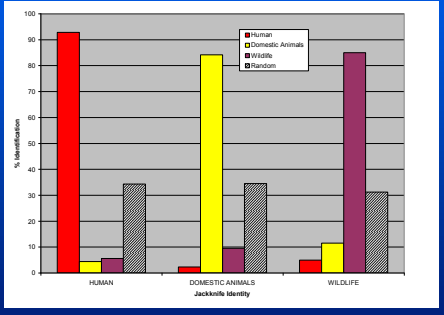
Texas *E. coli* BST Library (ver. 1.0)
Self –validated, combined
Waco + San Antonio Libraries

Texas Library Composition



1009 isolates
from 891 different samples

Cross-Validation Accuracy of Texas Library

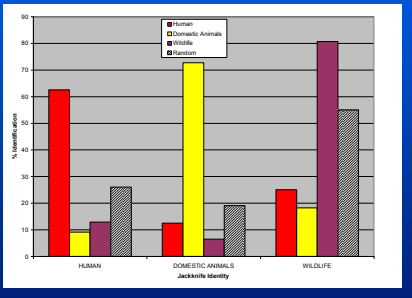


87% average rate
of correct classification (ARCC)

Challenge of Version 1.0 Library With Lake Granbury Source Isolates

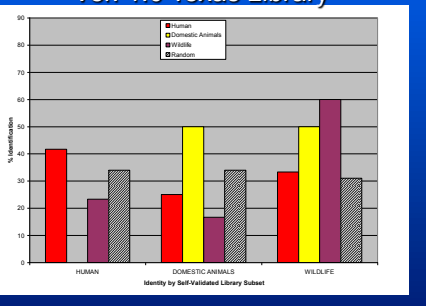
Lake Granbury Local Library
RCCs

80 isolates from 59 source samples



72% ARCC
28% unidentified

Treat as Unknowns (EXTERNAL isolates)
Identification Accuracy
for LG Source Isolates Using
Ver. 1.0 Texas Library



51% ARCC (avg 18% BOR)
38% unidentified

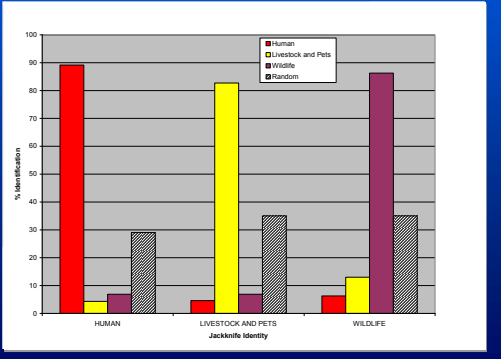
Well, better than random—NEED TO PONDER and EXPAND

Texas *E. coli* BST Library (v. 8-10)

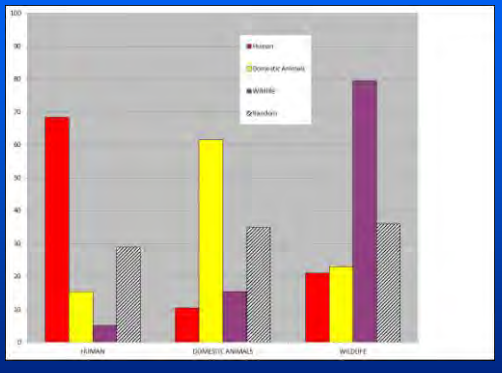
Self –validated isolates from 7 Texas watersheds

1309 isolates from 1185 source samples

Thousands of *E. coli* isolates screened from
Lake Waco; Belton Lake; San Antonio River;
(44+16) Lake Granbury; Buck Creek;
Upper Trinity River; Upper Oyster Creek



Lake Granbury Isolates Revisited
Texas *E. coli* BST library v. 8-10 (inclusive)



73% ARCC
11% unidentified

- Include self-validated local source isolates to represent watershed quirks
- Results Similar to Small Local Library
- Fewer unidentified isolates: ↑ water IDs

Three-Way vs. Six-Way Split of Sources

- Using the results in BMPs
  - Is it from human sources?
  - Is it from livestock?
  - Is it from wildlife?
- Biology
  - Cross identification between livestock
  - Large variety of wildlife
  - Cosmopolitan strains
  - Geographical and temporal differences
- Statistics
  - Number of water isolates per sampling station

1. Human
2. Domestic Animals
3. Wildlife

vs.

Human 1.
Pets 2.
Livestock, avian 3.
Livestock, non-avian 4.
Wildlife, avian 5.
Wildlife, non-avian 6.

Lake Granbury Source Isolate Identification with Texas Library v.8-10 (Inclusive) 6-Way Split

Source Class	Number of Isolates	Number of Samples	Library Composition and Expected Random Rate of Correct Classification	Calculated Rate of Correct Classification (RCC)	Left Unidentified (unique patterns)	RCC / Random Ratio*
Human	21	17	29%	68%	10%	2.4
Pets	3	2	8%	0%	33%	0.0
Avian Livestock	6	3	5%	50%	0%	10.1
Non-Avian Livestock	6	5	22%	60%	17%	2.7
Avian Wildlife	5	3	18%	100%	20%	5.7
Non-Avian Wildlife	39	29	18%	66%	10%	3.6

* An RCC/Random Ratio greater than 1.0 indicates that the rate of correct classification is better than random. For example, the rate of correct classification for Human is 2.4-fold greater than random chance.

Texas *E. coli* BST Library With Limited Local Isolates Added

- Decreases number of unidentified isolates
- Supplements difficult-to-get wildlife isolates
- Decreases known source sampling and processing from 1000s to 100s

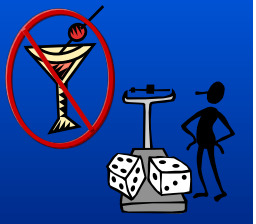
SAVES TIME and MONEY

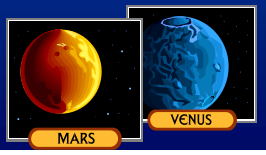
Future of
The Texas *E. coli* BST Library

Continued

EXPANSION CHALLENGE REFINEMENT

- Ver. 11-11 with Gentry & ongoing TSSWCB studies
- Identify and eliminate cosmopolitan strains
- Develop probabilities for strains frequently, but not always, associated with specific sources
- Explore synergy of library and library independent tools for best of both worlds





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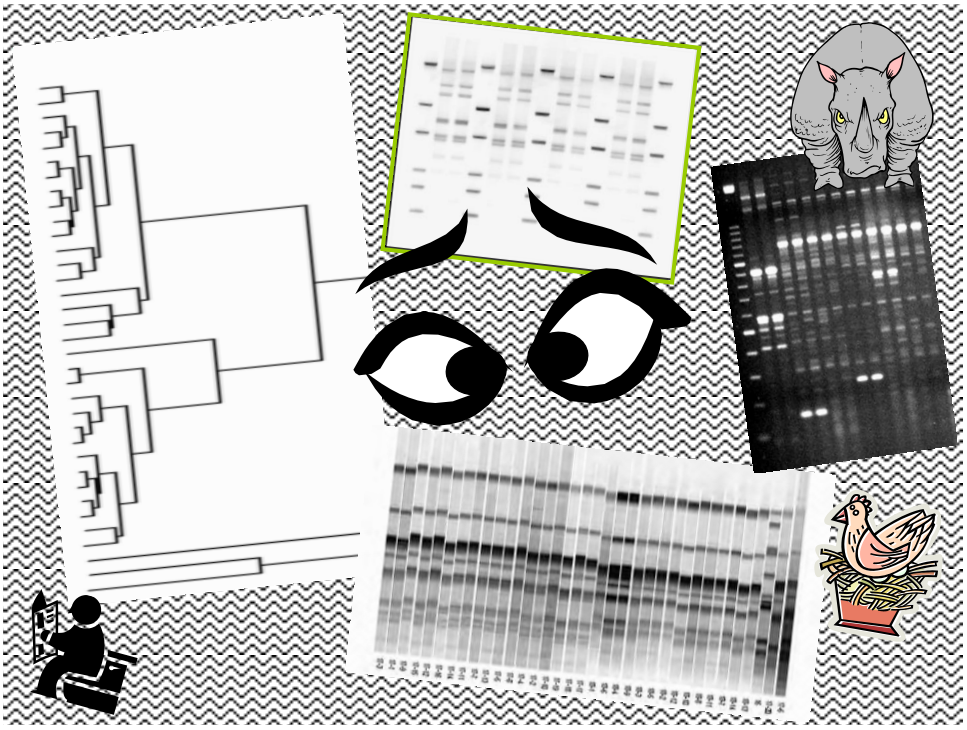

Section 3: Presentations

Wednesday, February 29

Exploration of Library-Independent BST for Texas

Terry Gentry
Texas A&M University

George Di Giovanni
University of Texas School of Public Health, El Paso

February 29, 2012

AgriLIFE RESEARCH
Texas A&M System

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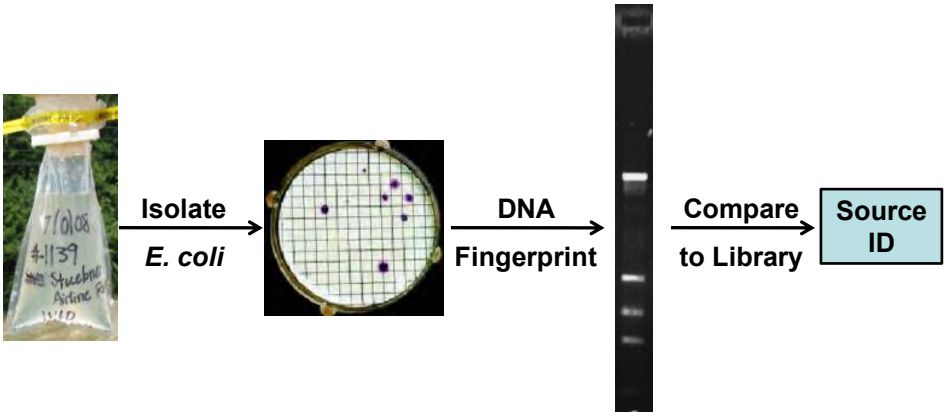
Outline

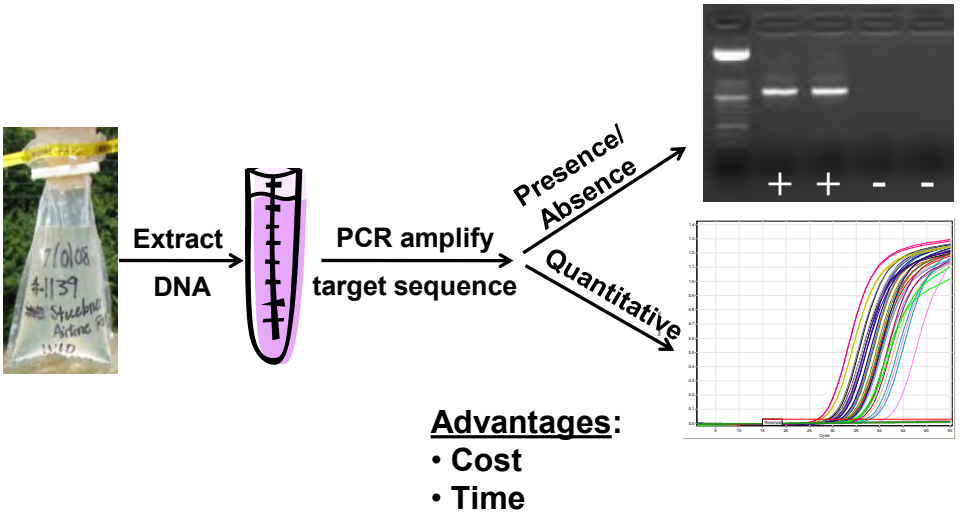
- Background
- Overview of BST projects
  - Characterization of watersheds
  - Evaluation/development of feral hog marker
  - Evaluation of grazing management practices

Library-Dependent BST



3

Library Independent BST



Advantages:
- Cost
- Time

4

Library Independent Screening of Pollution Sources Using *Bacteroidales* PCR

- Most common approach targets *Bacteroidales*
- *Bacteroidales* – human and animal fecal bacteria, more abundant than *E. coli*
- Markers available for
  - Ruminants (cattle, deer, elk, sheep, horses, llama)
  - Humans
  - Horses (needs optimization and validation)
  - Birds - (needs optimization and validation)
  - Hogs (including feral hogs – in development)
- Highly (but not 100%) specific
- Limited markers for wildlife
- Relationship to *E. coli* and pathogens uncertain

5

Library-Independent BST in Texas

- Six watersheds in Texas
  - Lake Granbury (UT)
  - Buck Creek (UT)
  - Little Brazos River Tributaries (TAMU)
  - Big Cypress (TAMU)
  - Attoyac Bayou (TAMU)
  - Leona River (TAMU)
- Edge-of-field runoff (BMP evaluation)
  - Dairy manure (UT)
  - Grazing systems (TAMU)
- Oklahoma City (UT; waterborne disease outbreak)

6

BST for Little Brazos River Tributaries

- Tier 2 BST
  - Library-dependent (limited) & library-independent approaches
    - Limited library-dependent
      - Analyzed *E. coli* from 81 water samples from across the study area using both ERIC-PCR and RP fingerprinting
      - Best match ID against Texas *E. coli* BST Library

7

BST for Little Brazos River Tributaries

- Library-independent
  - Analyzed 259 water samples from across the study area using *Bacteroidales* PCR (Presence/Absence)
    - Human (HF183F – Bernard and Field, 2000)
    - Ruminant (CF128F – Bernard and Field, 2000)
    - Hog (PF163F – Dick et al., 2005)
    - Horse (Ho597F, Dick et al., 2005)

8

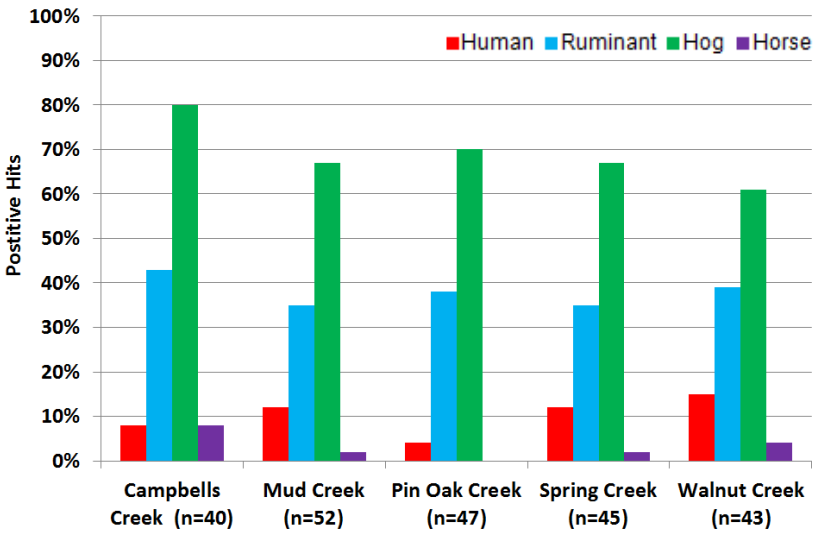
9

BST Samples

	2009								2010				
Parameter	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	Total Analyzed
Bacteroidales													
Stream (10)	10	17	8	10	5	0	20	10	10	20	10	10	130
WWTFs (3)		0			2			2	2	4	2	2	14
Storm - Stream (10)		0	6		14	50		10	10		10		100
Storm - WWTFs (3)		0			1	8		2	2		2		15
Bacteroidales Total													*259*
E. coli (ERIC-RP)													
Stream (10)					5		10		10		10		35
WWTFs (3)					2			2	2		2		8
Storm - Stream (10)			6		10	8					10		34
Storm - WWTFs (3)					1	2					1		4
E. coli Total													*81*

10

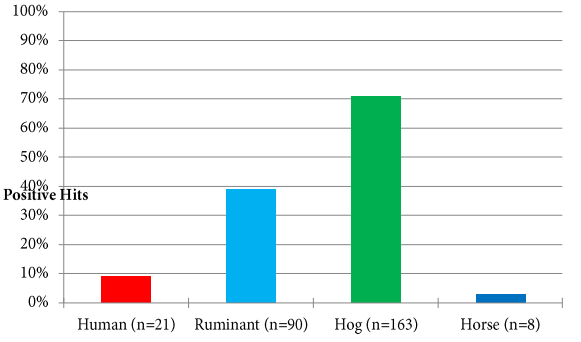
Bacteroidales BST Results
Sub-Watershed Stream Samples



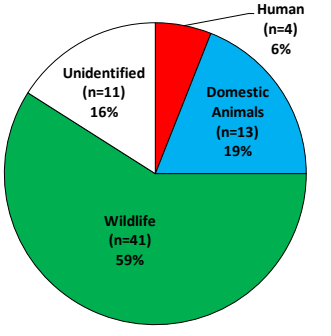
11

BST Results
Overall Stream Samples

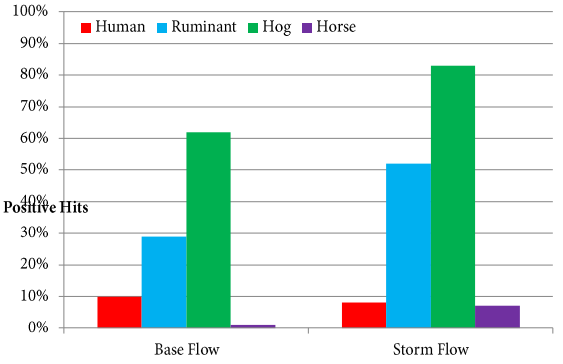
Library Independent



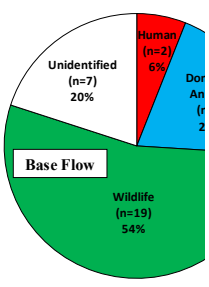
Library Dependent

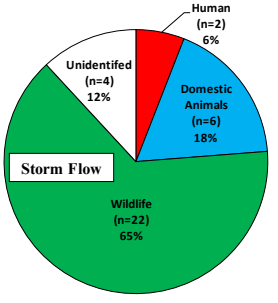


12



Library Independent





Library Dependent

13

BST Summary

- **Limited Library-Dependent Analysis**
  - **Existing Texas *E.coli* BST Library appears to work relatively well (84% of isolates identified)**
  - **Major sources in watershed appear to be wildlife (feral hogs, deer, avian wildlife, and small mammals) and to lesser extent domestic animals (livestock and pets)**
- **Library-Independent Analysis**
  - **Hog marker detected most frequently (71%) followed by ruminant marker (39%)**
  - **Small percentage of human (9%) and horse (3%) hits**

14

Use of BST Results

- Reconciled with:
  - Land use
  - Watershed source survey
  - Modeling
- Information provided to stakeholders for watershed protection planning process

15

Impacts of Feral Hogs

- Observed in many states – Texas and Southeastern states, Michigan, Iowa, Nebraska, New York, Pennsylvania, Wisconsin, and Hawaii
- Texas has a population of nearly 2 million
- Inhabit bottomlands such as rivers, creeks, and drainages
- Compete directly with livestock, game and nongame for food, destroy native plants,
- Approx. $52 million in damage every year in Texas alone
- Concerns over water quality impacts

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16

Evaluation of PCR for Hog Marker

Source	# of Samples	Location	Positive for Hog Marker	%Positive
Domestic Pig	7	Buck Creek	7	100%
Feral Hog	22	Buck Creek	21	95%
Feral Hog	18	Sinton, TX	18	100%
Domestic Pig	10	West Virginia	10	100%
Domestic Pig	5	Lake Granbury	5	100%
Feral Hog	7	Lake Granbury	7	100%

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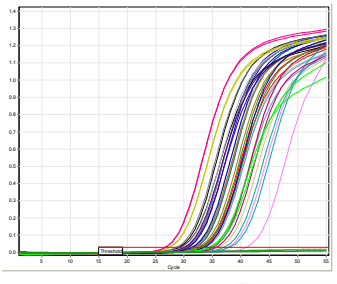
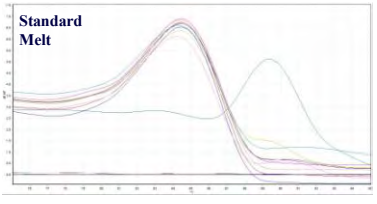
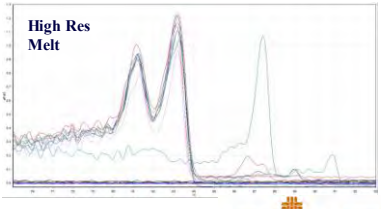
17

Bacteroidales qPCR and Melt Curve Characterization of PCR Products



No.	Colour	Name	Type	Ct
1		BC04 - 11/07/07	Unknown	32.77
2		BC04 - 11/07/07 (DUP)	Unknown	33.00
3		BC05 - 11/07/07	Unknown	30.66
4		BC05 - 11/07/07 (DUP)	Unknown	30.37
5		BC10A - 11/07/07	Unknown	37.67
6		BC10A - 11/07/07 (DUP)	Unknown	40.96
7		BC10C - 11/07/07	Unknown	31.98
8		BC10C - 11/07/07 (DUP)	Unknown	32.07
9		BC11 - 11/07/07	Unknown	33.30
10		BC11 - 11/07/07 (DUP)	Unknown	32.91
11		BC05 - 12/05/07	Unknown	29.21
12		BC05 - 12/05/07 (DUP)	Unknown	29.46
13		BC06 - 12/05/07	Unknown	34.80
14		BC06 - 12/05/07 (DUP)	Unknown	36.17





Di Giovanni et al., unpublished

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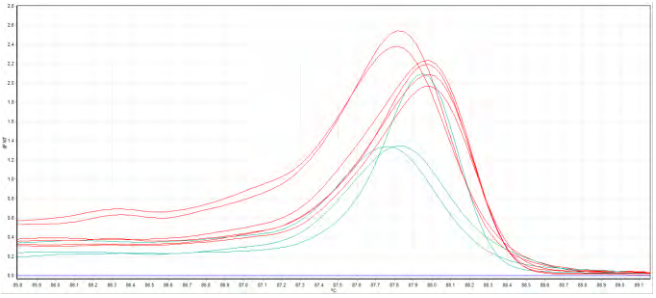
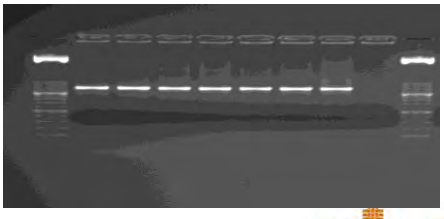
18

Bacteroidales PCR/qPCR Feral Hog Fecal Marker



qPCR high resolution melt curves

Conventional PCR



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Grazing Management Evaluation

- **Objective**

  Evaluate effects of grazing management on bacteria runoff from rangeland and improved pasture

- **3 Treatments Tested (7 total sites)**

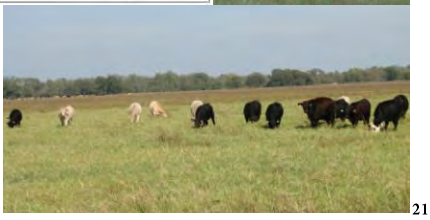
  1: No grazing – 3 locations

  2: Moderately stocked (at recommended rates) – 3 locations

  3: Heavily stocked (2 x moderate stocking rate) – 1 location

Grazing Management Evaluation

- Three locations:
  - Welder Wildlife Refuge
    - Sinton
    - Chaparral-mixed grass communities
  - USDA-ARS
    - Riesel
    - Native prairie & bermudagrass
  - Texas A&M Beef Center
    - College Station
    - Tifton 85



21

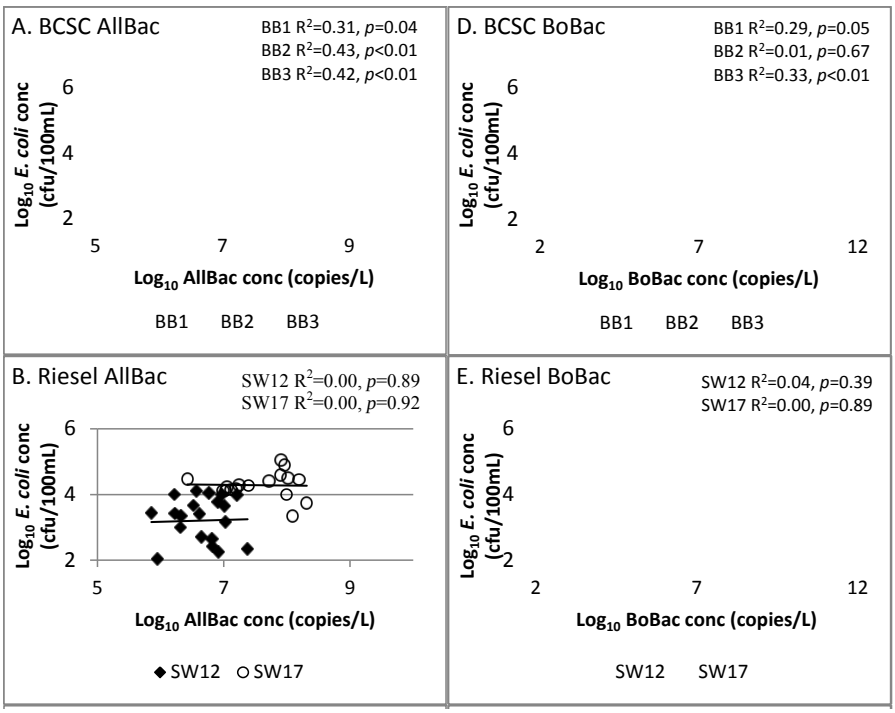
Grazing Management Evaluation

- Edge-of-field runoff collected over three years
- *E. coli* - EPA Method 1603
- *Bacteroides* (Layton et al., 2006)
  - Total *Bacteroides* spp. (AllBac)
  - Bovine-associated *Bacteroides* spp. (BoBac)

22

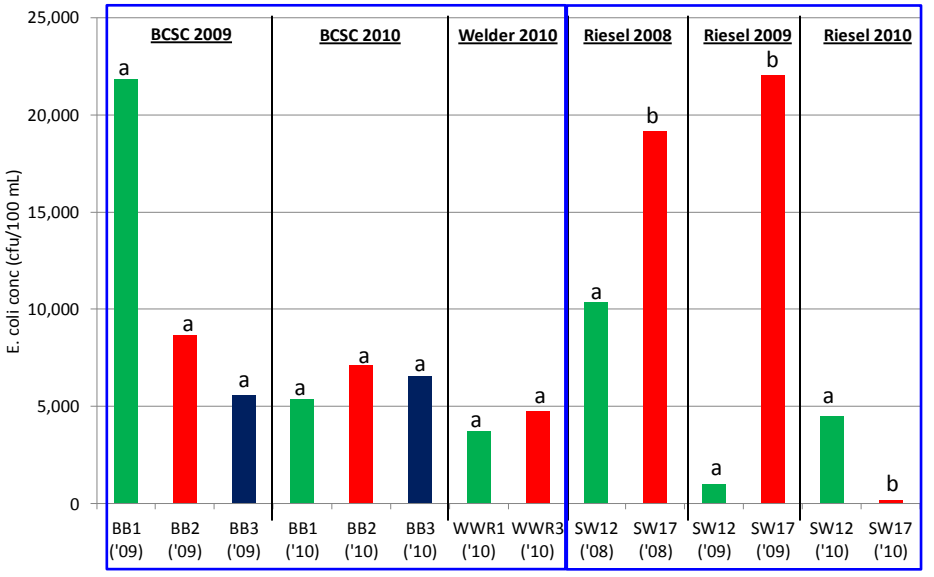
Site-Yr[1]	AllBac Median	BoBac Median	Grazing Management	Annual AUD/ha	Cattle on site during runoff-%[2]
Beef Cattle Systems Center					
BB1-09	9.49E+06	6.18E+03	Ungrazed	0	No-0%
BB2-09	4.30E+06	4.59E+03	Properly stocked	147	No-0%
BB3-09	3.30E+06	6.13E+03	Overstocked	312	No-0%
BB1-10	3.58E+06	1.12E+05	Ungrazed	17	Yes[3]-20%
BB2-10	4.74E+06	8.87E+05	Properly stocked	301	Yes-67%
BB3-10	1.45E+07	2.90E+06	Overstocked	543	Yes-75%
USDA-ARS Riesel watersheds					
SW12-08	7.61E+06	1.51E+03	Ungrazed	0	No-0%
SW17-08	5.22E+07	5.45E+06	Properly stocked	124	Yes-100%
SW12-09	4.18E+06	2.17E+03	Ungrazed	0	No-0%
SW17-09	1.58E+07	6.95E+06	Properly stocked	341	Yes-100%
Welder Wildlife Refuge					
WWR1-10	2.74E+06	7.93E+04	Ungrazed	0	No-0%
WWR3-10	6.99E+05	1.73E+04	Properly stocked[4]	0	No-0%

23



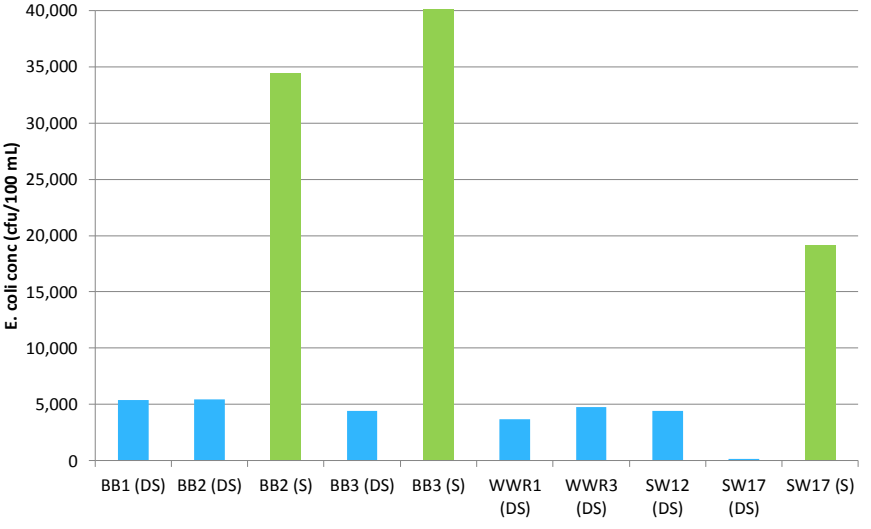
24

Annual *E. coli* Conc. At Each Site

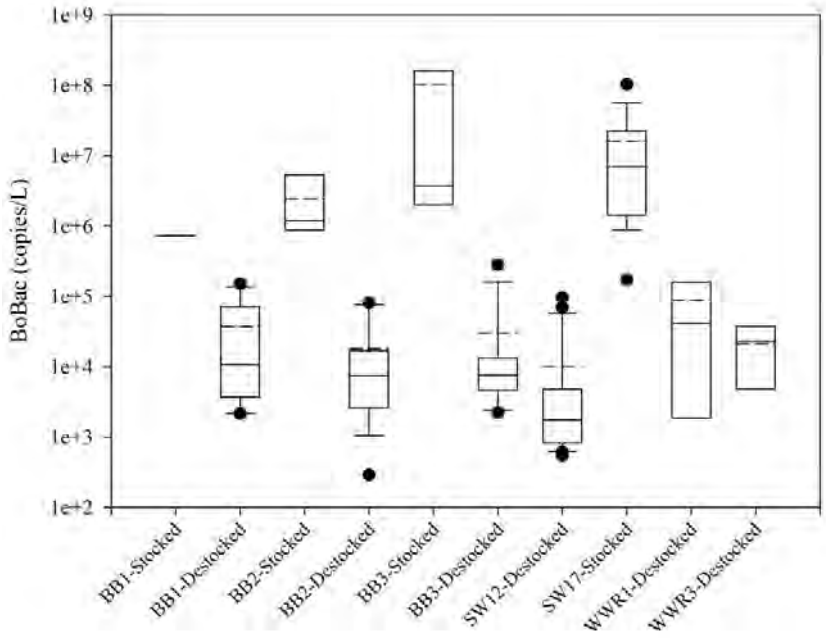


25

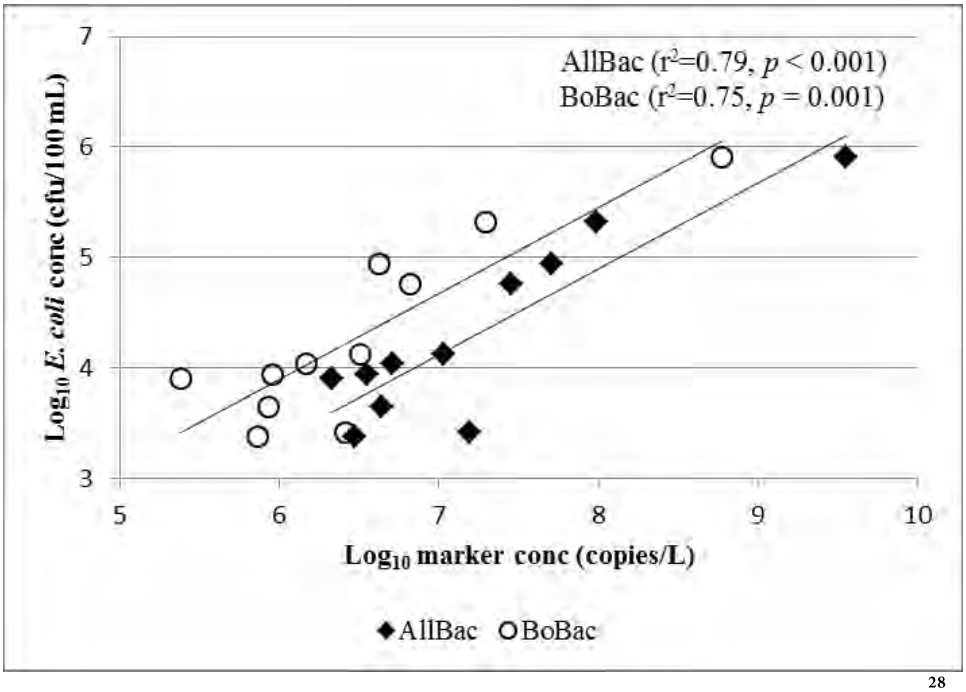
Comparison of Median *E. coli* Levels
While Sites Stocked (S) & Destocked (DS)



26


BoBac (copies/L)
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
BB1-Stocked
BB1-Destocked
BB2-Stocked
BB2-Destocked
BB3-Stocked
BB3-Destocked
SW12-Destocked
SW17-Stocked
WWR1-Destocked
WWR3-Destocked

27


AllBac (r^2=0.79, p < 0.001)
BoBac (r^2=0.75, p = 0.001)
Log10 E. coli conc (cfu/100 mL)
7
6
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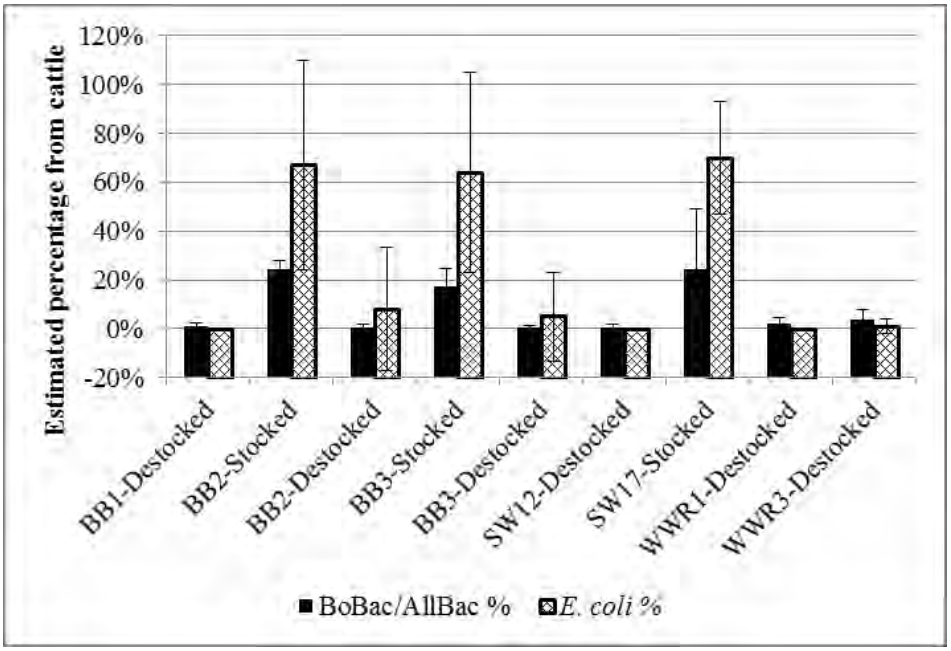
Log10 marker conc (copies/L)
AllBac
BoBac

28



29

Grazing Management Evaluation Summary

- **Both markers higher in runoff while sites stocked suggesting they provide good indicator of recent fecal contamination from cattle.**
- **BoBac/AllBac ratios generally aligned with stocking rate but may have underestimated percentage of bovine-associated fecal contamination.**
- **Differing results in various watersheds**
  - **Geographic variability markers?**
  - **Markers correlated well with *E. coli* at one location**
  - **Standard curve**
  - **1/3 ain't bad?**

30

Additional Library-Independent BST Research

- Development and evaluation of markers
  - Geographic variability
  - New species-specific markers
    - Feral hogs
    - Deer
    - Poultry
  - Validation

31

Acknowledgments

- Joy Truesdale (UT)
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- NRCS

32

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Library-independent MST for Coastal Waters

Joanna Mott
James Madison University

(formerly at
Texas A&M University-Corpus Christi)

Introduction

- BST – LDM, multiple coastal watersheds
  - FIB – relates to regulatory assessment
    - *E. coli* for shellfish harvesting waters, (fecal coliforms)
    - enterococci for tidal watershed
  - Methods
    - (PFGE)
    - ARA by Kirby Bauer – automated image analysis provides zone diameter data
    - Toolbox – added CUP by Biolog – well color intensity
    - Composite data sets ARA/CUP
    - Statistical toolbox - DA and Random Forests
- LIM to add to toolbox

Outline

- LIM used in Texas coastal watersheds
- USF collaboration (Harwood and Gordon): field testing of 3 human-specific molecular markers
  - Marine water – CC Bay beach locations
  - Fresh water - river locations
- Esp marker
  - Marine water – CC Bay beach locations (GLO/CBBEP)
  - Freshwater – part of upper Oso Creek study (TSSWCB)
- Future directions - qPCR markers

Library Independent Methods

- Do not require a reference database (library)
- Identify the presence of a specific target
- Examples
  - *Bacteroidales* host-specific 16S rRNA gene markers
  - F-specific RNA bacteriophage genotyping
  - Human polyomavirus (McQuaig *et al.* 2006)
  - *esp* gene (Scott *et al.* 2005)

Host Specific Molecular Markers (PCR – USF study)

- Three human-specific markers:
  - Human associated *Bacteroides* spp.
  - *Methanobrevibacter smithii*
  - human polyomaviruses
- Evaluated for specificity and sensitivity in Gulf of Mexico setting (Harwood et al. 2009)
- Use of single marker can fail to detect fecal contamination (Ahmed et al. 2006)

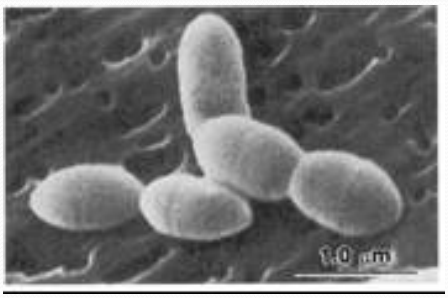
Human associated *Bacteroides*

- Gram negative bacilli, obligate anaerobes
- Primers target 16S ribosomal subunit DNA
- Widely used
  - Mississippi, Florida (Harwood et al. 2009), Oregon (Bernhard and Field 2000a, Bernhard and Field 2000b), France (Gourmelon et al. 2007, Gourmelon et al. 2010), Belgium (Seurinck et al. 2006), and Australia (Ahmed et al 2008)

Courtesy American Society for Microbiology

Methanobrevibacter smithii

- Methanogenic archaean
- Primary methanogen in human digestive tract
- Rod shaped and often found in chains
- Primers target *nifH* gene which encodes a non functional nitrogenase



http://www.uprm.edu

Human polyomaviruses (JCV and BKV)

- Icosahedral viruses in family polyomaviridae
- Primary infections occur in early (BKV) to late (JCV) childhood
- Infections are latent in renal tissue and shed in urine
- Primers target the conserved T-antigen of both viral strains
- 100% specific to human feces, detected in 100% of samples containing human fecal material (Harwood et al. 2009)

http://zhuang.harvard.edu

Methods

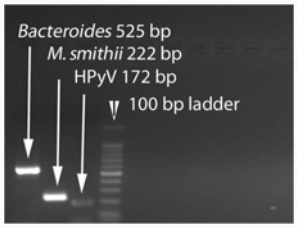
- PCR protocols courtesy of Dr. Valerie Harwood, USF
- Samples for PCR adjusted to pH 3.5 with 1.0 N HCl
- 500 ml vacuum filtered onto 0.45µm nitrocellulose membrane
- DNA extracted with PowerSoil™ DNA Isolation Kit
- All PCR reactions conducted in duplicate

Methods

Primers and references for marker organisms

Indicator organism	Primer sequence	Size of PCR product	References
Human Polyomaviruses	SM2: 5'-AGT CTT TAG GGT CTT CTA CCT TT-3' P6: 5'-GGT GCC AAC CTA TGG AAC AG-3'	172 bp	McQuaig et al. 2009
Human *Bacteroides*	HF183f: 5'ATC ATG AGT TCA CAT GTC CG 3' Bac708r: 5'CAA TCG GAG TTC TTC GTG 3'	525 bp	Bernhard and Field 2000b
Methanobrevibacter smithii	Mnif-342f: 5'AAC AGA AAA CCC AGT GAA GAG 3' Mnif-363r: 5'ACG TAA AGG CAC TGA AAA ACC 3'	222 bp	Ufnar et al. 2006

- All PCR products visualized on 2% agarose gel with ethidium bromide



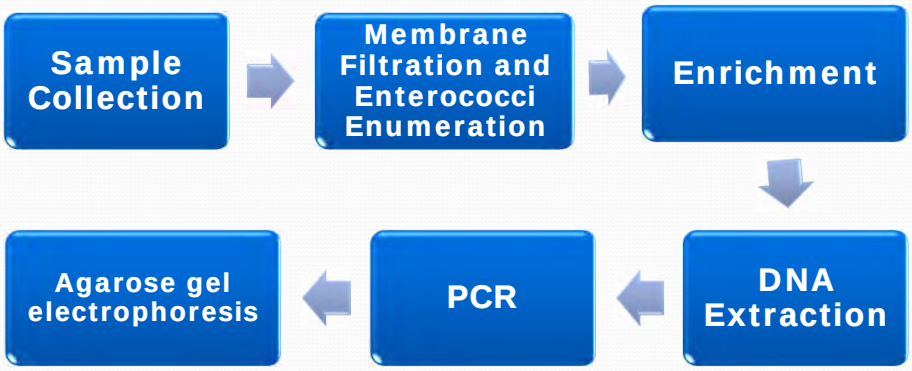
Additional human-associated marker used:

- **Enterococcal Surface Protein (Esp)**
  - High molecular weight surface protein found in *Enterococcus* species associated with human intestinal tract
  - Involved in biofilm formation by *E. faecium* and *E. faecalis*
  - *esp* gene used in several library-independent MST studies (Scott *et al.* 2005; McDonald *et al*. 2006; Brownell *et al.* 2007; Ahmed *et al*. 2008; Korajkic *et al.* 2009; Abdelzaher *et al.* 2010)

esp Gene as a LIM

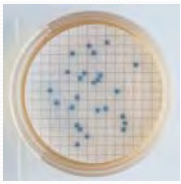
- Indicates the presence of human-associated *E. faecium*
  - PCR-based detection
  - Primers target *esp* gene from human-specific *E. faecium,* not *E. faecalis*
- Specificity: conflicting research
  - Not detected in any animal fecal samples (Scott *et al.* 2005; Ahmed *et al*. 2008; Ahmed *et al*. 2009)
  - Detected in non-human sources, dog and gull (Whitman *et al.* 2007)

Esp Methods



Methods

- Enterococci enumeration followed EPA Method 1600: Enterococci in water by membrane filtration using membrane-*Enterococcus* Indoxyl-β-D Glucoside agar (mEI)

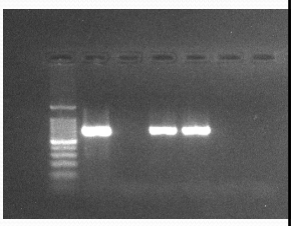


- Esp protocol courtesy Harwood and Gordon, based on McQuaig et al. (2006).
  - 300 ml sample filtered, incubated on mEI, transferred to 15 ml tubes for enrichment in azide dextrose broth, incubated for 3 h on shaking table at 41°C

Methods: *esp* Analysis

- DNA Extraction
  - Qiagen DNeasy Blood and Tissue Kit and ASL buffer
  - InhibitEX tablets to prevent inhibition
- PCR
  - GoTaq Green mix (Promega)
  - *esp* forward primer (5'-TAT GAA AGC AAC AGC ACA AGT T-3') (Scott *et al*. 2005) and *esp* reverse primer (5'-ACG TCG AAA GTT CGA TTT CC-3') (Hammerum and Jensen 2002)

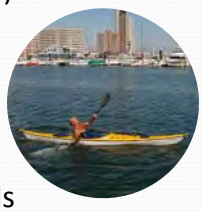
Methods: *esp* Analysis



- Agarose gel electrophoresis
  - 2.0% agarose gel
  - Promega 100 bp ladder
  - 680 bp product
- Controls
  - 1: positive control for PCR - *E. faecium* C68 DNA
  - 2: negative control for PCR; no DNA added to reaction
  - 3: inhibition spike (SP1) - *E. faecium* C68 DNA to spike a composite environmental sample
  - 4: inhibition spike (SP2) - *E. faecium* C68 DNA to spike dilution water
  - 5: method blank (MB); dilution water carried into the field and processed like environmental samples
  - 6: extraction blank (EB)

Corpus Christi Area

- Human population: 550,000 in 2000 census (CBBEP 2010)
- Tourism in the coastal bend
  - 13,000 jobs and $1.1 billion (CBBEP 2010)
  - Nature and wildlife activities account for 40% of visitors' trips (CBBEP 2010)
- Estuary of national significance (USEPA 1999)
  - Commercial and sport fisheries
  - Recreational use
  - Discharge points for industry and municipalities
- Segments impaired for bacteria – CC Bay – Ropes/Cole Parks, several coastal watersheds

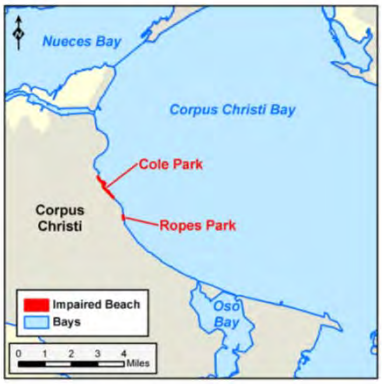


LIM Objectives

- Can human-specific molecular markers be used as an MST method for south Texas coastal waters – marine and freshwaters ?
- Is there a human source contribution to the contamination of Corpus Christi Bay (Ropes and Cole parks) marine waters, under both dry conditions and following rainfall?
- Is there a human source contribution to the contamination of the upper Oso Creek, using the *esp* gene as an indicator of human fecal contamination ?

Corpus Christi Bay study

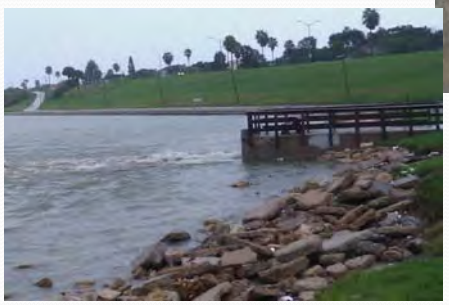
- Cole Park and Ropes Park beaches: data from Texas Beach Watch Program indicated bacteria concentrations higher than EPA criteria for protecting contact recreation use.
- Included in 2010 Draft TCEQ 303(d) list as impaired water segment 2481CB (TCEQ 2010) for bacteria contamination
- Six sites sampled (TBW sites)



Texas Commission on Environmental Quality
Oct 2011

Corpus Christi Bay

- Rope and Cole sites routinely monitored by Texas Beach Watch
- Each park contains outfalls which discharge storm water



Corpus Christi Bay

Sites in Ropes Park

Sites in Cole Park

Collection – beach samples

- Monthly collections, February-July 2010
- Four additional sampling events after significant rainfall – two of these in Sept
  (≥ 2.5 cm rain in 24 h or > 7.5 cm rain in 7 day period)
- Three samples per site
- Salinity, pH, dissolved oxygen, and water temperature measured in field with YSI multi probe instrument

Summary results:

Table 1: Marine samples from Corpus Christi Bay (HBac, *M. smithii*, HPyVs, and *esp*)

Date (* 24 hr rainfall ≥ 1 in)	Average enterococci cfu/100ml	Total sites with marker detected (of 6)	Markers detected
2/17/2010	6	2	HBac and *M. smithii*
3/24/2010	150	4	*M. smithii* & HPyVs
4/28/2010	17	0	0
5/16/2010*	424	1	HPyVs
5/26/2010	14	0	0
6/03/2010	504	1	HPyVs
6/09/2010*	14	1	HBac
7/06/2010	8	3	HBac and HPyVs
9/10/2010*	127	6	HBac and *M. smithii*
9/22/2010*	1144	5	HBac, *M. smithii*, and *esp*

Results for Ropes and Cole Parks hbac/smithii/hpyv

Date	Total Detects (of 18)	Total Sites with Marker Detected (of 6)	7 Day Rainfall (cm)	Average Enterococci (cfu/100ml)
2/17/2010	2 (hbac, *smithii*)	2	2.25	6
3/24/2010	6 (hpyv, *smithii*)	4	0.7	150
4/28/2010	0	0	0	17
5/16/2010	1 (hpyv)	1	2.5	424
5/26/2010	0	0	0	14
6/03/2010	1 (hpyv)	1	3.5	504
6/09/2010	1 (hbac)	1	4.25	14
7/06/2010	4 (hbac, hpyv)	3	5.75	8
9/10/2010	11 (hbac, *smithii*)	6	9	127
9/22/2010	7 (hbac, *smithii*)	5	20.75	1144

Results for Ropes and Cole Parks hbac/smithii/hpyv

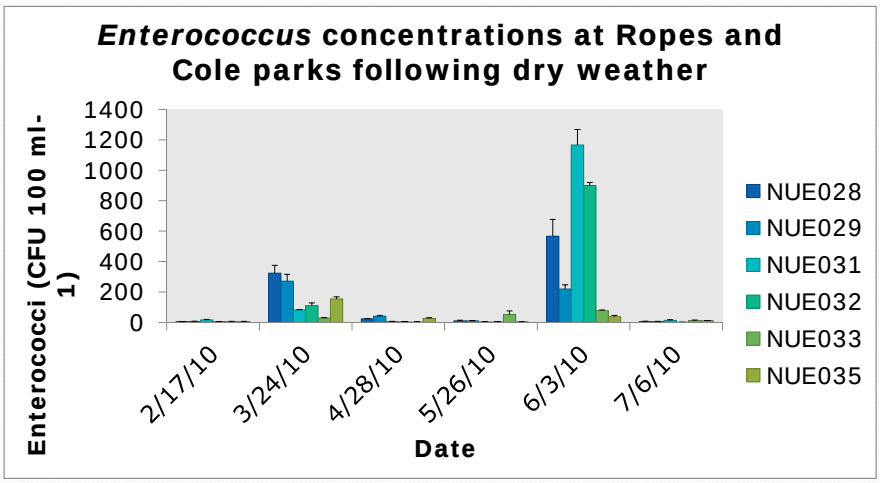
- Detection of human-specific markers was significantly associated with:
  - three and seven day rainfall (p= 0.045 and p=0.000)
  - concentration of enterococci (p= 0.030)
- Harwood examined relationship based on exceedance/not (104 cfu/100mL) and scored for detection of at least one marker. Three labs found association, 2 did not (TAMU-CC, UWF).

Results for Ropes and Cole Parks hbac/smithii/hpyv

Frequency of Detection for Human Markers at Ropes Park and Cole Park

Site	Frequency of Human Marker Detection (%)	Distance from outfall (m)
NUE028 - Ropes	50	48
NUE029 - Ropes	40	133
NUE033 - Cole	40	48
NUE035 - Cole	40	60
NUE031 - Cole	30	583
NUE032 - Cole	30	198

- Highest frequency of detection at NUE028 Ropes Park (50% of sample events)
- Lowest frequency of detection at NUE031 and NUE032 at Cole Park (30% of sample events)

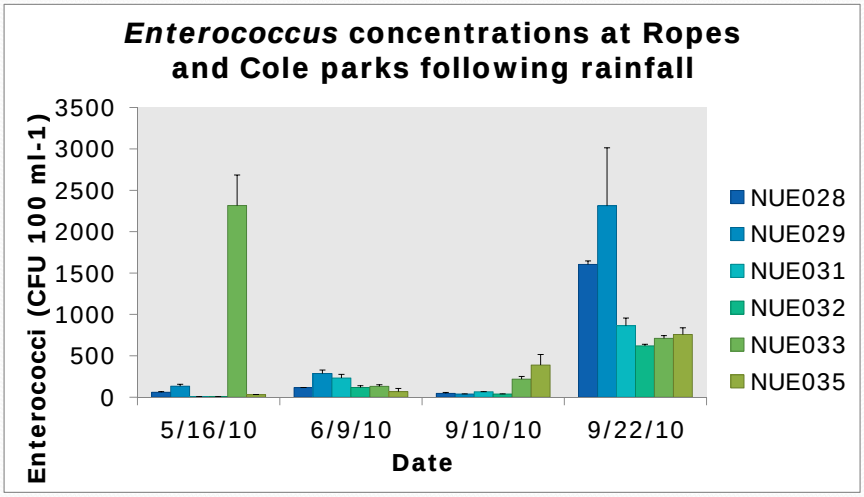


Values are the mean of enterococci counts from three subsamples at each site, and error bars represent the standard error (n=3).

esp Results: Marine Water Following Dry Weather

Sample number	2/17/10 Ent* CFU 100 ml^{-1}	*esp*	3/24/10 Ent* CFU 100 ml^{-1}	*esp*	4/28/10 Ent* CFU 100 ml^{-1}	*esp*	5/26/10 Ent* CFU 100 ml^{-1}	*esp*	6/3/10 Ent* CFU 100 ml^{-1}	*esp*	7/6/10 Ent* CFU 100 ml^{-1}	*esp*
NUE028A		-		-		-		-		-		-
NUE028B	4.3 ± 0.9	-	323.3 ± 52.1	-	23.3 ± 1.7	-	10.3 ± 3.9	-	566.7 ± 110.5	-	6 ± 1.5	-
NUE028C		-		-		-		-		-		-
NUE029A		-		-		-		-		-		-
NUE029B	5.7 ± 2.2	-	271 ± 45.4	-	41 ± 4.9	-	10 ± 1.7	-	220 ± 26.5	-	5.7 ± 1.5	-
NUE029C		-		-		-		-		-		-
NUE031A		-		-		-		-		-		-
NUE031B	16 ± 3.2	-	81.3 ± 3.0	-	6.3 ± 0.7	-	3 ± 1.5	-	166.7 ± 101.7	-	13.7 ± 3.5	-
NUE031C		-		-		-		-		-		-
NUE032A		-		-		-		-		-		-
NUE032B	5 ± 0.6	-	110.3 ± 17.6	-	2.7 ± 0.3	-	1.3 ± 0.3	-	900 ± 20.1	-	1 ± 0.6	-
NUE032C		-		-		-		-		-		-
NUE033A		-		-		-		-		-		-
NUE033B	5.3 ± 1.2	-	27.7 ± 3.7	-	1.7 ± 0.3	-	51.7 ± 23.1	-	77.7 ± 3.9	-	12 ± 3.1	-
NUE033C		-		-		-		-		-		-
NUE035A		-		-		-		-		-		-
NUE035B	4.7 ± 1.9	-	154 ± 13.4	-	25.3 ± 5.4	-	3.7 ± 0.7	-	37.3 ± 8.8	-	10.7 ± 0.3	-
NUE035C		-		-		-		-		-		-

*Ent CFU 100 ml^{-1} values are expressed as mean ± standard error (n=3).



Values are the mean of enterococci counts from three subsamples at each site, and error bars represent the standard error (n=3).

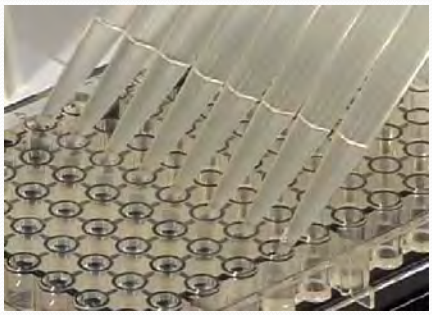
esp Results: Marine Water Following Rainfall

Sample number	5/16/10 (2.5 cm 24 h rainfall) Ent* CFU 100 ml^{-1}	*esp*	6/09/10 (3.6 cm 24 h rainfall) Ent* CFU 100 ml^{-1}	*esp*	9/10/10 (9.1 cm 7 d rainfall) Ent* CFU 100 ml^{-1}	*esp*	9/22/10 (21.0 cm 7 d rainfall) Ent* CFU 100 ml^{-1}	*esp*
NUE028A		-		-		-		+
NUE028B	60 ± 6.4	-	115.7 ± 1.3	-	46 ± 11.5	-	1603.3 ± 43.7	-
NUE028C		-		-		-		-
NUE029A		-		-		-		+
NUE029B	133.3 ± 21.9	-	286.3 ± 40.8	-	38 ± 2	-	2313.3 ± 700.8	+
NUE029C		-		-		-		-
NUE031A		-		-		-		-
NUE031B	5 ± 1.5	-	232.3 ± 43.8	-	65.3 ± 2.7	-	863.3 ± 94.0	-
NUE031C		-		-		-		-
NUE032A		-		-		-		-
NUE032B	5.7 ± 0.9	-	116.7 ± 21.9	-	37.7 ± 3.2	-	620 ± 20	-
NUE032C		-		-		-		-
NUE033A		-		-		-		-
NUE033B	2315.7 ± 368.5	-	131.3 ± 18.4	-	217 ± 33.1	-	710 ± 34.6	-
NUE033C		-		-		-		-
NUE035A		-		-		-		-
NUE035B	30 ± 1.2	-	67 ± 39.5	-	389 ± 126.0	-	756.7 ± 80.9	-
NUE035C		-		-		-		-

*Ent CFU 100 ml^{-1} values are expressed as mean ± standard error (n=3).

Enterococcus faecium ?

- 140 colonies isolated after rainfall 9/22/10
- 58 grew on mEI or in TSB and inoculated in GP2 Microplates™ (Biolog, Inc.)
- 36 genus *Enterococcus*
- **13 *Enterococcus faecium***
- (Other isolates identified as: *Pediococcus, Alloiococcus, Streptococcus)*



http://www.biolog.com/mID_technical.shtml

Results for Ropes and Cole Parks

- Human markers were detected:
  - at all sites sometime(s) during the study
  - on eight of ten sample events
- *M. smithii* was most frequently detected (8.3% of samples)
- Human associated *Bacteroides* spp. detected in 5.6% of samples
- Human polyomaviruses detected in 4.4% of samples
- *Esp* detected only after rainfall, one event

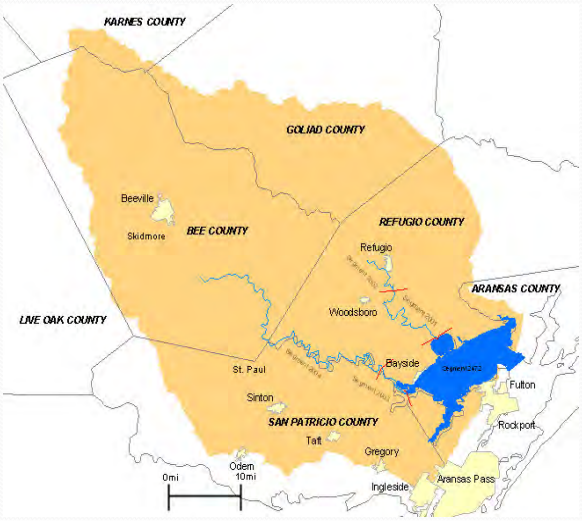
Fresh water sites

hbac/smithii/hpyv

- Freshwater effluent from ten waste water treatment plants in the Copano Bay watershed - Mission and Aransas Rivers, provided by Nueces River Authority
- Portions of Copano Bay and tidal segments of Mission River and Aransas Rivers included on 2010 Draft TCEQ 303(d) list (TCEQ 2010) for bacteria contamination
- LDM BST study of Mission and Aransas Rivers had suggested human contribution

Waste water treatment plants

- City of Taft
- City of Skidmore
- City of Bayside
- City of Beeville
- City of Odem
- City of Sinton
- Town of Refugio
- Town of Woodsboro
- St. Paul WSC
- Pettus MUD



http://copanobay-wq.tamu.edu

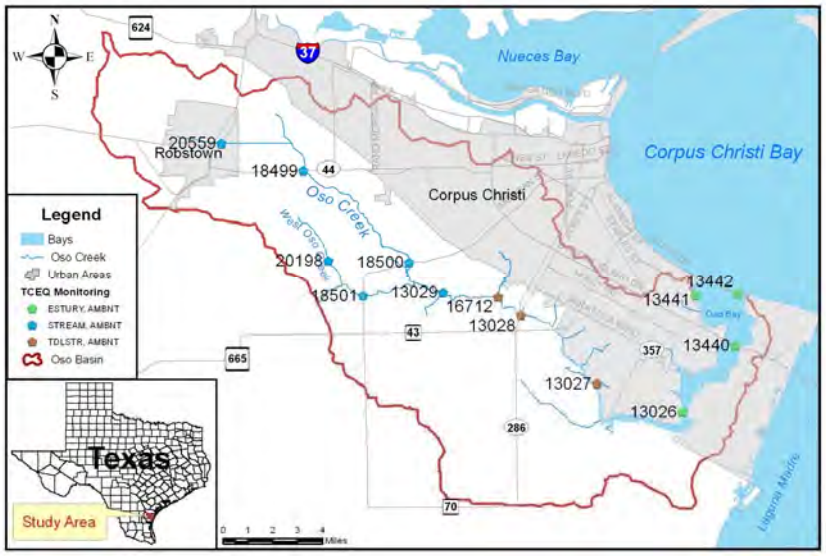
Results for Fresh Water Effluent (one time)

Human marker and fecal indicator bacteria results for waste water treatment plants

Source	Fecal coliforms	*Escherichia coli*	Enterococci	Markers detected
City of Taft	<1	<1	<1	0
City of Skidmore	<1	<1	<1	**3**
City of Bayside	73	69	950	0
Town of Refugio	<1	<1	<1	0
City of Beeville	<1	7	24	2 (HBa*c, smithii*)
City of Odem	<1	<1	35	0
Town of Woodsboro	<1	60	7	*2* (HBa*c, smithii*)
St. Paul WSC	127	560	1390	**3**
City of Sinton	65	152	1490	0
Pettus MUD	<1	<1	<1	0

Esp study in fresh water - Oso Creek

- Segment 2485A, flows into Oso Bay and ultimately Corpus Christi Bay
- Included on the Texas 303 (d) list of impaired waters for bacteria since 2002
- Receives effluent from Robstown Wastewater Treatment Facility
- Urban and agricultural runoff
- Elevated levels of enterococci starting at upper section of creek



Upper Oso Creek

- Larger study examining sources of contamination in upper section of Oso Creek – physical and animal sources.
- Library dependent BST – composite ARA/CSU profiles showed little human contribution, majority avian and non-avian wildlife with some livestock
- *Esp* analysis added as independent 'human' source evaluation
- Freshwater samples collected quarterly, three samples per site, from 5 sites in the upper Oso Creek

Site	07/07/10	09/13/10	10/18/10	12/06/10	01/19/11	03/09/11	04/20/11	08/08/11
18499A	-	-	-	-	-	-	-	-
18499B	-	-	+	-	-	-	-	-
18499C	-	-	-	-	-	-	-	-
18500A	-	-	-	-	-	-	+	-
18500B	-	-	-	-	-	-	+	+
18500C	-	-	-	-	-	-	+	+
18501A	-	-	-	-	-	-	-	NA
18501B	-	-	-	-	-	-	-	NA
18501C	-	-	-	-	-	-	-	NA
20559A	NA	-	NA	-	NA	-	NA	NA
20559B	NA	-	NA	+	NA	-	NA	NA
20559C	NA	-	NA	-	NA	+	NA	NA

esp Results: Freshwater

	7/7/10		9/13/10		10/18/10		12/6/10		1/19/11		3/9/11		4/20/11	
	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*	Ent* CFU 100 ml^{-1}	*esp*
OST18499A OST18499B OST18499C	988.3 ± 61.9	- - -	405.7 ± 81.9	- - -	513.3 ± 121.4	- + -	978 ± 160.2	- - -	3188.7 ± 433.0	- - -	633.3 ± 117.2	- - -	152.3 ± 14.7	- - -
OST18500A OST18500B OST18500C	1477.3 ± 219.1	- - -	1088.7 ± 178.9	- - -	983.3 ± 37.2	- - -	363.3 ± 31.8	- - -	2600 ± 96.4	- - -	503.3 ± 48.4	- - -	572 ± 36.0	+ + +
OST18501A OST18501B OST18501C	1044 ± 174.4	- - -	426.7 ± 43.3	- - -	423.3 ± 95.3	- - -	383.3 ± 235.5	- - -	3189 ± 48.5	- - -	97 ± 7.4	- - -	19.3 ± 8.7	- - -
OST20198A OST20198B OST20198C	NA		8233.3 ± 2281.3	- - -	NA		DRY†		NA		DRY†		NA	
OST20559A OST20559B OST20559C	NA		317.7 ± 29.9	- - -	NA		89.7 ± 6.7	- + -	NA		191 ± 18.2	- - +	NA	

*Ent CFU 100 ml^{-1} values are expressed as mean ± standard error (n=3).
† The sample location was dry, so water could not be collected.

Upper Oso Creek

- *esp* marker detected at three of the five sites
- 20559, immediately downstream of the Robstown WWTF
  - One sample positive for *esp* on 12/6/10 - enterococci 83 cfu/100 mL.
  - One sample positive on 3/9/11 - enterococci 160 cfu/100 mL[1].
- 18500 was the only site where the *esp* gene was detected in all three samples on 4/20/11 with *Enterococcus* 500 to 608 cfu/100 ml,
  - also detected in two samples from this site on 8/8/11.
  - Neither of these sampling events were preceded by any rainfall.

Summary

- All the human-specific molecular markers tested could be detected in fresh and marine waters of the Coastal Bend area of Texas
- Toolbox approach critical – LIM or LIM/LDM
  - Use of multiple markers increases the likelihood of detecting human fecal pollution when present
    - All markers never detected in the same sample at a marine site
  - Use of *esp* with LDM, added confidence in results of the LDM study for upper Oso Creek – both suggest that human contribution is not a major source of contamination

Summary - beaches

- Human fecal pollution appears to contribute to bacteria contamination at the beach sites
- *esp* detected only after rainfall, and with enterococci levels >1600 cfu/100 mL
- No advisory issued in 53% of instances human markers were detected

Summary (Esp)

- Limited detection of *esp* in marine samples may have been due to:
  - Non-human sources of enterococci during dry weather
  - PCR inhibition
  - Presence in concentrations below the minimum detection limit
  - Possible absence or low concentration in feces of local populations
- Freshwater:
  - lowest enterococci concentration of an *esp* positive sample was 83 cfu/100mL
  - InhibitEX effective (shown by controls)
  - Absence in samples with high levels of *Enterococcus* suggests that other sources are contributing to enterococci levels in the creek

Lessons learned and future directions

- Complexity of factors affecting results – what do they mean ? How much sampling/analysis needed to answer questions ?
- Need for stable funding to construct adequate study design, especially for initial testing of markers in geographic regions
- Develop a more comprehensive study using LIM in Coastal Texas watersheds - qPCR and markers from different hosts to quantify human and animal contributions
- Further investigate *esp* for potential use in conjunction with beach monitoring

Acknowledgements

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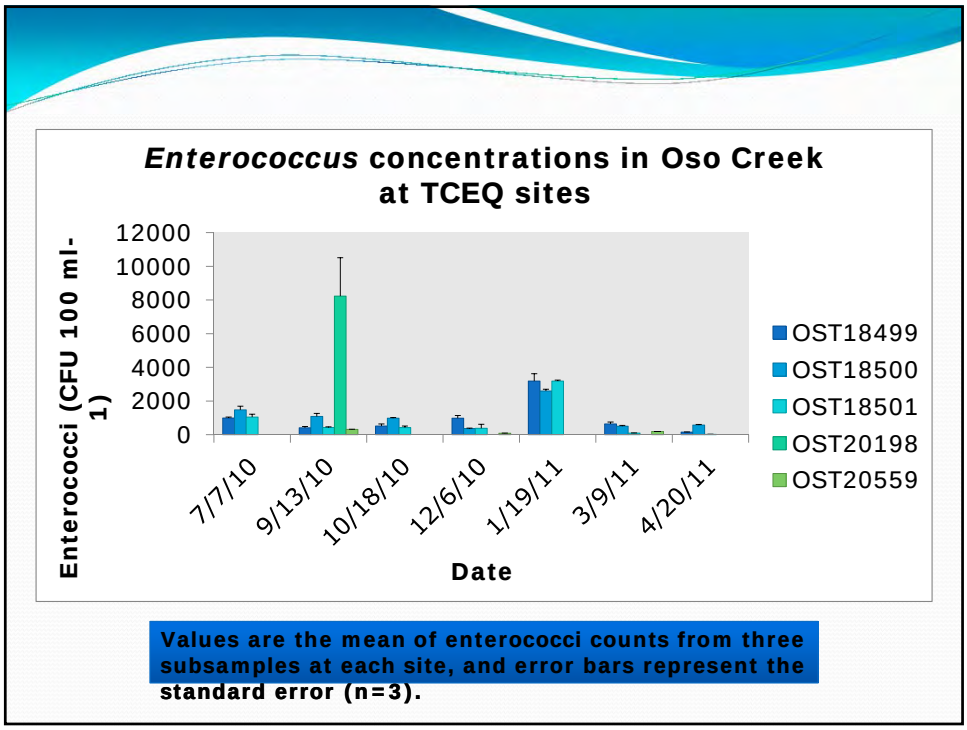
- Dr. Gregory W. Buck
- Richard Hay
- Roger Sealy and Michelle Lindsey
- Members of the Environmental Microbiology Lab., TAMU-CC


Enterococcus concentrations in Oso Creek at TCEQ sites
Enterococci (CFU 100 ml-1)
12000
10000
8000
6000
4000
2000
0
7/7/10
9/13/10
10/18/10
12/6/10
1/19/11
3/9/11
4/20/11
Date
OST18499
OST18500
OST18501
OST20198
OST20559
Values are the mean of enterococci counts from three subsamples at each site, and error bars represent the standard error (n=3).

Review of Water Quality Models:
Current Capabilities & Limitations

R. Srinivasan, Jeff Arnold, A. Sadeghi, Claire Buffet
Texas A&M University and USDA-ARS

Water Quality Models

- *Spatially Explicit Statistical Models (LDC, ArcHydro, SELECT and SPARROW)*
- *Mass Balance (MB) Methods (BLEST, BSLC, BIT)*
- *Mechanistic Hydrologic/Water Quality Bacteria Models (HSPF, SWAT, SWMM, WSAP)*

Bacteria Modeling Matrix

Model		LDC	Spatial Explicit Statistical Models			Mass Balance Models			Mechanistic/Hydrologic/WQ			
			ArcHydro	SPARROW	SELECT	BLEST	BSLC	BIT	HSPF	SWAT	SWMM	WASP
Watercourse Type	Watersheds		x	x	x	x	x	x	x	x	x	
	River/Stream	x	x	x	x	x	x	x	x	x		x
	Lake/Reservoir		x	x	x	x	x	x				x
	Fresh/Saltwater Estuarine		x	x	x	x	x	x				x
TMDL Phase	Development	x	x	x	x	x	x	x	x	x		x
	Implementation		x			x			x	x		x
Model Type	Analytical	x	x	x	x	x	x	x				
	Numerical								x	x	x	x
Spatial Dimensions	1-D			x	x				x	x	x	x
	2-D											x
	3-D											x
Time Scale	Steady-state			x						x		x
	Time Varying								x	x	x	x
	Single Storm Event				x				x	x	x	
	Continuous in time			x					x	x	x	x
Watershed Characteristics	Rural	x	x	x	x	x	x	x	x	x		
	Urban	x	x	x	x	x	x	x	x	x	x	
	Sediment transport			x	x				x	x		x
In-Stream Processes	Bacteria Regrowth											
	Bacteria Die-off			x					x	x		
	Settling								x	x		
	Re-suspension					x			x	x		
WLA Sources	WWTF			x	x	x			x	x		x
	Storm Sewers			x	x	x			x	x		x
LA Sources	Septic Tanks			x	x	x	x	x	x	x		
	Direct Deposition					x	x	x	x	x		x
	Bed Sediment					x			x			x

Sources of Pathogens

- **Water Pollution (nutrients, pathogens & sediment)**
  - **Point Source – direct entry of wastes into water supply, easier to identify & control**
  - **Non-point source - more difficult to identify & control**
    - **The source of bacterial pollution in stream can potentially originate from various sources**
    - **The effective treatment and control normally demand a more comprehensive solution that usually necessitates the consideration of many watershed or basin factors**

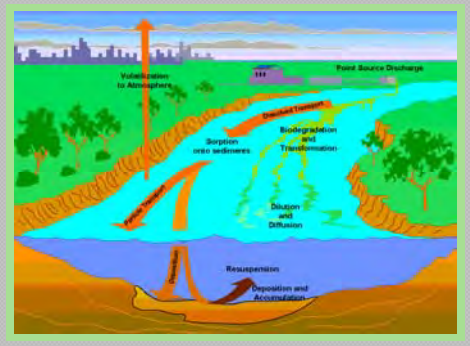
Source of Pathogens

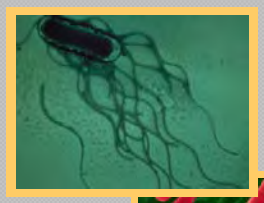
- Despite the many potential sources of release of pathogenic organisms into the environment, agronomic practices that utilize animal manures, contaminated with pathogenic or parasitic organisms, appear to be the major contributors to watershed or basin contaminations (USEPA, 1998).

- **The Animal Feeding Operations (AFOs) have been cited as one of the agricultural activities that can adversely impact environmental and public health (USEPA, 1994). High rates of land-applied manure increase the risks of surface or ground water contamination, both from excess nutrients and pathogenic organisms.**
- **Unfortunately, current technologies are not adequate for handling large-scale treatment processes. Therefore, modeling capabilities should be extended to account for individual and cumulative impacts of various pollutants and pollutant sources.**

Approach

- Existing model was modified by incorporating a comprehensive microbial fate and transport sub-model and validate the resulting model at the field and watershed levels.



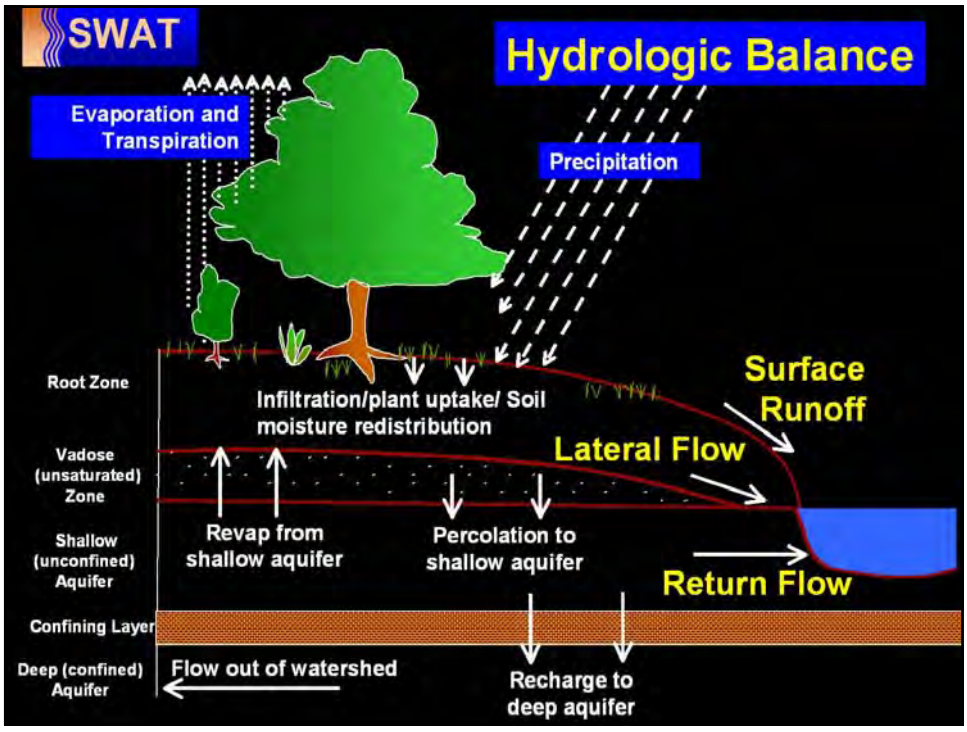


Salmonella sp.

Escherichia coli

Major Components of Deterministic Models

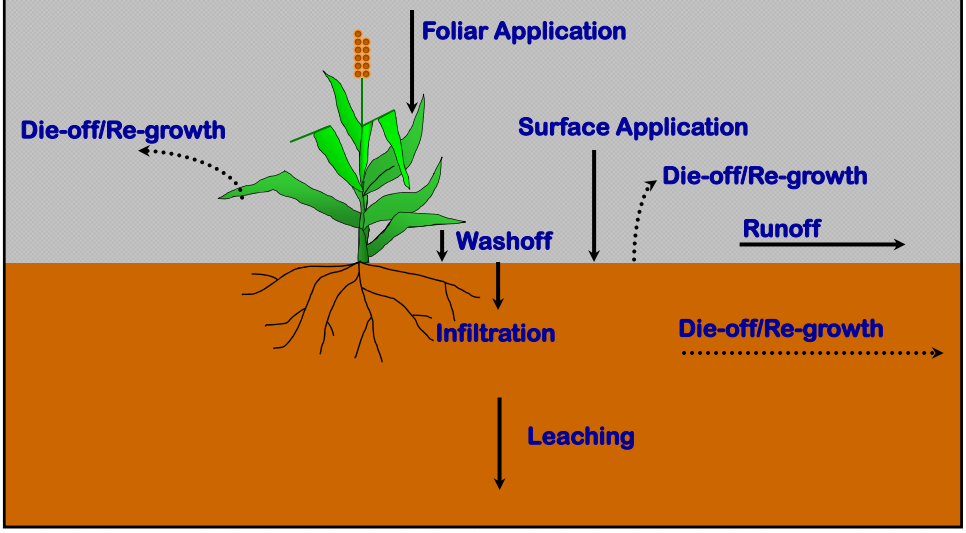
- Hydrology (water balance)
- Weather (actual/simulated)
- Sediment
- Crop Growth
- Nutrients
- Pesticides
- Groundwater & Lateral Flow
- Management Scenarios
- Bacteria



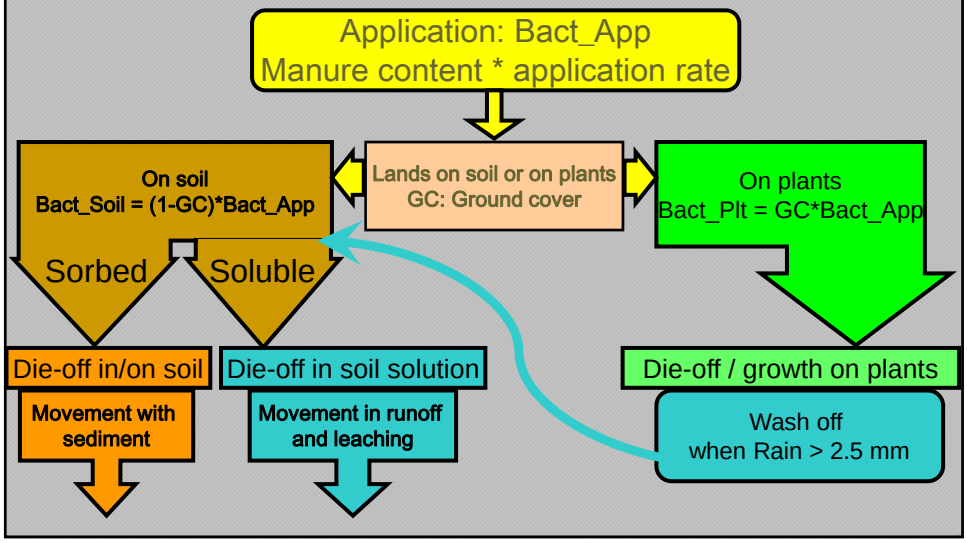
Fate and Transport of Pathogens

- Surface loadings
- Direct stream inputs
- Adsorption coefficient BactKdDB
- Runoff extraction coefficient BactKdQ
- Enrichment coefficient
- Decay rates (soil solution, sediment, streams, reservoirs)

Bacteria Fate



Bacteria Processes



Removal of Bacteria from the Soil

- Dissolved bacteria can be removed by runoff and leaching.
- Adsorbed bacteria can be transported by moving sediment.

What Happens After That?

- Bacteria arrives in the stream.
- There is more decay, at a rate characteristic of the stream.

Fate and Transport in-stream Processes

- Deposition → **Manure spreading, grazing, point sources**
- Adsorption → $\frac{Bacteria\_solution}{Total\_Bacteria} = K_d DB$
- Extraction by runoff → $Bact\_runoff = \frac{Bacteria\_solution * Runoff}{bulk\_density * 10 * Kd\_Q}$
- Sediment extraction → **Enrichment ratio concept**
- Decay → **Hick's Law: 1st order decay**

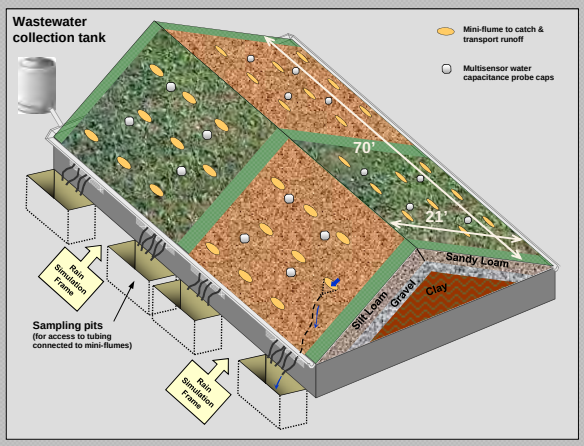
Degradation

- First-order kinetics
- Different degradation rates:
  - In the soil, attached to sediment
  - In soil solution
  - On foliage (i.e. when exposed to air)
  - In the stream
  - In a pond or reservoir

Model Testing & Validation

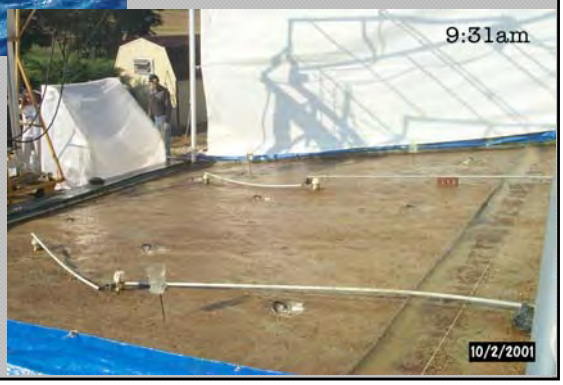
➢**For further "fine-tuning" of the pathogenic fate and transport functions in the model, data from two large-scale lysimeters (ca. 20 x 14 x 3 m), were used.**

➢**The lysimeters were modified to allow examination of leaching and runoff as a function of soil texture, vegetation cover, and slope.**



Lysimeter in Beltsville, Maryland used for model validation

Manure application and overland flow sampling in grass and bare plots



Each lysimeter has a gutter at the lower edge to collect surface runoff.

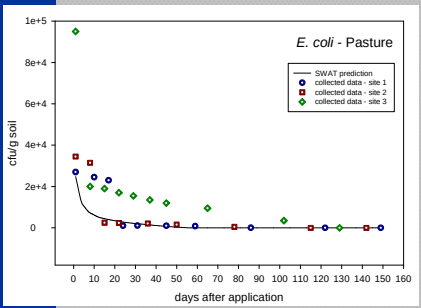
Soil sample collected for pathogen analysis within the top 20 cm soil depths.

Runoff sample volumes were recorded prior to sub-sampling for laboratory analysis.

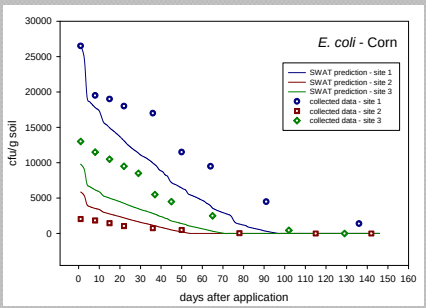
Model Testing & Validation

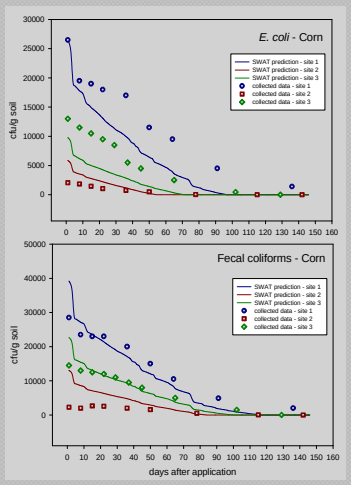
E.Coli Concentrations in Topsoil After Manure Application

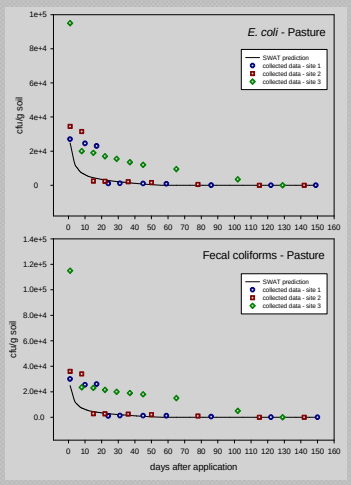
Pasture



Corn







For the initial testing and validation of the model, data from field and watershed studies for both "pasture" and "crop field" conditions in Virginia (Virginia Polytechnic Institute & State University) have been used. Considering the complexity of the processes involved, the model predictions appear to portray the general patterns of the fate and transport of bacteria observed in the three sites examined.

Important Considerations for Bacteria Modeling

- **The model used will only be as good as the data used to develop it.**
- **Models should be used as part of the TMDL framework (not as an only tool for decision-making)**
- **Models should continually evolve as the knowledge base develop.**
- **Bacteria regrowth and decay are not well represented.**
- **Detailed models allow for spatial and temporal analysis.**
- **Sensitivity and uncertainty in data, parameters and models**

Verification of *E. coli* Sources in Watersheds using GIS Tools & Bacterial Source Tracking

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Biological and Agricultural Engineering

Texas A&M University

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T. A. Burthold
K. Wagner
TWRI

Brazos River Authority
GBRA

Texas State Soil and Water Conservation Board

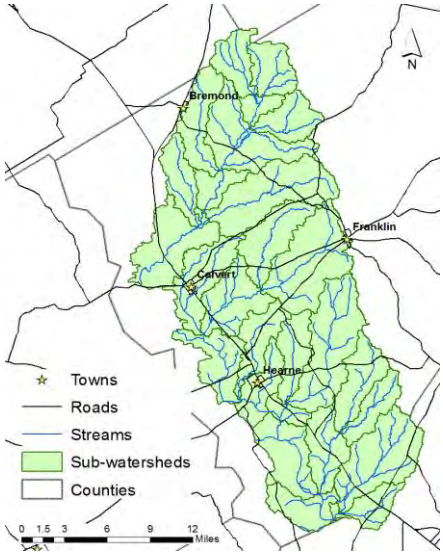
Spatially Explicit Load Enrichment Calculation Tool (SELECT)

- Bacteria load assessment tool
- Characterizes potential *E. coli* sources
- Estimates daily potential *E. coli* loads
- Utilizes spatial data in GIS to pinpoint areas of concern for bacterial contamination

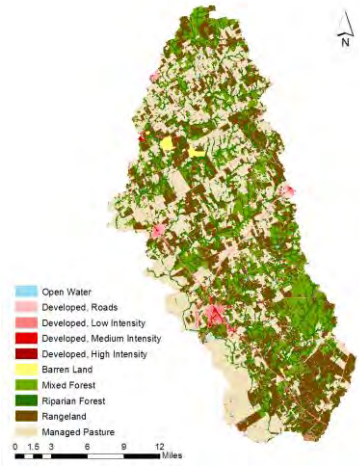
SELECT Input Data

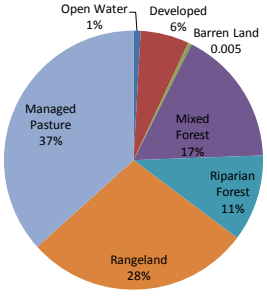
- Census Blocks (US Census Bureau)
- Soils (USDA-NRCS)
- Digital Elevation Map (BASINS)
- Urban Areas (TCEQ)
- Sub-watersheds & stream network (BASINS)
- Livestock
  - Stakeholder input
  - Agricultural densities (USDA)
  - Poultry Operations within the watershed (TSSWCB)
- Wildlife
  - Stakeholder input
  - Wildlife experts input, Resource Management Unit data for Deer (TPWD)

Little Brazos River Watershed



Little Brazos River Watershed - Land Use





Land Use Classification	Acres
Managed Pasture	98183
Rangeland	75187
Mixed Forest	45526
Riparian Forest	29015
Developed, Roads	11750
Developed, Low Intensity	3644
Open Water	2387
Barren Land	1242
Developed, Medium Intensity	616
Developed, High Intensity	203

E. coli Source - Cattle

Range Cattle

- Density: 5 acres per animal
- Estimated Population: 28238
- Land Use
  - Rangeland
  - Mixed Forest
  - Riparian Forest

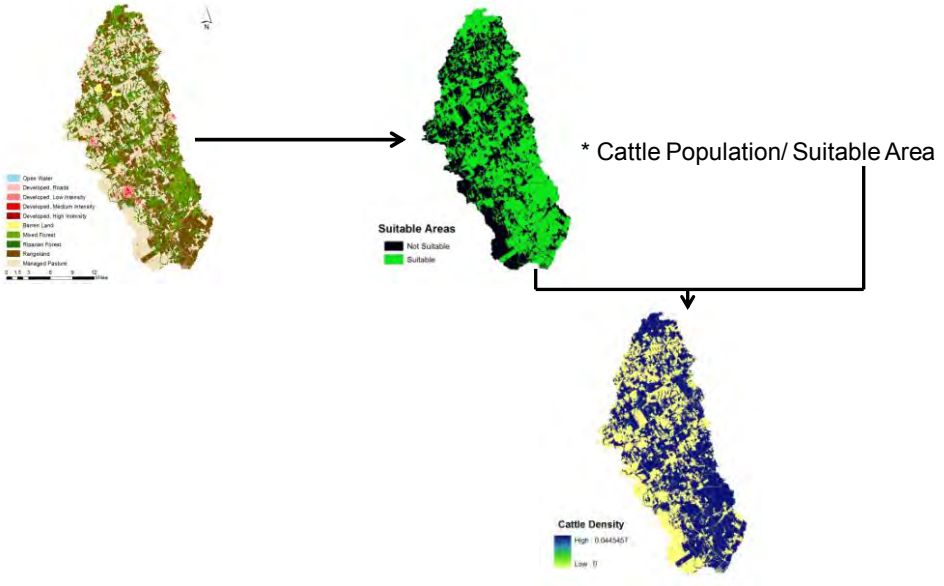
Pasture Cattle

- Density: 2 acres per animal
- Estimated Population: 44603
- Land Use
  - Managed Pasture

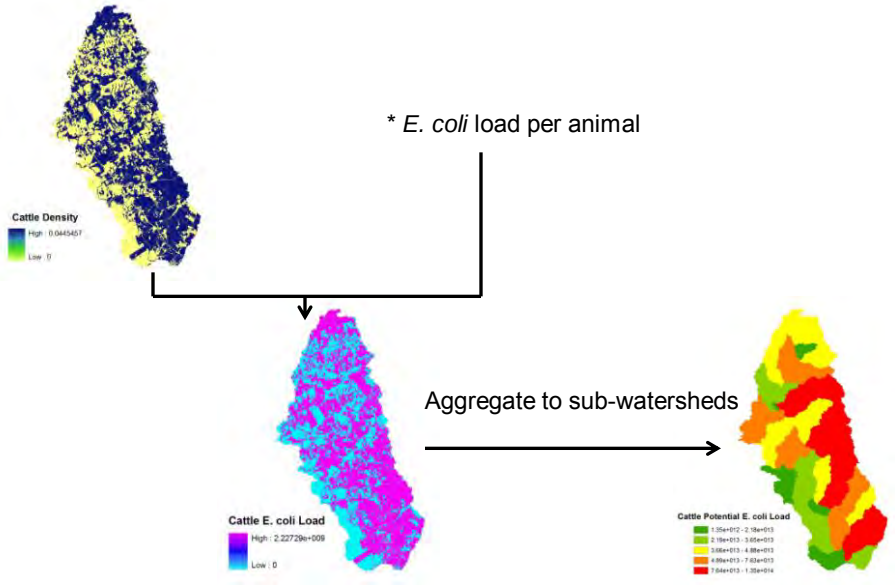
E. coli Load per head of cattle

- 10 x 10^{10} Fecal Coliform = 5 x 10^{10} *E. coli*

Distributing Cattle Over Suitable Areas



Calculating *E. coli* Load from Cattle



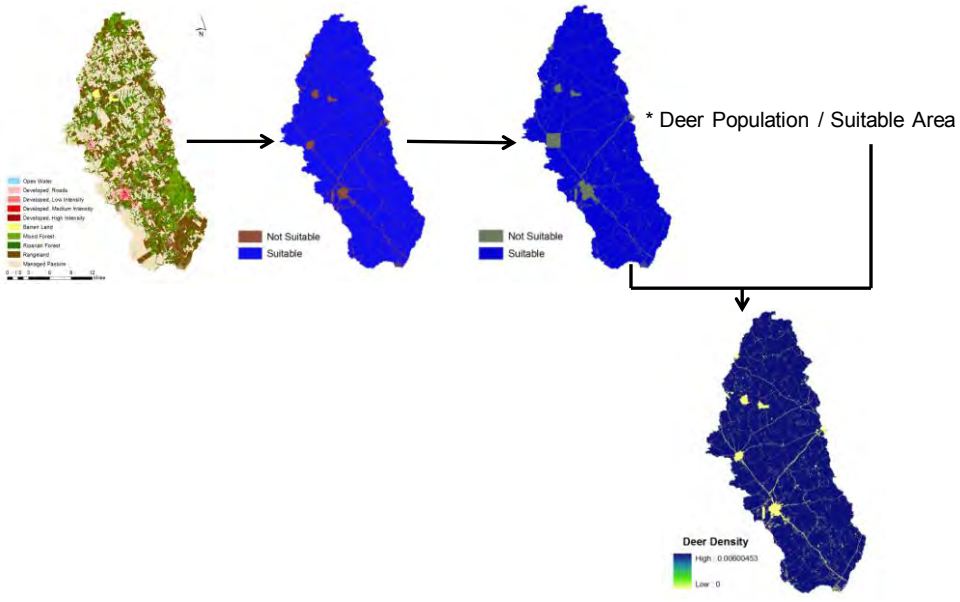
E. coli Source - Deer

- Density: 37 acres per animal
- Land Use
  - Rangeland
  - Managed Pasture
  - Mixed Forest
  - Riparian Forest

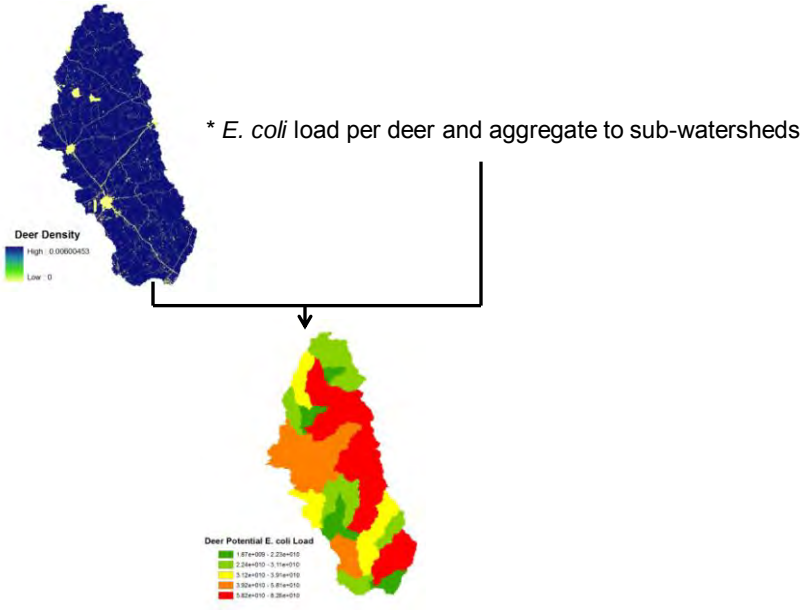
E. coli Load per Deer

•3.5 x 10^8 Fecal Coliform = 1.75 x 10^8 *E. coli*

Distributing Deer Over Suitable Areas



Calculating *E. coli* Load from Deer

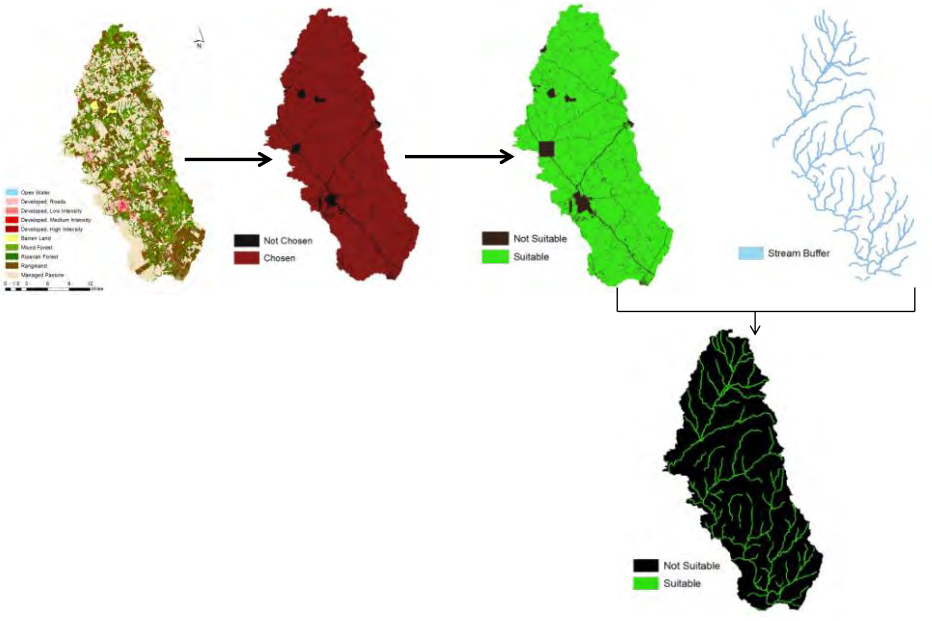


E. coli Source – Feral Hog

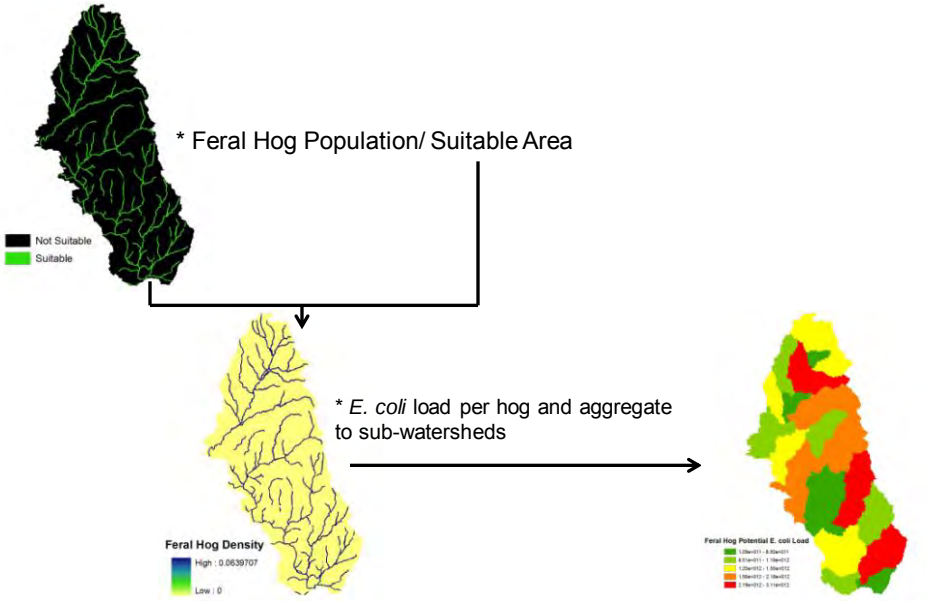
- Density: 20 acres per animal
- Land Use
  - Rangeland
  - Managed Pasture
  - Mixed Forest
  - Riparian Forest

E. coli Load per Hog
- 1.1 x 10^9 Fecal Coliform = 5.5 x 10^8 *E. coli*

Distributing Feral Hog Over Suitable Areas



Calculating *E. coli* Load from Feral Hogs

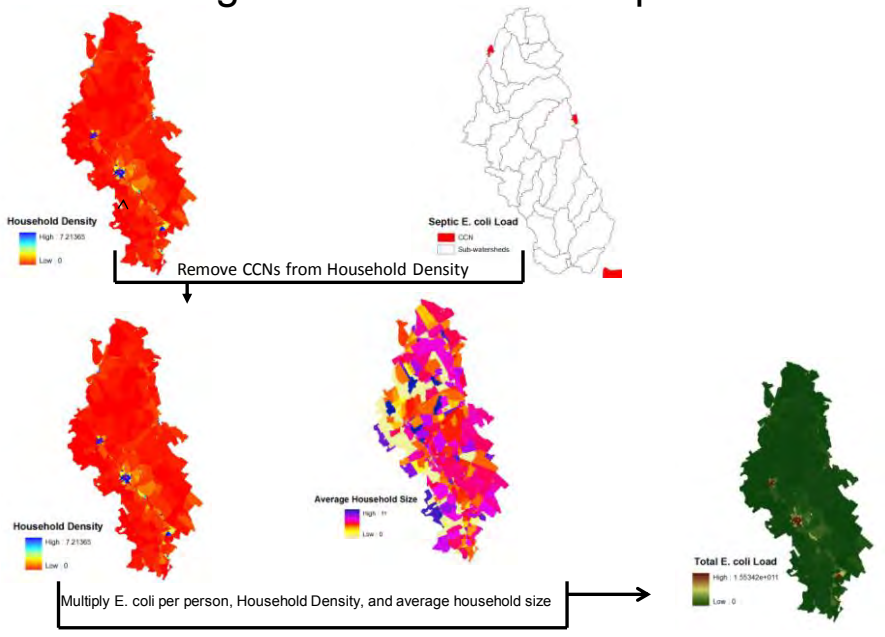


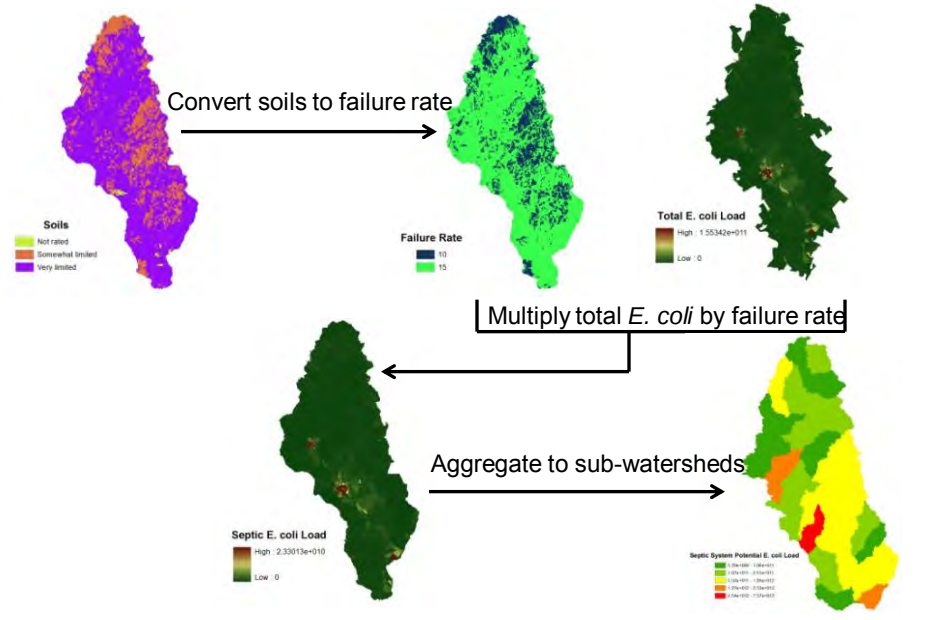
E. coli Source – Human (*septic system*)

E. coli Load = Number of systems failure rate people per home discharge concentration

- Number of systems: 2000 Census data
- Failure rate: SSURGO soils drain-field limitation class
  - Very limited: 15%
  - Somewhat limited: 10%
  - Slightly limited: 5%
  - Not rated: 15%
- People per home: 2000 Census data
- Discharge: 60 gallons per person
- *E. coli* Concentration: 5 10^6/100 mL

Calculating *E. coli* Load from Septic Failure

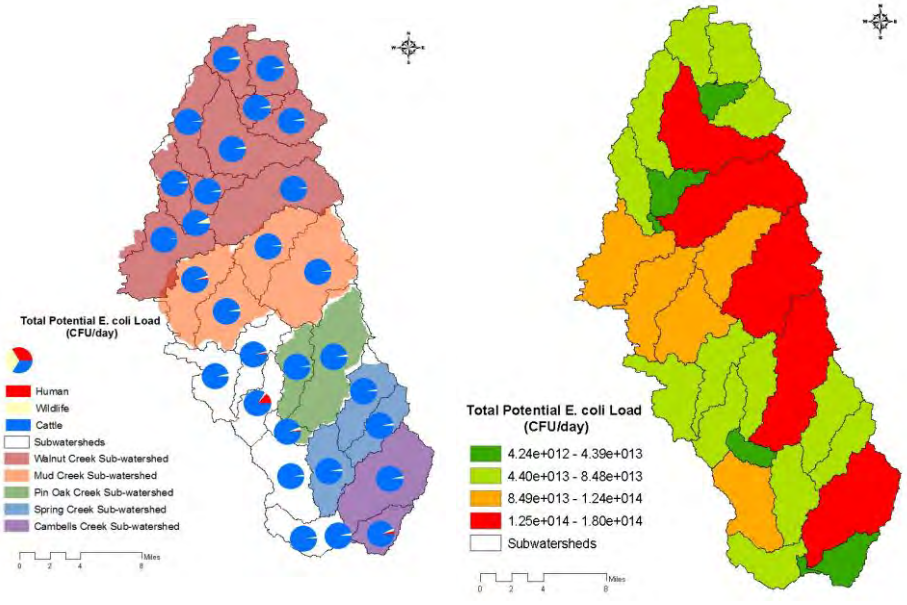




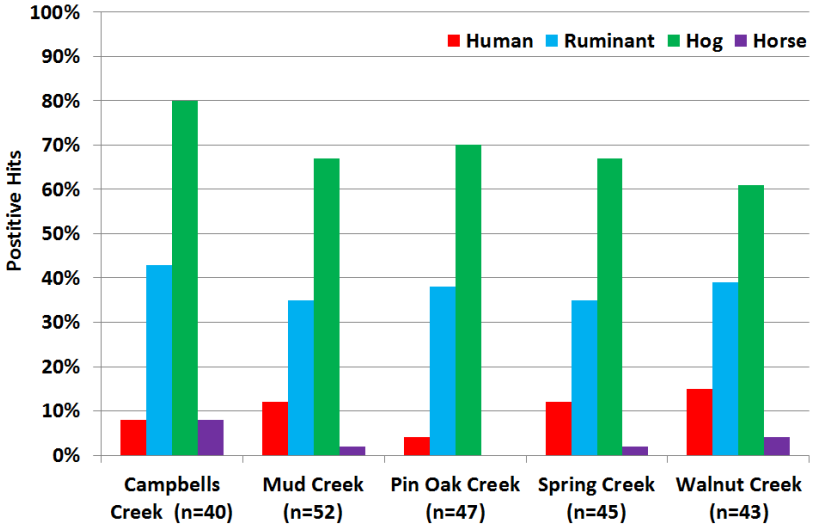
E. coli Source – Human (*WWTPs*)

- Wastewater treatment plants (WWTPs)
- A concentration of 126 CFU/100 mL was applied
- The maximum permitted discharge was used

Total daily potential *E. coli* load



Bacteroidales BST Results
Sub-Watershed Stream Samples



#

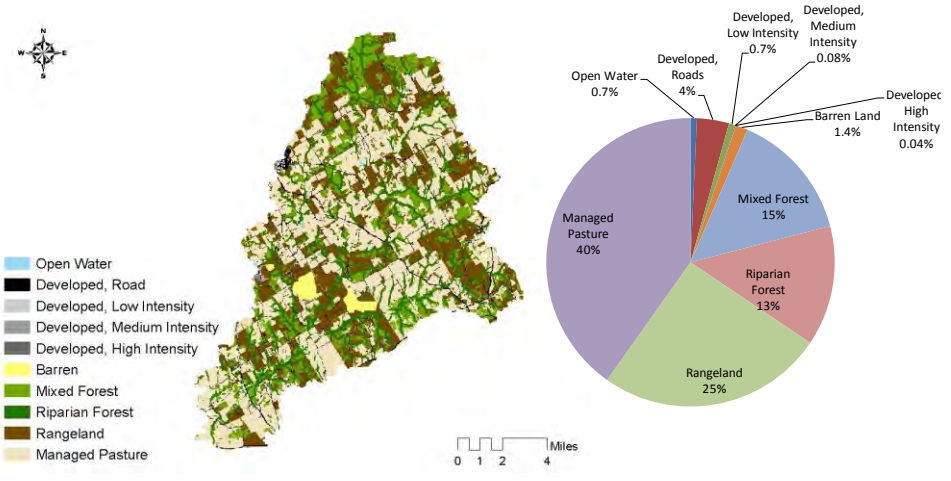
BST Summary

- Limited Library-Dependent Analysis
  - Existing Texas *E.coli* BST Library appears to be working relatively well (84% of isolates identified)
  - Major sources in watershed appear to be wildlife (feral hogs, deer, avian wildlife, and small mammals) and to lesser extent domestic animals (livestock and pets)

- Library-Independent Analysis
  - Hog marker detected most common (71%) followed by ruminant (39%)
  - Small percentage of human (9%) and horse (3%) hits

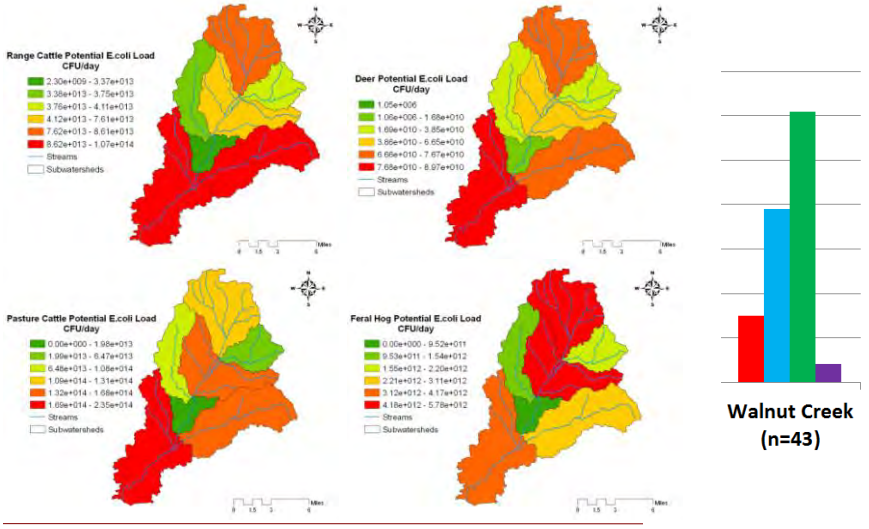
#

Walnut Creek – Land Use



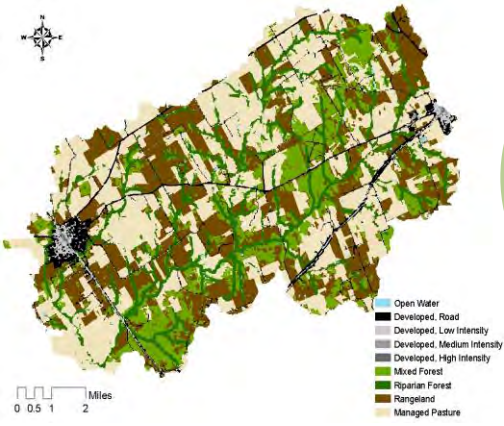
23

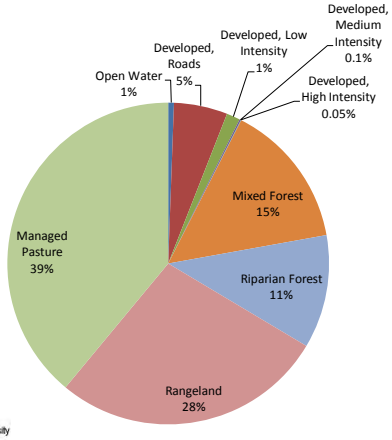
Walnut Creek - Potential *E. coli* loads



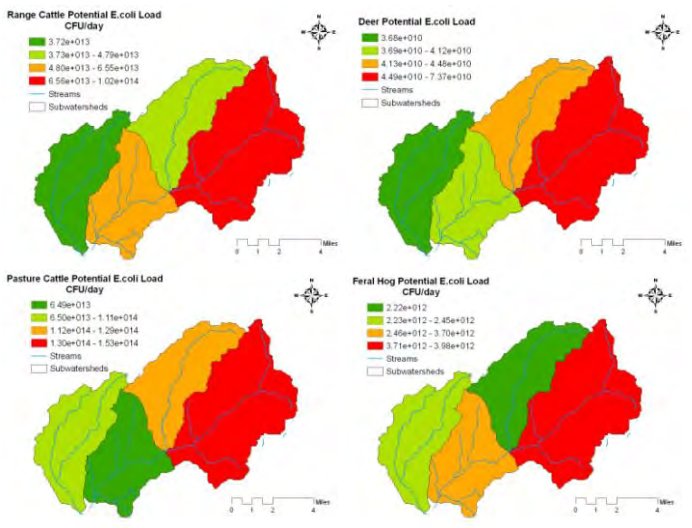
24

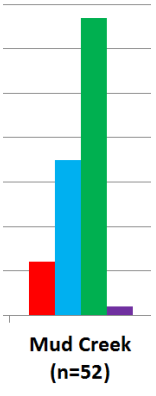
Mud Creek – Land Use





Mud Creek - Potential *E. coli* loads





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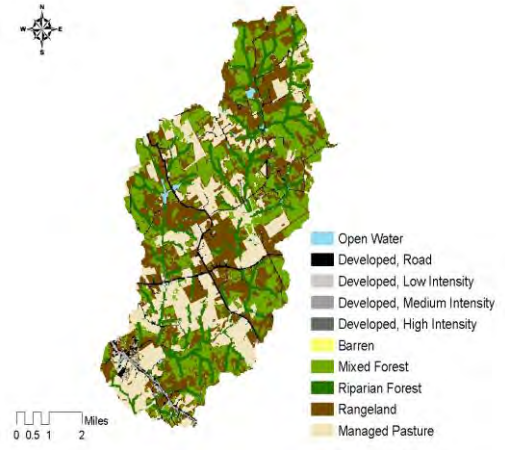
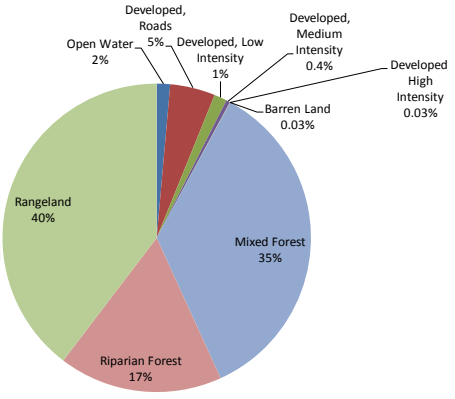
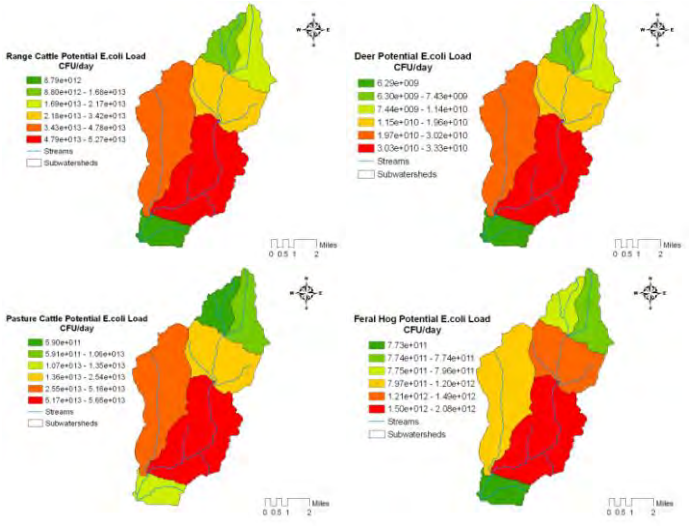
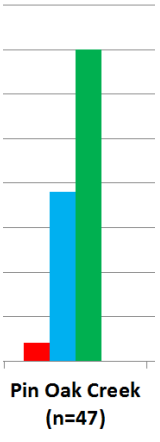




Pin Oak Creek - Potential *E. coli* loads





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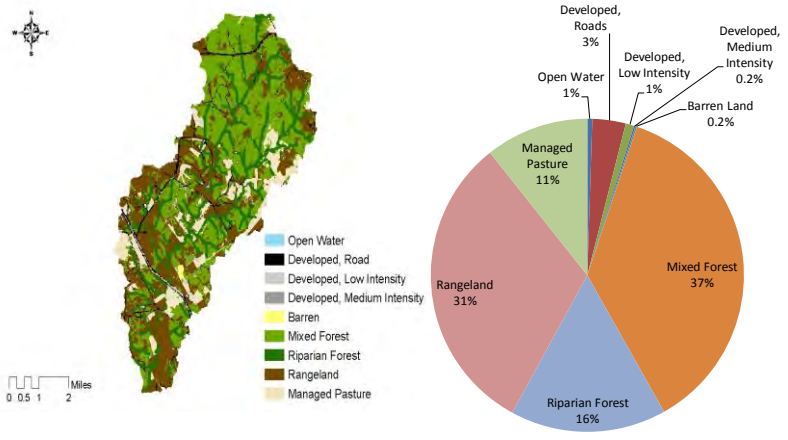
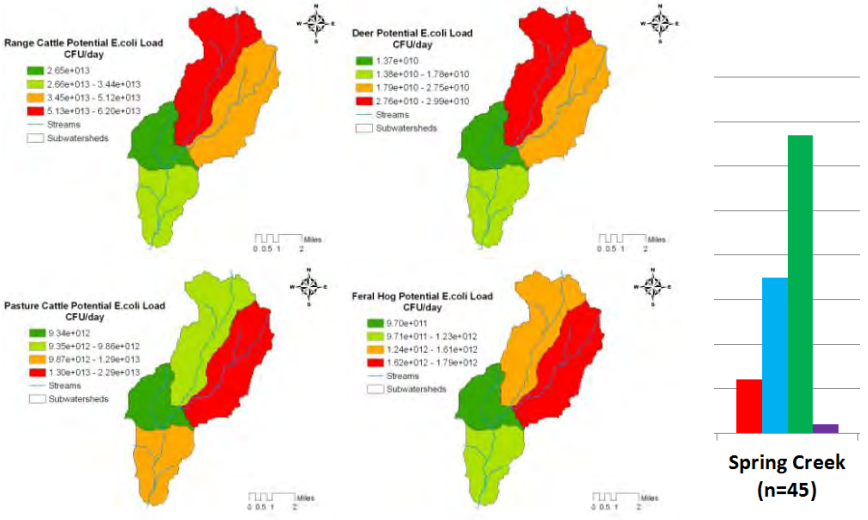
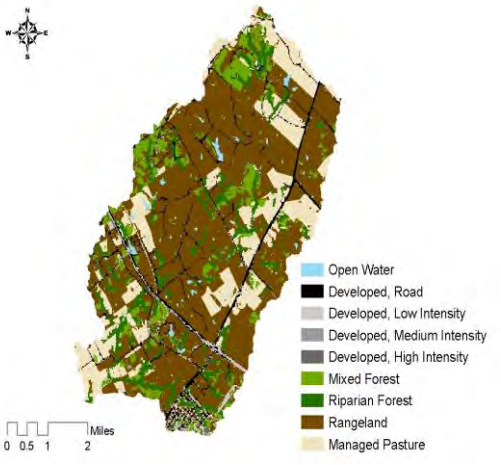
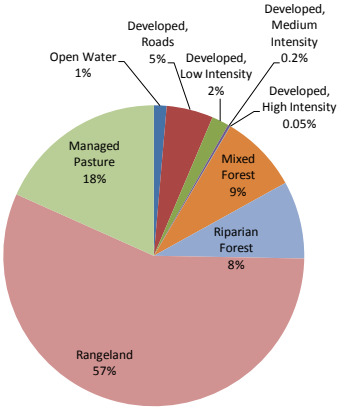


Spring Creek - Potential *E. coli* loads

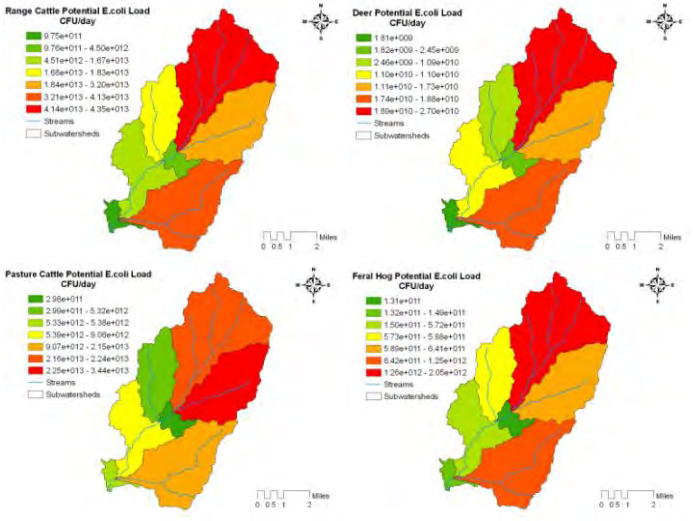


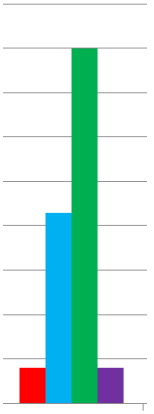
Campbell's Creek – Land Use





Campbell's Creek - Potential *E. coli* loads





Section 4: Poster Abstracts

Evaluation of Bacteroides qPCR for Assessing Cattle Fecal Contributions in Runoff from Grazing Lands

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Excessive levels of fecal indicator bacteria (e.g. *E. coli*, *Enterococcus*, and fecal coliforms) are a major cause of water quality impairment. Better analytical methods are needed to quantify the proportion of bacterial loading contributed by the various sources of bacteria so appropriate restoration goals can be established and restoration efforts targeted. This study evaluated (1) the ability of quantitative polymerase chain reaction (qPCR) analysis of the bovine-associated *Bacteroides* marker, BoBac, to accurately assess the percentage of bovine-associated fecal contamination at the small watershed scale and (2) the relationship between the total *Bacteroides* marker, AllBac, and *E. coli* levels and its relevance as a fecal indicator.

Data suggest the AllBac and BoBac markers are good indicators of recent fecal contamination from cattle. However, although elevated BoBac/AllBac ratios generally aligned well with the presence of cattle, the ratio appeared to underestimate the percentage of bovine-associated fecal contamination. *E. coli* levels were strongly correlated with the AllBac and BoBac markers for one watershed (from which the feces used to generate gene copy curves were collected), but they were not well correlated for the other two watersheds in the study. This suggests a geographic bias in the markers and that feces for development of gene copy curves for future studies should be collected from the watershed being assessed in order to reduce potential errors resulting from geographic variability in *Bacteroides* populations.

These markers appear to be useful tools for identifying sources of fecal contamination; however, more work is needed to improve their ability to accurately quantify total and source-specific bacterial loading before implementation at the watershed scale.

Rapid Real-Time PCR Method for Bacterial Source Tracking Using DNA FRET Probes

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One subspecies of the bacterium *Xylella fastidiosa* is known to cause Pierce's disease (PD), which is the major factor limiting winegrape production in Texas. Other subspecies and strains of *X. fastidiosa* are difficult to discriminate from PD-causing types, and have hindered accurate epidemiological assessments of disease threat. We have developed a 10 locus genotyping method using real-time PCR with adjacent-hybridizing DNA Fluorescence Resonance Energy Transfer (FRET) probes that quickly and accurately distinguishes between *X. fastidiosa* subspecies and strains. The method is very rapid (1.5 hours), inexpensive (~$0.50/sample), and could be applied to fecal indicator bacteria for the purpose of microbial source tracking.

Keywords: real-time PCR, FRET, Microbial Source Tracking

Tracking Non-point Fecal Pollution in the Guadalupe River: Distinguishing Urban and Rural Influences upon Water Quality

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Non-point fecal pollution is a problem in water bodies influenced by agricultural as well as urban runoff; tracking non-point pollution sources has always presented a challenge. Molecular markers for source-specific fecal bacteria can be used to identify and manage such sources. We attempted to distinguish between agricultural and urban influences upon the river water quality by analyzing coliform bacteria in the Guadalupe River at four locations from Seguin to Victoria. Goff Bayou at Highway 35 served as a control sampling point. Molecular fingerprints were produced by membrane filtration, EMB cultivation, and rep-PCR of coliform-like colonies with BOXA1R primers, followed by agarose gel electrophoresis. Digitized fingerprints were subjected to maximum likelihood treeing analysis. We detected three major clusters of coliforms; representatives of one were found in both urban and rural locations, while the remaining two were unique to urban stations only. Our results indicate that urban areas present their own unique fecal pollution sources, necessitating site-specific management strategies.

Evaluation of Human and Cattle Host Specific Genetic Markers for Bacterial Source Tracking in a Small Urban Watershed

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Accurate identification of sources and the extent of fecal contamination in an impaired watershed is crucial for developing best management practices. In this study, we evaluated human- and cattle-specific Bacteroidales genetic markers for their applicability in Alabama and used the most suitable primer sets in qPCR assays to assess fecal contamination in environmental samples. Four human- and seven cattle-specific genetic markers were evaluated. HF183, targeting the 16S rRNA gene of Bacteroidales, and CowM3, targeting the sialic acid-specific 9-O-acetylesterase secretory protein gene, appeared to be the best human and cattle markers, respectively. DNA extracted from water samples collected from an urban stream was amplified with general Bacteroidales primers as well as human- and cattle-specific primers. E. coli were enumerated simultaneously Results indicate that *E. coli* were present in all samples and the numbers varied from 40 to 5340 CFU/100 ml. The general Bacteroidales marker was also positive for all samples, with gene copies ranging from 366 to 1,289,898 copies/100 ml. A positive correlation between *E. coli* and Bacteroidales was observed. The human-specific genetic marker was detected in 90% of the water samples, while only 23% of the samples contained cattle-specific markers above the detection limit. The HF183 and CowM3 qPCR assays appeared to be suitable for identification of fecal contamination sources in Alabama.

Turtle Populations as a Potential Source of *E. coli* in Lake Elmendorf

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Fecal coliform bacteria, including *E. coli*, are commonly used as an indicator to assess water quality. Lake Elmendorf, an urban water source on the west side of San Antonio in the San Antonio River watershed, has had historically poor water quality, including high levels of *E. coli*. There are many potential sources for the bacterial pollution, and a bacterial source tracking project has been proposed to identify the primary sources. Fecal coliforms are reported to colonize only the gastrointestinal tracts of warm-blooded animals (birds and mammals). However, some studies have indicated that coliforms may also colonize the gastrointestinal tracts of some reptiles, including turtles. As part of a microbial source tracking study of Lake Elmendorf, we asked whether the local turtle populations are a potentially important source of *E. coli*. In summer 2011, we initiated a study in which we collected 30 turtles representing 3 of the 4 species residing in the lake. We rinsed each turtle in fresh water and obtained negative-control and cloacal swabs, which were used inocula for a presence/absence test for coliforms and *E. coli* using Colilert™ medium. Of the 23 turtles with an appropriate negative control result, 17 turtles (73%) produced a cloacal swab that was positive for *E. coli*. Although there are some limitations of our study, these results suggest that, at least in certain environments, turtles should be considered a potential source of *E. coli* and possibly other fecal coliforms.

Keywords: coliform, *E. coli*, Colilert, San Antonio, turtle, reptile, Trionyx, softshell, Trachemys, slider, Sternotherus, musk turtle

Large Heronries Contribute *E. coli* and Nutrient Loads to Waterbodies

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The impairment of rivers and streams by pathogens as indicated by the detection of high levels of *Escherichia coli* has been a problem in Texas for many years. Over half of the waterbodies designated for contact recreation in Texas are listed as impaired by bacteria. Although several analytical techniques have been used, there remains a moderate level of difficulty in identifying and quantifying *E. coli* sources. Herons and egrets such as cattle egrets (*Bubulcus ibis*) are known to establish large colonies in coastal areas and inland in close proximity to water. No information is available on the *E. coli* and nutrient loads contributed to Texas watersheds by these colonial waterbirds. The objectives of this preliminary study were to determine the potential contribution of *E. coli* and nutrient loads from large heronries located near selected waterbodies in Texas. In the summer of 2011, three colonies were studied (Murphy Park, Taylor, TX; Lake Conroe, Conroe, TX; and Richland Creek, Streetman, TX) The size of each colony was estimated and fecal material was collected from each colony. Water samples were collected beneath and from two sides of the colonies. All samples were enumerated for *E. coli* and concentrations of nutrients were quantified. Geometric means of *E. coli* in all water samples taken from both Murphy Park (130 to 8,400 cfu/100ml) and Richland Creek (75,000 cfu/100ml) exceeded the criteria for primary contact recreation set by the Texas Commission on Environmental Quality (126 cfu/100ml). Nutrient concentrations in the fecal samples were found to be approximately 4 orders of magnitude greater than that of the water samples. At Murphy Park, the average nitrogen (N) and phosphorus (P) concentrations in the fecal samples were 95,916.7 and 7,191.3 mg/L respectively compared to 3.5 and 0.4 mg/L in the water samples. At Lake Conroe, the average N and P concentrations in the fecal samples were 92,845.7 and 9,705.6 mg/L respectively compared to 1.3 and 0.1 mg/L in the water samples. These preliminary results establish a foundation for improving our understanding of the potential contribution of *E. coli* and nutrients from heronries to Texas watersheds and clearly demonstrate the need for further investigation. Such results will also contribute to the development of best management practices and other strategies to address bacterial and nutrient loads to Texas watersheds.

Comparison of the Diversity of *E. coli* Isolates Obtained from Surface Water Samples using Different Enumeration Methods

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Surface water contamination due to excessive levels of fecal indicator bacteria is a cofounding problem throughout the United States. Many bacterial source tracking (BST) projects rely on the library-dependent construction of an *E.coli* library from both known fecal sources as well as the impacted environmental area in order to identify a source(s) of the contamination. Multiple standard methods are widely accepted and utilized to enumerate and then isolate *E.coli*. These include traditional most probable number assays as well as membrane filtration methods, and are often used in combination or interchangeably in library construction. However, if different enumeration methods select for different *E. coli* populations, this could bias and/or confound BST results. To our knowledge, no evaluation of *E.coli* community compositional effects of these accepted methods has been conducted. The objective of this study was to evaluate differences in *E.coli* community composition across three standard water quality assessments including EPA Standard Method 1603, Colilert®, and mColiBlue24®. Enterobacterial repetitive intergenic consensus sequence-polymerase chain reaction (ERIC-PCR) fingerprinting was used to characterize a collection of 1000 isolates from three diverse environmental water samples and a known fecal source sample (cattle). Enumeration results show variability across the three techniques, with the EPA Standard Method 1603 and mColiBlue24® being most comparable while Colilert® indicated lower numbers of *E.coli*. Diversity analysis of the fingerprint library revealed the Colilert® communities to be much less diverse than the other media types. Similarity analysis shows very limited overlap in the communities across the three enumeration techniques with only approximately 10% of the isolates occurring in all three media types. Results of this study confirm the need for standardization of enumeration and isolation techniques utilized in library-dependent microbial source tracking applications.

Appendix A

Conference Participant List

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Appendix B

Conference Primer Materials

Conference Introductory Materials

Title	Author	Summary
Microbial Source Tracking presentation	Orin C. Shanks U.S. EPA Region 5	This presentation provides an overview of microbial source tracking including method classifications; library dependent and library independent methods; and an overview of the U.S. EPA's Microbial Source Tracking Guide Document.
http://water.rutgers.edu/Source_Tracking/MicrobialSourceTracking/MicrobialSourceTrackingEPApresentation.pdf		
Statewide Bacterial Water Quality Impairment Reduction Initiative	Texas State Soil and Water Conservation Board	The website lists the Texas State Soil and Water Conservation Board's efforts to address bacteria impairments across the state.
http://www.tsswcb.texas.gov/managementprogram/initiatives/bacteria		
Microbial Source-Tracking and Detection Techniques	U.S. Geological Survey	Links are provided on this website to general information on microbial source-tracking and detection techniques, such as ribotyping (DNA fingerprinting), genetic enterovirus detection using PCR/rtPCR and IC/PCR, and pulse field gel electrophoreses (PFGE).
http://water.usgs.gov/owq/microbial.html		
Microbial Source Tracking Fact Sheet	Michigan State University Center for Water Sciences	This document provides information on microbial source tracking; how it's done; and includes advantages and disadvantages of microbial source tracking.
http://cws.msu.edu/documents/Fact_sheet4_final.pdf		

Microbial Source Tracking and the TMDL (Total Maximum Daily Loads) Process	Charles Hagedorn, Brian L. Benham, Sara C. Zeckoski Virginia Tech Virginia Cooperative Extension	This website provides an introduction to microbial source tracking; methods; methods used in Virginia; how MST is used in the TMDL Process; and the future of MST.
http://pubs.ext.vt.edu/442/442-554/442-554.html		
US EPA Microbial Source Tracking Guide Document	US EPA Office of Research and Development	The intent of this guide document is to provide the reader with insight into various tools and approaches used to track sources of fecal contamination impacting water quality in streams, rivers, lakes, and marine beaches. Descriptions of research and several case studies gathered through workshops, literature searches, and phone interviews are also provided. An effort was made to showcase programs, activities, and analyses that incorporated diverse microbial source tracking approaches and tools.
http://www.ces.purdue.edu/waterquality/resources/MSTGuide.pdf		
Microbial Source Tracking: Library Based Methods	Thomas Atherholt New Jersey Department of Environmental Protection	This paper gives an overview of available microbial source tracking methods and includes advantages and disadvantages for each.
http://www.state.nj.us/dep/dsr/wq/technology-critique-dec.pdf		
Research Area: Microbial Source Tracking	Southern California Coastal Water Research Project	This website provides an overview of microbial source tracking projects as part of the Southern California Coastal Water Research Project.
http://www.sccwrp.org/ResearchAreas/BeachWaterQuality/MicrobialSourceTracking.aspx		

Texas Watershed Coordinator Roundtable: Bacteria Dynamics, Assessment Methods, and BMPs	Texas Water Resources Institute	Videos, presentations and summary notes are available on this website from a meeting hosted by the Texas Water Resources Institute in regards to bacteria dynamics, assessment methods, and best management practices.
http://watershedplanning.tamu.edu/developing/roundtable/july-27-2011/		
Bacteria Total Maximum Daily Load Task Force Final Report	C. A. Jones, K. Wagner, G. Di Giovanni, L. Hauck, J. Mott, H. Rifai, R. Srinivasan, G. Ward	The Task Force report describes the characteristics, as well as some of the strengths and weaknesses of several models that have been used and/or are under development to assist bacteria TMDL and I-Plan analysis. The report also describes and makes recommendations for effective use of BST methods that have been used in Texas and elsewhere for TMDL development.
http://twri.tamu.edu/publications/reports/2009/tr-341/		

Publications for Review		
Microbial Source Tracking: Current Methodology and Future Directions	Troy M. Scott, Joan B. Rose, Tracie M. Jenkins, Samuel R. Farrah, Jerzy Lukasik	Appl Environ Microbiol. 2002 December; 68(12): 5796–5803. DOI: 10.1128/AEM.68.12.5796-5803.2002

Microbial Source Tracking: State of the Science	Joyce M. Simpson, Jorge W. Santo Domingo, and Donald J. Reasoner U.S. Environmental Protection Agency, Office of Research and Development, Water Supply Water Resources Division, Cincinnati, Ohio	Environ. Sci. Technol., 2002, 36 (24), pp 5279–5288 DOI: 10.1021/es026000b Publication Date (Web): November 15, 2002
Microbial Source Tracking: Methods, Applications, and Case Studies	Charles Hagedorn, Anicet R. Blanch, and Valerie Harwood (eds.)	2011, 656 p., Springer http://www.amazon.com/Microbial-Source-Tracking-Methods-Applications/dp/1441993851/ref=sr_1_1?ie=UTF8&qid=1327943310&sr=8-1
Microbial Source Tracking	Jorge W. Santo Domingo and Michael J. Sadowsky (eds.)	2007, 300 p., American Society for Microbiology Press http://estore.asm.org/viewItemDetails.asp?ItemID=666

Appendix C

Speaker Biographies

Speaker Biographies

Dr. Elizabeth Casarez, a native of West Texas, is a Research Associate in Dr. George Di Giovanni's Environmental Microbiology laboratory at the University of Texas - Houston School of Public Health, El Paso Regional Campus. She received her Ph.D. in Toxicology with a minor in Soil, Water, and Environmental Sciences from the University of Arizona. She began studying bacterial source tracking as a post-doctoral research associate with Dr. Di Giovanni in 2004, using molecular techniques to determine the sources of water fecal pollution in Texas watersheds. That research was honored with a Texas Environmental Excellence Award in 2007. Her main research interest is the diversity of *E. coli* from different host sources and geographical regions. She is currently the curator of the Texas *E. coli* Bacterial Source Tracking Library.

Dr. George Di Giovanni is a Professor of Environmental and Occupational Health Sciences with the University of Texas - Houston School of Public Health, El Paso Regional Campus. He received his Ph.D. from the University of Arizona and did postdoctoral work as a National Research Council Associate with USEPA. Prior to joining UTHealth, he was a Professor and Faculty Fellow with the Texas A&M System and Senior Environmental Scientist for the American Water Works Company. His research program focuses on the detection and molecular analysis of waterborne pathogens including *Cryptosporidium*, *Giardia*, and viruses; and microbial source tracking to determine the sources of water fecal pollution. He is past Chair of the AWWA Microbiological Contaminants Research Committee and is a member of the Organisms in Water Committee. He and his research team have been honored with a Texas Environmental Excellence Award and he recently received the University of Arizona Alumni Professional Achievement Award.

Dr. Terry Gentry graduated from the University of Arkansas in 1993 with a B.S. in Agronomy and in 1998 with a M.S. in Agronomy (Soil Microbiology). He attended the University of Arizona where he completed his Ph.D. in Microbiology and Immunology in 2003. He did postdoctoral training from 2003-2005 in Environmental Microbiology at Oak Ridge National Laboratory. Since 2006, he has been an Assistant Professor of Soil and Aquatic Microbiology at Texas A&M University in the Department of Soil & Crop Sciences. Dr. Gentry's research program focuses on the development and use of molecular technologies to enhance the detection and remediation of environmental contamination. This includes the detection and identification of microbial pathogens from animal, human, and natural sources and also the characterization of microbial populations and communities contributing to applied remediation processes such as the bioremediation of organic and metal contaminants.

He has authored or co-authored 43 peer-reviewed journal articles, 123 abstracts of poster and oral presentations, and 4 book chapters. Dr. Gentry has developed and instructed a graduate-level course in Environmental Microbiology, co-developed and co-instructed an undergraduate/graduate course on Biofuels and the Environment, and also instructed an undergraduate/graduate Environmental Soil Science course. Dr. Gentry has served as major advisor or co-advisor for 5 postdoctoral associates and 16 graduate students and has served on 17 other graduate student committees during his tenure at Texas A&M.

Sally C. Gutierrez has been recently appointed as the Director of Environmental Technology Innovation Cluster Development and Support Program for the U.S. Environmental Protection Agency's Office of Research and Development. This new effort seeks to advance environmental protection in tandem with economic development

through the formation of public private partnerships among environmental technology companies, investors, researchers, economic development agencies, federal government agencies and others. Over the past year, she has been instrumental in the formation of the Cincinnati regional Water Technology Innovation Cluster. Prior to her appointment, she was the Director of the National Risk Management Research Laboratory (NRMRL) in Cincinnati, Ohio. NRMRL is one of three Federal research laboratories within the EPA's Office of Research and Development. The Laboratory is responsible for conducting engineering and environmental technology research to support the Agency in development of policy, regulations and guidance to further environmental protection in the U.S. The research staff consists of 400 environmental and chemical engineers, chemists, microbiologists, economists, hydrologists and other scientists and support staff. Key areas of research include: treatment and control of contaminants in drinking water, restoration of ecosystems, control of air pollutants, remediation of contaminated sites, environmental sustainability and environmental technology testing and development.

Sally was born and raised in Houston. She received a Master of Science degree from the University of Texas, School of Public Health in Houston. Her area of expertise is water resource management. She has spoken extensively on the topic of sustainable water resource management to a variety of technical and other audiences domestically and abroad.

She was appointed NRMRL's Director in 2005. Prior to this appointment she was the Director of the Water Supply and Water Resources Division with the Laboratory. During her tenure as Director of the Water Supply and Water Resources Division, she was responsible for leading a national technology demonstration program for control of arsenic in drinking water. Prior to coming to EPA, she was responsible for administering water programs for the State of Texas environmental agency in the areas of drinking water, water monitoring, wastewater treatment permitting, and utility rates.

As a member of the Senior Executive Service, she holds the highest career rank in the Federal government. She is a Registered Sanitarian in the State of Texas and a member of the American Water Works Association, the American Society of Civil Engineers and past President of the Texas Environmental Health Association.

Dr. Valerie (Jody) Harwood is an environmental microbiologist and a Professor in the Department of Integrative Biology at the University of South Florida, Tampa. She earned her Ph.D. in Biomedical Sciences at Old Dominion University and Eastern Virginia Medical School in Norfolk, Virginia. One of Dr. Harwood's major areas of expertise is microbial source tracking (MST), which endeavors to determine the source(s) of fecal pollution in water. She is a major contributor to the USEPA Guide Document on MST (*http://www.epa.gov/nrmrl/pubs/600r05064/600r05064.pdf*), and is the co-editor of Microbial Source Tracking: Methods, Applications and Case Studies (Springer Scientific, 2011). She is also interested in the persistence and ecology of enteric organisms in secondary habitats such as water and sediments. Harwood is the author of over fifty peer-reviewed papers on various areas of environmental micro and microbial ecology, including the efficacy of treatment for reclaimed water, the biochemistry of the hyperthermophile *Pyrococcus furiosus*, on *Vibrio* genetics, physiology, and detection in environmental waters, on phylogeny and antibiotic resistance of *Enterococcus* spp., and on MST and environmental persistence of fecal indicator bacteria and pathogens.

Dr. Charles Hagedorn is a professor in the Department of Crop and Soil Environmental Sciences at Virginia Tech. His research and outreach program at Virginia Tech addresses the public health aspects of pathogens in the environment, management of fecal microbes in waste treatment and application, the impacts of environmental release of genetically modified organisms, and determining sources of fecal pollution in water.

Dr. Hagedorn's scientific expertise has been recognized by awards of 78 state, private, and federal competitive research grants; publication of 136 refereed journal articles; 18 invited review articles; 10 invited book chapters; co editor of two books; 75 invited presentations at international, national, and state conferences; 23 invited

memberships on proposal review panels; 12 refereed bulletins; and 142 abstracts and presentation papers. Fourteen Ph.D. and twenty-two M.S. students have completed degrees under his direction and he has generated in excess of $5,135,000 in external grants and contracts to support his environmental microbiology program.

Over the past sixteen years, Dr. Hagedorn has been involved in the development of microbial source tracking methods and protocols, and has deployed these to determine sources of fecal pollution in 40+ projects in Virginia and 16 in other states and the District of Columbia, plus projects in Puerto Rico, Canada, Egypt, Spain, Tanzania, and China. His research program on microbial source tracking has been supported by competitive awards from the National Science Foundation, US Dept. of Agriculture-National Research Initiative, the EPA, the National Oceanic and Atmospheric Administration, and the US Geological Survey. His program has also been supported by contracts from state agencies, counties, municipalities, the private sector, and not-for-profit organizations including the Chesapeake Bay Foundation and the Friends of Rivers.

Part of his Professorship at Virginia Tech includes serving as a water quality specialist for the Virginia Cooperative Extension Service. In this regard, he has worked with the Virginia Department of Environmental Quality and the Virginia Department of Health over the past 20 years to perform on-site pollution and water quality evaluations at farms, homes, and communities throughout Virginia.

Dr. R. Karthikeyan is an Associate Professor in the Biological & Agricultural Engineering Department at Texas A&M University. He received his Ph.D. from Kansas State University. His research interests focus on engineering biochemical processes for water quality control and resource recovery. Dr. Karthi is currently serving as an Associate Editor for Transactions of ASABE and Applied Engineering in Agriculture and Section Editor for Journal of Natural and Environmental Sciences. He has received the College of Engineering BP Teaching Excellence Award, Excellence in Teaching Award in the Biological and Agricultural Engineering Department, and the Texas AgriLife Extension Service Superior Service Team Award for Plum Creek Watershed Protection Plan. He is also a Motague Teaching Scholar in the Center for Teaching Excellence.

Katherine McElhaney is a Research Associate in the Food & Environmental Microbiology Laboratory at Texas A&M University, where she works on microbiology projects associated with food, the environment, wastewater, and various types of irradiation. She completed her B.S. in Biology from Texas A&M University in 2008 and her M.S. in Food Science & Technology from Texas A&M in 2010. Her Master's thesis, "*16S rRNA-Based Tag Pyrosequencing of Complex Food and Wastewater Environments: Microbial Diversity and Dynamics*", focused on next-generation deep sequencing analysis of microbial communities in milk and sewage sludge. In addition to her laboratory-based work, she also works closely with the National Center for Electron Beam Research at Texas A&M University, assisting companies in commercializing E-Beam and X-ray irradiation technologies.

Dr. Joanna Mott is a Professor and Head of the Biology Department at James Madison University. She received her B.S. in Biological Sciences from the University of Aston in England, M.S. in Biology from the University of Waterloo, Canada and Ph.D. in Soil Sciences (Microbiology) from Texas A&M University. Dr. Mott previously held faculty and Chair positions at Texas A&M University-Corpus Christi in the Department of Life Sciences and affiliations with Texas A&M University and the Harte Research Institute.

As an environmental microbiologist, Dr. Mott's research in Texas focused on fecal contamination of coastal surface waters and estuarine pathogens, primarily *Vibrio vulnificus*. Her accredited laboratory (NELAP) worked on TMDL related issues for multiple coastal watersheds and monitored 52 beach stations for the Texas Beach Watch Program. She has utilized a variety of phenotypic and genotypic bacteria source tracking techniques to identify sources of contamination in coastal watersheds and continues to study survival, persistence and movement of fecal bacteria in the environment. She and co-PIs recently completed a multi-year investigation of sources of fecal bacteria in the upper section of Oso Creek, a watershed in the Coastal Bend area of Texas. Dr. Mott served on the Texas Joint

Technical Task Force on Bacteria TMDLs and is a member of Interstate Sanitary Shellfish Conference committees, the Gulf of Mexico Alliance Water Quality Team and Pathogens Working Group.

Dr. Michael J. Sadowsky is a Professor in the Department of Soil, Water and Climate; and Director of the BioTechnology Institute at the University of Minnesota in St. Paul. He studied at the Department of Bacteriology at the University of Wisconsin-Madison, and received his Ph.D. in Microbiology from the University of Hawaii in 1983. Between 1983 and 1985, Dr. Sadowsky did postdoctoral research at the McGill University in the plant-microbe interactions group of the Plant Molecular Biology laboratory. He worked shortly for Allied Corporation as a Molecular Biologist and then worked for the USDA in Beltsville, Maryland for several years in the Nitrogen Fixation and Soybean Genetics Laboratory. He joined the faculty at the University of Minnesota in 1989, where he is currently a Distinguished McKnight Professor in two departments and a member of 7 graduate faculties.

In addition to his teaching and research efforts, Dr. Sadowsky is Director of Graduate Studies for the Microbial Ecology Program. He was editor of the journal Applied and Environmental Microbiology (where he has served on the editorial board for 20 years) and is currently and editor for Molecular-Plant Microbe Interactions. He also is an editorial board member of the journals Symbiosis and Microbe and Environments.

Dr. Sadowsky has authored or coauthored more than 168 articles in scientific journals and books, was elected fellow of the American Academy of Microbiology in 1999 and fellow of the American Association for the Advancement of Science in 2008. Dr. Sadowsky's research efforts are directed towards the development and use of molecular tools to determine sources of fecal bacteria in the environment and is active in several metagenome studies involving humans, animals and the environment. He is developing new metagenomic tools to determine microbial sources in waterways and web based applications for analysis of fecal sources from metagenomic data. He is also specifically interested in studying *Rhizobium* and *Bradyrhizobium* genes that play a prominent role in host/microbe recognition and in the establishment of symbiotic, nitrogen-fixing nodules.

Dr. Orin C. Shanks is a geneticist at the US Environmental Protection Agency in the Office of Research and Development. His primary research area is the application of DNA-based molecular technologies for environmental microbiology. Projects focus on the identification of host-associated genetic markers of fecal pollution, development of quantitative real-time PCR methods, fate and transport of nucleic acids, as well as utility of molecular methods for water quality management. Other research activities employ next generation sequencing and computational biology to elucidate the influence of host age, diet, and geographic locality on the shedding of fecal indicator bacteria.

Dr. Shanks received his undergraduate and Master's degrees from the University of Wyoming and his Ph.D. from Oregon State University.

Dr. Raghavan Srinivasan is a professor at Texas A&M University and director of the Spatial Sciences Laboratory at Texas A&M. He has become known and respected throughout the world for his developmental work with spatial sciences and computer-based modeling, especially the Soil and Water Assessment Tool or SWAT model. His research and its applications have contributed to long-lasting changes in natural resource assessments and development of management system options, currently being used in more than 90 countries.

Over the past nine years, he has conducted more than 60 international workshops for students and professionals in more than 20 countries and the demand is increasing each year. Currently, more than 50 graduate students worldwide are using the SWAT model as a central focus of their graduate research work and more than 20 universities have adapted the SWAT model as part of their graduate curriculum.

Dr. Don Stoeckel is a microbiologist based in Columbus, Ohio. His formal education includes a Bachelor of Science degree in Microbiology (the Ohio State University), a Master of Science degree in Environmental Microbiology (University of Cincinnati) and a Doctor of Philosophy degree in Soil Microbiology (Auburn University). His professional career, to date, includes 10 years as a research hydrologist (public health) at the US Geological Survey and various instruction and outreach positions in public health microbiology and environmental microbiology at colleges and universities. He currently works at Battelle, an international not-for-profit research institute, in research related to purposeful contamination of food and water along with other public health issues.

Like most adults, Don was 75% water at birth but currently is down to about 60% water. Water, in various forms, remains a major part of his diet and environment. He spends as much of his time as possible to floating on water, attempting live-capture of aquatic vertebrates, and processing water-based beverages. He currently is working with probabilistic models and statistical methods for better interpretation of water quality data.

Dr. Kevin Wagner has 18 years' experience in watershed assessment and planning, project implementation, and program management. His experience ranges from water sampling and analysis to developing projects and policies to restore impaired water bodies. His previous research includes stratigraphical analysis of sedimentary inorganics to determine paleo-productivity trends in lakes, development of lake health indicators, evaluation of effects of off-stream watering facilities on cattle behavior and instream *E. coli* levels, assessment of cattle grazing effects on *E. coli* runoff, and evaluation of *Bacteroides* qPCR for assessing cattle fecal contributions in runoff from grazing lands.

Dr. Wagner currently serves as Associate Director of the Texas Water Resources Institute where he provides leadership and administration for institute water programs. Wagner works with internal and external stakeholders in developing priorities for water resources research and extension programs and develops interdisciplinary teams for addressing these high priority issues. Before joining the Texas Water Resources Institute in 2005, he served as the Nonpoint Source Team Leader and Assistant Director of Programs at the Texas State Soil and Water Conservation Board.

He received a bachelor of science in biology from Howard Payne University, master of science in environmental science from Oklahoma State University, and doctorate in agronomy from Texas A&M University.

Aaron Wendt currently serves as the Statewide Watershed Planning Coordinator for the Texas State Soil and Water Conservation Board (TSSWCB), supporting the administration of the Texas Nonpoint Source Management Program. Headquartered in Temple, Texas, the TSSWCB is the lead agency in Texas responsible for planning, implementing, and managing programs and practices for preventing and abating agricultural and silvicultural nonpoint sources of water pollution.

As point for the agency's Total Maximum Daily Load Program, he works closely with stakeholders across the state and staff from other agencies in the development and implementation of TMDLs which seek to attain water quality standards through load allocation of agricultural and silvicultural nonpoint sources of water pollution. The TSSWCB is actively engaged in mitigating bacteria, atrazine, dissolved oxygen, phosphorus and salinity impairments through TMDLs for nearly four dozen priority waterbodies.

Through leadership of the agency's Watershed Protection Plan Program, he provides technical guidance to local watershed coordinators and stakeholders across the state in the development and implementation of integrated water quality protection and restoration strategies that holistically address sources of impairments and threats to water resources within a watershed. The TSSWCB is currently supporting the development and implementation of WPPs in nearly two dozen prominent watersheds.

Additionally, he provides technical support in implementing the agency's Environmental Data Quality Management Program to ensure data generated and processed through TSSWCB-funded activities is accomplished through the application of sound science and appropriate quality assurance standards and quality control mechanisms. TSSWCB water quality data is used to understand the fate and transport of environmental pollutants, to evaluate effectiveness of best management practices, and to assess the State's water resources for the biennial federal Clean Water Act §305(b) Water Quality Inventory and §303(d) List of Impaired Waters.

Additionally, he facilitates agency involvement in, and represents the agency on, water quality committees and work groups associated with the Texas Clean Rivers Program, the National Estuary Program, and the Association of State and Interstate Water Pollution Control Administrators. And he provides direction to agency efforts associated with the Clean Water Act §319(h) Nonpoint Source Grant Program, the Texas Groundwater Protection Committee and the Coastal Coordination Council.

Wendt previously served the TSSWCB as the Regional Watershed Coordinator in the agency's Wharton Field Office where he implemented a regional coordinated watershed protection strategy in southeast and south central Texas and facilitated the Regional Watershed Coordination Steering Committee.

He is a graduate of Texas A&M University in College Station, where he earned a Bachelor of Science in Renewable Natural Resources Management in December 1999. Before joining the TSSWCB staff in November 2004, he served with Texas Parks and Wildlife Department, Texas Tech University, and Texas Agricultural Experiment Station (now known as Texas AgriLife Research).

BST

Bacterial Source Tracking

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UTHealth
The University of Texas
Health Science Center at Houston
School of Public Health

Soil
Water
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