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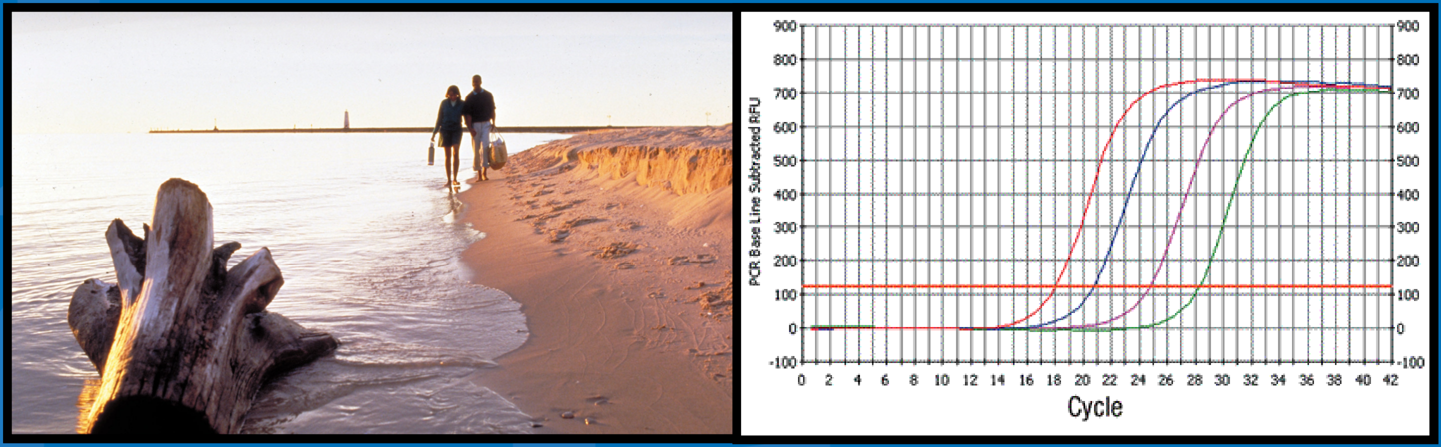
Fecal Waste Contaminates our Waterways:

Molecular technologies offer new solutions

Orin C. Shanks



Office of Research and Development
National Risk Management Research Laboratory, Water Supply and Water Resources Division

December 17, 2014

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Presentation Overview

1. Microbial Source Tracking
2. Identification of Host-Associated Indicators
3. Method Standardization
4. Field Demonstrations
5. Outreach and Resources

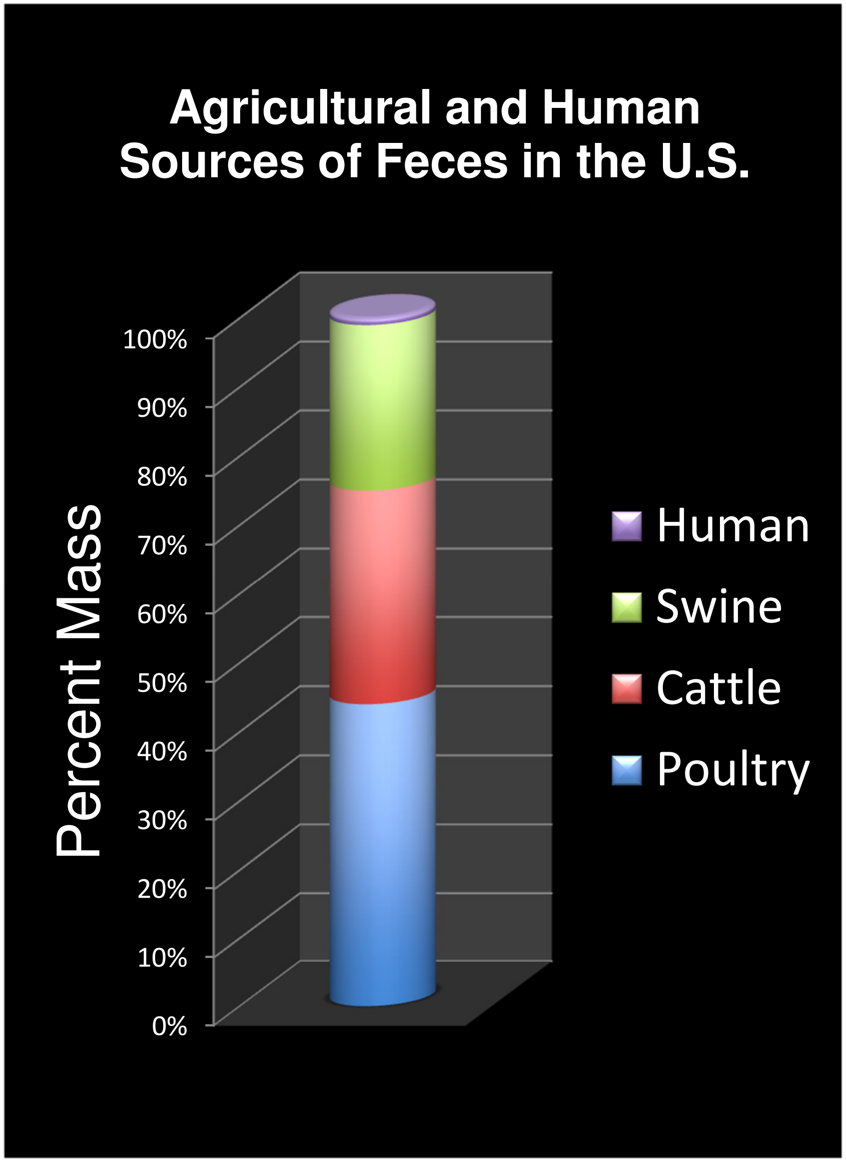
Fecal Pollution is a Nationwide Problem

- Fecal microbes are the most common biological contaminants in U.S. waters
- Public and ecological health risks
- Current National Ambient Water Quality Criteria
  - Based on general fecal indicators
  - Represent overall fecal pollution amount
  - Does not discriminate between sources
- Different animal sources have different risks

Fecal Pollution is a Nationwide Problem

- Estimated 1x10^9 tons of fecal material produced in U.S. each year
  - Human (0.01%)
  - Poultry
  - Cattle
  - Swine
  - Contributions from other agricultural animals and wildlife not included

- Fecal pollution source information can improve water quality management



RL Kellogg, CH Lander, DC Moffitt, N Gollehon - NRCS and ERS GSA Publ. No. NPS00-0579. Washington, DC: USDA, 2000

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Fecal Pollution in Surface Waters:

EPA Responsibilities

Protect and Restore Waters for Recreational Use

- Clean Water Act 1972

Risk Assessment of Beach Contaminants

- BEACH Act (2000)
- Development of new or revised ambient water quality criteria (AWQC)

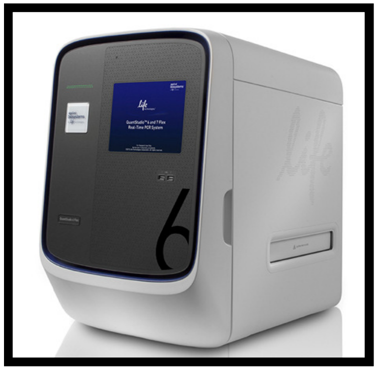
Management of Point and Non-Point Pollution Sources

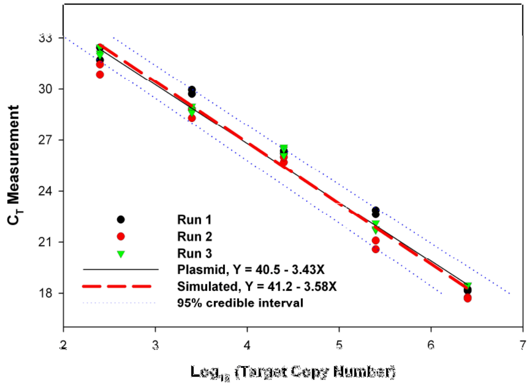
- Total Maximum Daily Load (TMDL) programs
- National Pollutant Discharge Elimination System (NPDES) programs
- National Estuary Program (NEP)

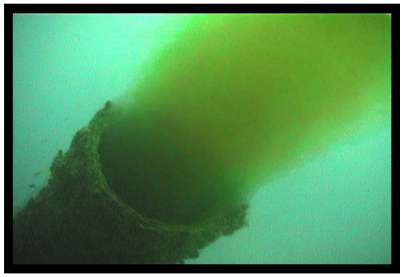
Microbial Source Tracking:

Concept Review

SOLUTION… Method designed to collect, isolate, identify, and measure a host-associated indicator from an environmental sample.







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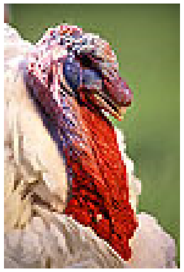
Microbial Source Tracking:

Scientific Premise

Host-associated indicators are expected to exist in different animal groups due to:

- **Gut conditions**
    - Temperature
    - Diet
    - Digestive physiology
- **Natural selection**
    - Space
    - Nutrients



Microbial Source Tracking:

Some Potential Applications

- Total Maximum Daily Load program
    - -Identification of non-point pollution sources
    - -Pollution source surveys
    - -Wet and dry weather risk assessments
- National Ambient Water Quality Criteria
    - -Beach eligibility for alternative criteria
- National Estuary Program
    - -Pollution impact assessments
- Impaired site prioritization for remediation
- Evaluation of a best management practice

Microbial Source Tracking:

Ideal Method Wish List

Attribute	Description
Host Specificity	Indicator closely associated with target host species
Host Distribution	Frequency and concentration of indicator in target and non-target species populations
Quantitative Technology	Measurements of indicator concentrations are accurate and reproducible
Expert Consensus	Agreement among majority of professional researchers on method choice
Standardization	Standard operating procedure with benchmark performance criteria available
Validation	Multiple laboratory confirmation that the method adequately meets application needs
Field Demonstrations	Comprehensive real-world example of application

Identification of Host-Associated Indicators:

Overview

- Goal to find a single DNA sequence in a fecal microbial community associated with a particular animal group

- Multiple step process
  - Comparison of fecal microbial communities
  - Adaption to a quantitative technology
  - Host distribution with reference sample collections

- Successful host-associated indicator identification for several fecal pollution sources:
  - Human
  - Cattle
  - Dog

Shanks *et al.* (2008). Quantitative PCR for Detection and Enumeration of Genetic Markers of Bovine Fecal Pollution. *Journal of Microbiological Methods* 74:745-752

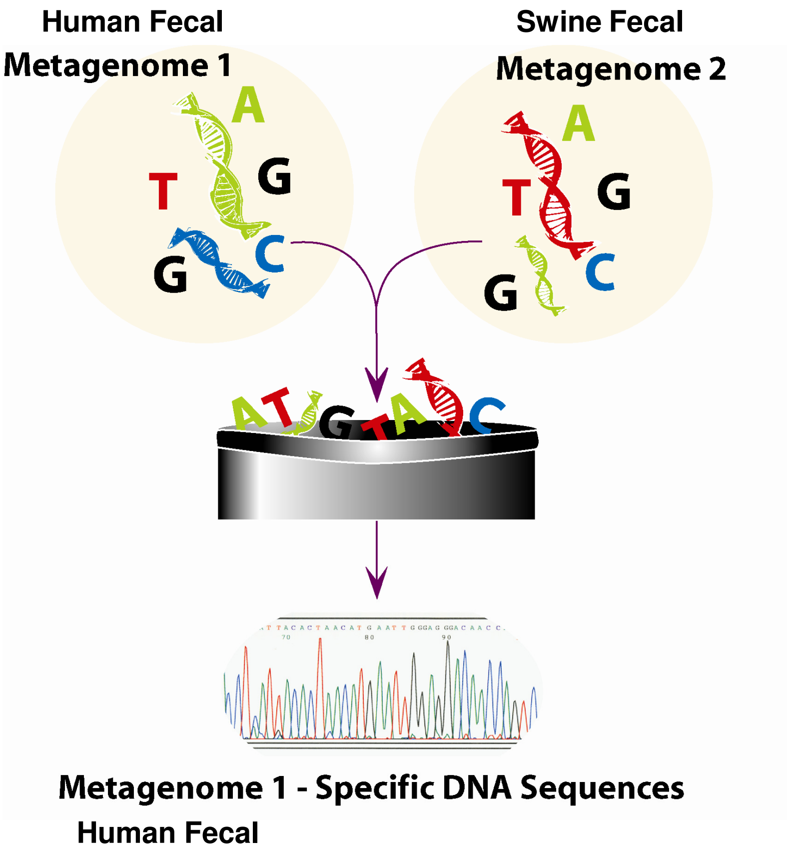
Shanks *et al.* (2009). Quantitative PCR for Genetic Markers of Human Fecal Pollution. *Applied and Environmental Microbiology* 75:5507-5513

Green *et al.* (2014). Development of Rapid Canine Fecal Source Identification PCR-Based Assays. *Environmental Science & Technology* 48:11453-11461

Identification of Host-Associated Indicators:

Comparison of fecal microbial communities

- Compare all microbial DNA from two different pollution sources with a DNA sorting technology
- DNA targets unique to one pollution source become candidate host-associated indicators
- Example: HumM2 human-associated indicator



Human Fecal Metagenome 1

Swine Fecal Metagenome 2

Metagenome 1 - Specific DNA Sequences
Human Fecal

Shanks *et al.* (2006). Use of Competitive DNA Hybridization to Identify Differences in the Genomes of Bacteria. *Journal of Microbiological Methods* 66:321-330

Identification of Host-Associated Indicators:

Adaption to a Quantitative Technology

- Ability to measure concentration of host-associated indicator
- Must be highly sensitive and specific
- Proven performance track record
- Quantitative real-time polymerase chain reaction (qPCR)

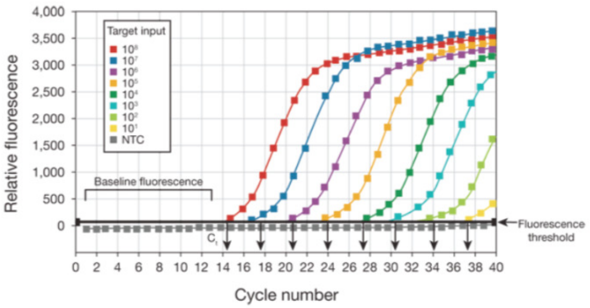
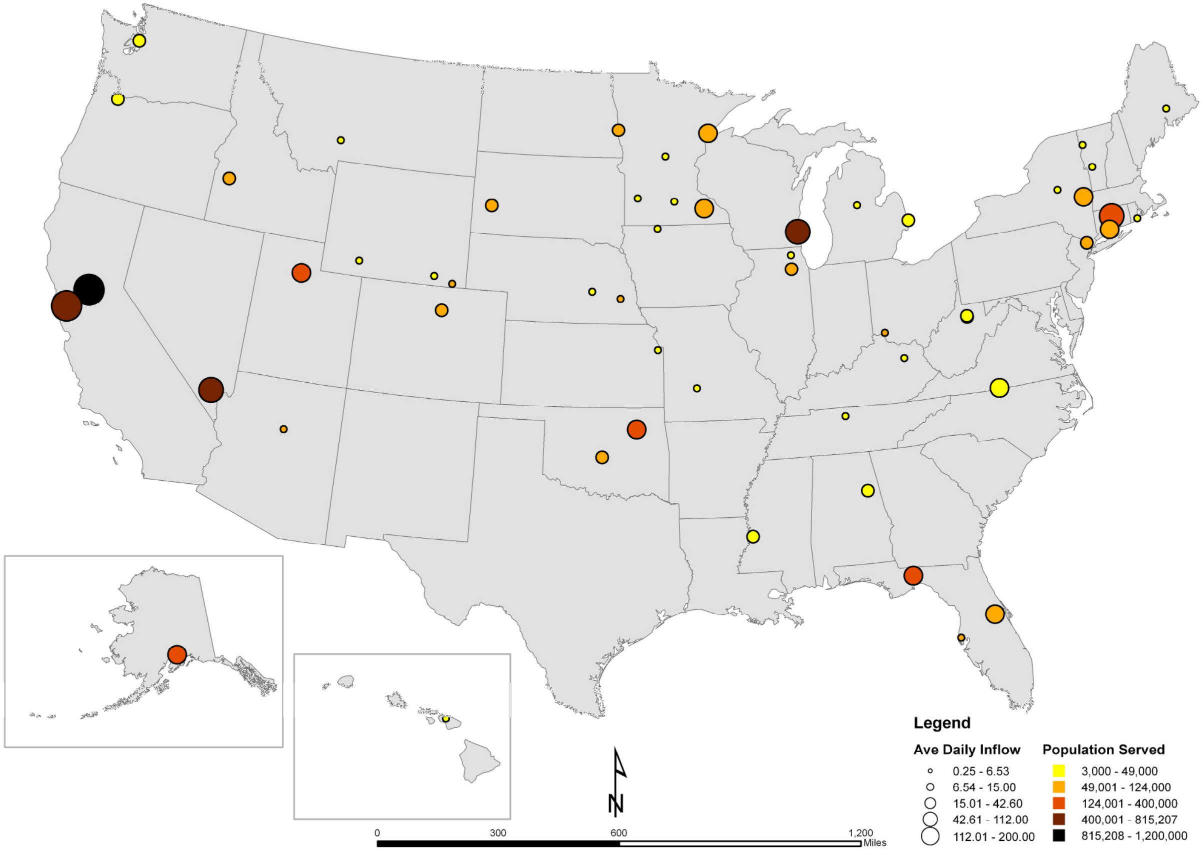


Figure 1. Relative fluorescence vs. cycle number. Amplification plots are created when the fluorescent signal from each sample is plotted against cycle number; therefore, amplification plots represent the accumulation of product over the duration of the real-time PCR experiment. The samples used to create the plots in this figure are a dilution series of the target DNA sequence.

Real-Time PCR Handbook (2014). *Life Technologies*

Identification of Host-Associated Indicators:

Host distribution with reference samples: target pollution source



- Evaluation on national scale
- Sewage reference collection
  - 54 Facilities
  - 39 States
  - 1,150 MGD
  - ~6.4 Million Individuals
- Tested 15 human-associated indicators
- All present at measurable levels in all samples

Kelty *et al.* (2006). Distribution of Genetic Marker Concentrations for Fecal Indicator Bacteria in Sewage and Animal Feces. *Applied and Environmental Microbiology.* 78:4225-4232

Shanks *et al.* (2010). Performance of PCR-Based Assays Targeting Bacteroidales Genetic Markers of Human Fecal Pollution in Sewage and Fecal Samples. *Environmental Science & Technology* 44:6281-6288.

Identification of Host-Associated Indicators:

Host distribution with reference samples: NON-target pollution sources

- Non-target reference collection
  - 22 animal species
  - 174 individual samples

PHOTO BY BRUCE SORENSEN

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Identification of Host-Associated Indicators:

Host distribution with reference samples: NON-target pollution source

Table 1: False positive detections in NON-target fecal reference samples

Reference Sample	HumM2	qHS
Antelope		🟥
Moose		🟥
Mule Deer		🟥
Whitetail Deer		🟥
Canadian Goose		🟥
Duck		🟥
Pelican		🟥
Racoon		🟥
Gull		🟥
Elk	🟥	🟥
Beef Cattle		🟥
Dairy Cattle		🟥
Goat		🟥
Pig		🟥
Turkey		🟥
Sheep	🟥	🟥
Chicken		🟥
Dog		🟥
Cat		🟥
Dolphin		🟥
Sea Lion		🟥
Elephant Seal		🟥

Test quantity = 1 ng total DNA/reaction

Table 2: Host-associated indicator average concentrations in target and NON-target hosts

Indicator	Concentration Target Host*	Concentration NON-Target Host*
HumM2	3.42	0.18
qHS	5.07	1.83

* Estimated $\log_{10}$ mean DNA target copy number; Test quantity = 1 ng total DNA/reaction

- Tested 15 human-associated indicators
- None perfect; range of false positives; some more suitable than others
- Host-associated indicator still useful if concentration is low in NON-target hosts

Shanks *et al.* (2010). Performance of PCR-Based Assays Targeting Bacteroidales Genetic Markers of Human Fecal Pollution in Sewage and Fecal Samples. *Environmental Science & Technology* 44:6281-6288.

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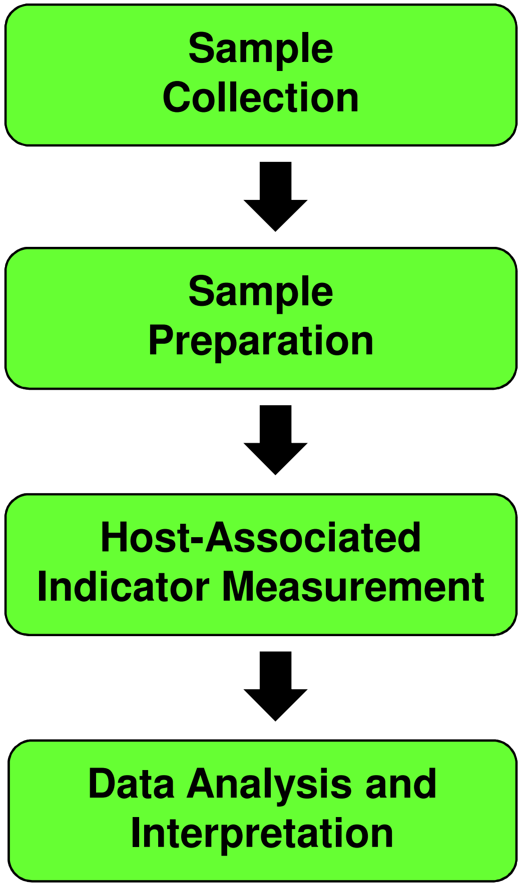
EPA Method Standardization:

Definition and Rationale

- **Anatomy of a Method Review**
  -Series of protocols linked together
  -Alterations in single step may change performance

- **Method Standardization**
  -Formal development of method protocol
  -Establish uniform performance benchmarks
  -Necessary for widespread adoption



Sample Collection

Sample Preparation

Host-Associated Indicator Measurement

Data Analysis and Interpretation

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EPA Method Standardization:

Development Plan

Method Selection

- Which pollution source?
- What detection technology?
- Which host-associated indicator?

Technical Evaluation

- Peer-reviewed

Method Validation

- Establish Standard Operating Procedure (SOP)
- Establish quality assurance metrics
- Conduct multiple lab study

EPA Method

- Decision made by EPA Office of Water
- If selected, then eligible for use in future EPA policies and programs

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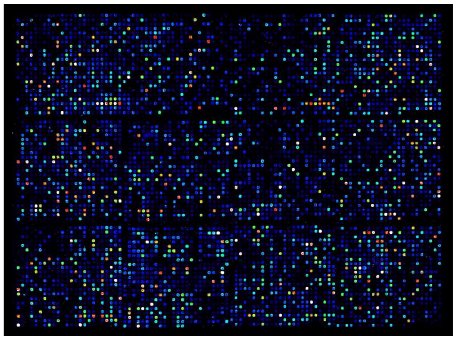
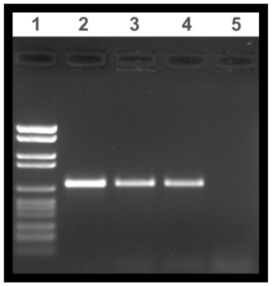
EPA Method Standardization:

Detection Technologies



- Microarray
- Deep sequencing
- End-point PCR
- Real-time quantitative PCR
- Digital PCR
- Immuno-magnetic separation
- Terminal restriction fragment length polymorphism
- Selective bacterial culturing
- Antibiotic resistance profiling
- Chemical detection
- Canine scent detection



EPA Method Standardization:

Selection by Expert Consensus

- Source Identification Protocol Project (SIPP)

  5 organizations formed technical lead team
  Public challenge via blinded study
  27 expert laboratories
  41 methods
  Special Issue of Water Research

- Majority of experts (>90%) favor a PCR-based technology

  Boehm, A. B. *et al.* (2013) Performance of forty-one microbial source tracking methods: a twenty-seven lab evaluation study. *Water Research* 47: 6812-6828.

- qPCR methods are highly reproducible across labs only when protocol is standardized

  Ebentier, D. L. *et al.* (2013) Evaluation of the repeatability and reproducibility of a suite of PCR-based microbial source tracking methods. *Water Research* 47: 6839-6848.

- Top performing host-associated indicators for pollution sources tested were DNA-based

  Boehm, A. B. *et al.* (2013) Performance of forty-one microbial source tracking methods: a twenty-seven lab evaluation study. *Water Research* 47: 6812-6828.

EPA Method Standardization:

Technical Evaluation

- Administered by team of experts
    - -Government sector
    - -Academic sector
- Rigorous assessment subject to peer-review
- Protocol adherence to Minimum Information for Publication of qPCR Experiments (MIQE)

Bustin, S. A. et al. (2009). The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*. *55*: 611-622.

- Optimization to reagents custom designed for environmental samples

Green, H. C. ***et al.*** (2014). Improved HF183 Quantitative Real-Time PCR Assay for Characterization of Human Fecal Pollution in Ambient Surface Water Samples. *Applied and Environmental Microbiology*. 80: 3086-3094.

EPA Method Standardization:

Multiple Laboratory Validation Study

- Formal study conducted by EPA
  - -Office of Water
  - -Office of Research and Development
- Human-associated qPCR method(s)
- 14 Laboratory Participants
- Anticipated Completion Date: FY15

EPA Method Standardization:

Multiple Laboratory Validation Study

	Description:	**Status:**
Phase I: **Lab Proficiency**	• Confirm each lab is properly implementing the method	✓
Phase II: **Water Matrix Spike Testing**	• Data used to evaluate performance in freshwater and marine locations	✓
Phase III: **Blinded Filter Testing**	• Blinded filter set • Data used to evaluate specificity, sensitivity, performance at different concentrations, and reliability of controls	✓
Phase IV: **Data Analysis**	• Establish uniform benchmark performance criteria • Finalize standard operating procedure (SOP)	**In Progress**

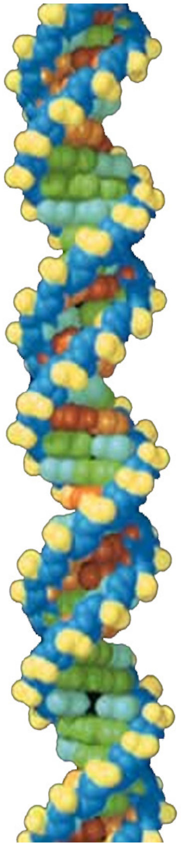
Field Demonstrations:

Rationale and Significance

- Many potential applications

- Applications may require additions to SOP
    - -Water sampling strategy
    - -Experimental set-up
    - -Data analysis
    - -Supporting data needs

- Demonstration studies provide a comprehensive, real-world guide for implementation for each application



Field Demonstrations:

Identification of Non-Point Pollution Sources

- **Question:** Does human fecal pollution originate from leaky sewer lines or failing septic systems in my watershed?

East Fork Little Miami Watershed

- 1,295 km^2 Southeastern Ohio watershed
- Range of septic/sewer use intensity
- 9 catchment areas
- Small stream sampling
- 24-month sampling period
- 3 human-associated qPCR methods
- Unsafe levels of fecal pollution > 40% of time

(*E. coli* and enterococci MPN cell counts)

Peed *et al.* (2011). Combining land use information and small stream sampling with PCR-based methods for better characterization of diffuse sources of human fecal pollution, *Environmental Science & Technology* **45:5652-5659**.

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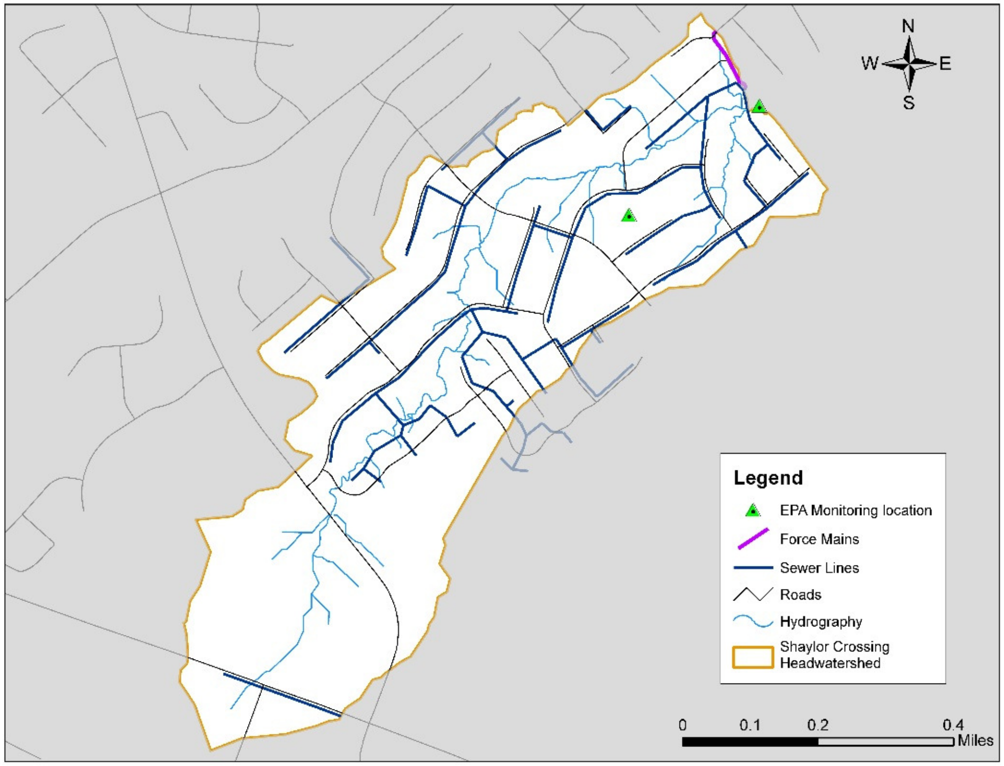
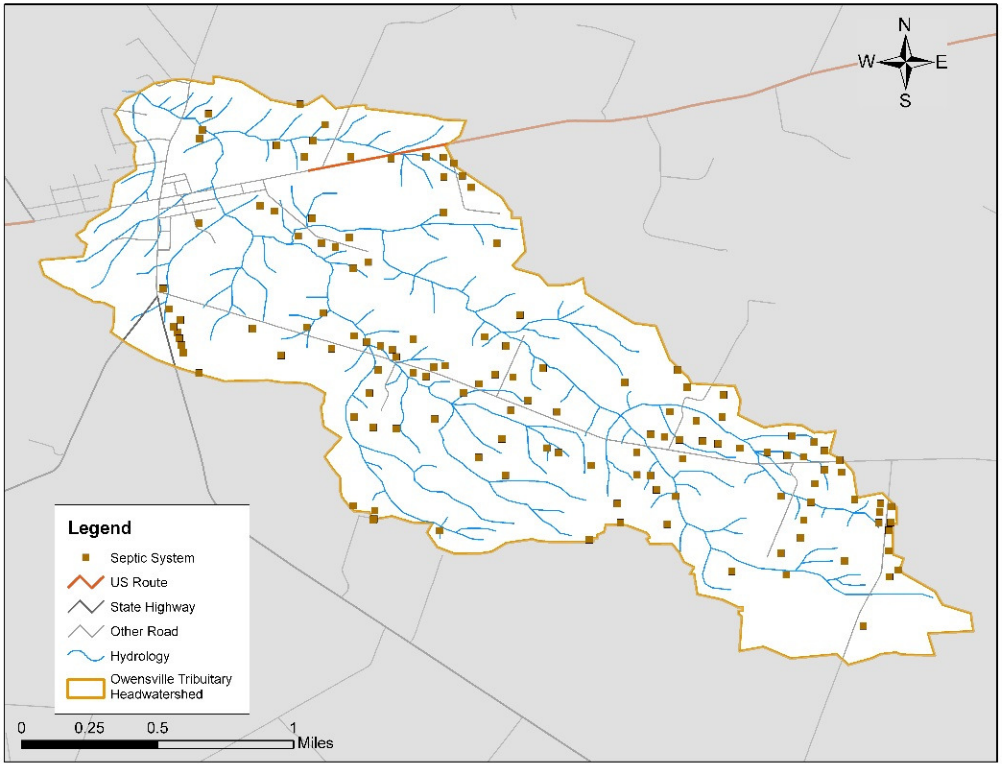
Field Demonstrations:

Identification of Non-Point Pollution Sources





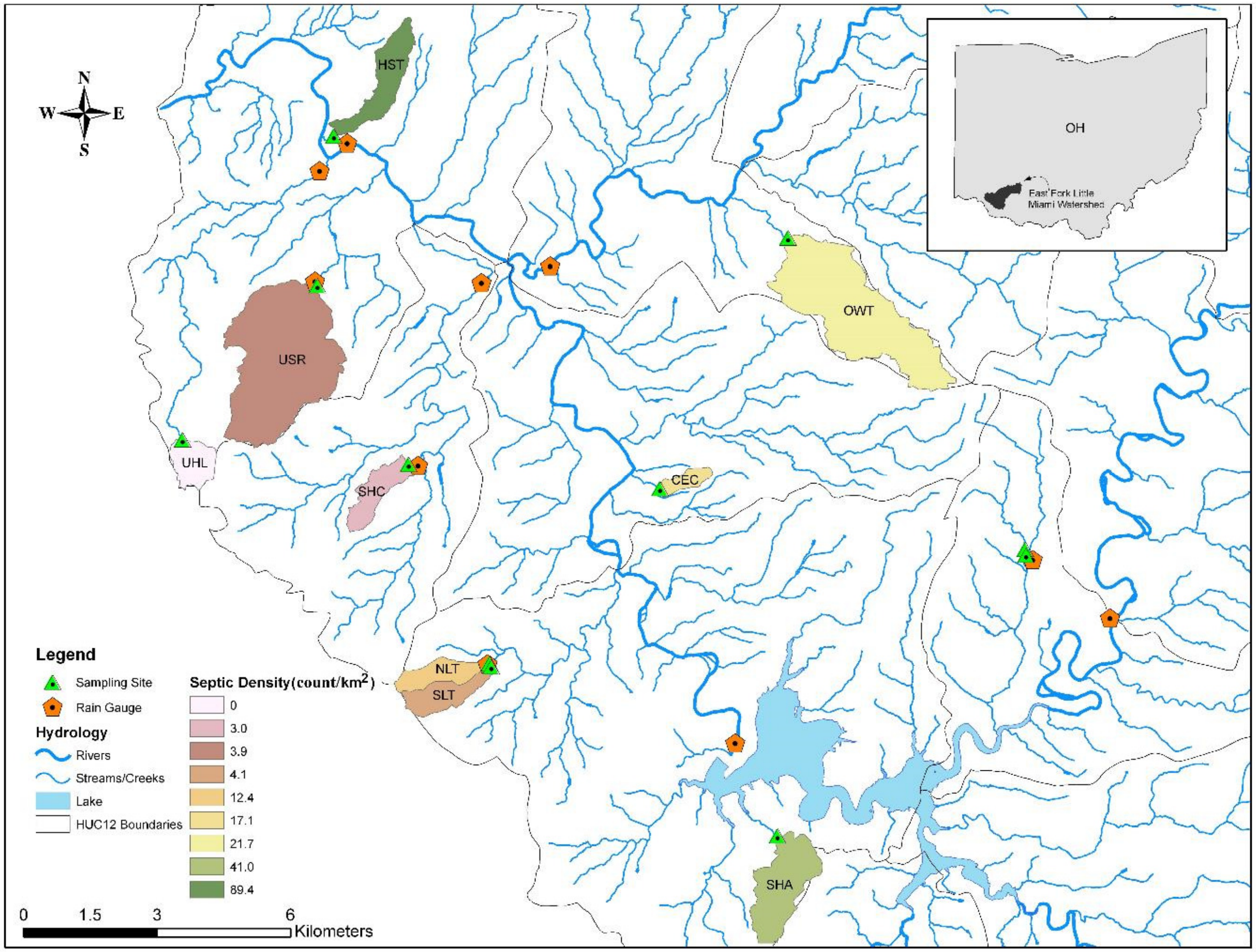
- GIS mapping to estimate sewer and septic densities
- Densities normalized by catchment area

Peed *et al.* (2011). Combining land use information and small stream sampling with PCR-based methods for better characterization of diffuse sources of human fecal pollution, *Environmental Science & Technology* **45:5652-5659**.

Field Demonstrations:

Identification of Non-Point Pollution Sources

- Catchments represent gradient of sewer and septic use
- Negative correlation between septic and sewer densities (R^2 = -0.69)
- Does human pollution trend with sewage, septic, or neither?



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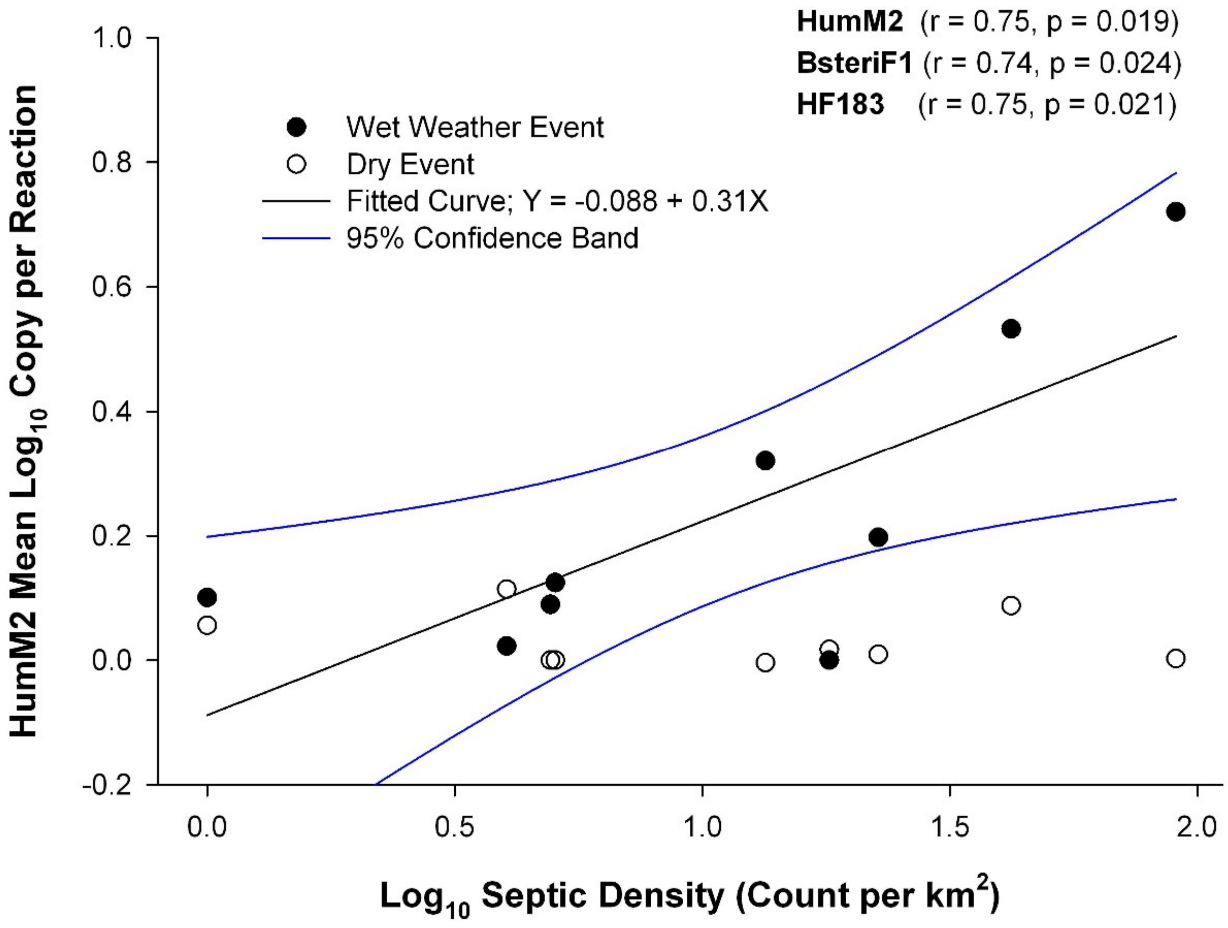
Field Demonstrations:

Identification of Non-Point Pollution Sources



- Human fecal pollution increases with septic density (wet weather events only)
- Trend supported by all 3 human-associated qPCR methods

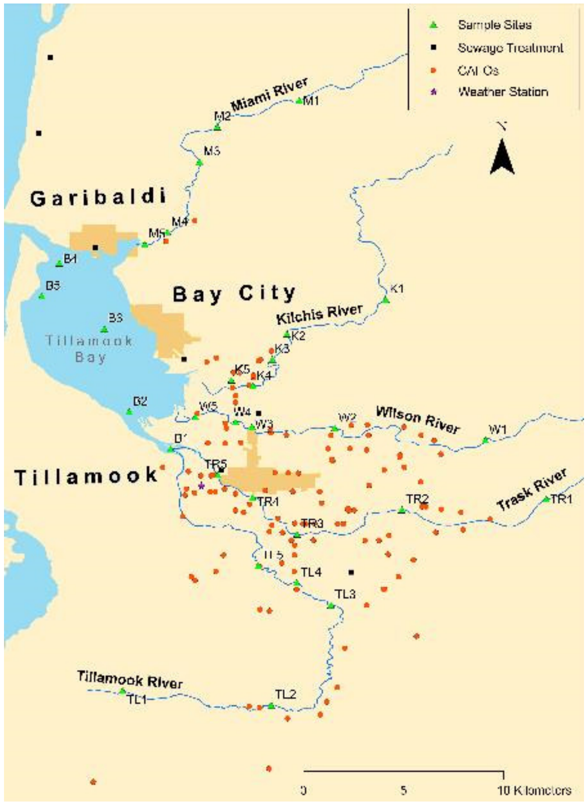
Peed *et al.* (2011). Combining land use information and small stream sampling with PCR-based methods for better characterization of diffuse sources of human fecal pollution, *Environmental Science & Technology* **45:5652-5659**.

Field Demonstrations:

Pollution Source Survey

- **Question:** What fecal pollution sources are present in my chronically impaired watershed?

- Tillamook Basin, Oregon
- Chronic water quality impairment at multiple sampling sites *(E. coli MPN)*
- Urban, residential, agricultural, and wildlife pollution sources
- 30 sites
- Sampled bimonthly for 12-months
- Collaborators
  - EPA Region 10 Laboratory
  - Oregon Department of Agriculture
  - Oregon Department of Environmental Quality
  - Tillamook Estuaries Partnership



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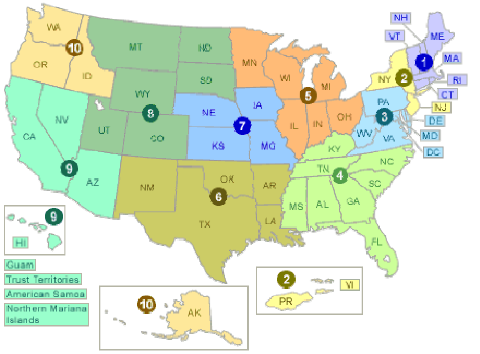
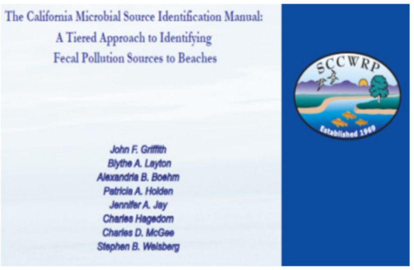
Outreach and Resources



- Regional and State Workshops

- The California Microbial Source Identification Manual: A Tiered Approach to Identifying Fecal Pollution to Beaches. Technical Report 804 (December 2013)


The California Microbial Source Identification Manual:
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Fecal Pollution Sources to Beaches

John F. Griffith
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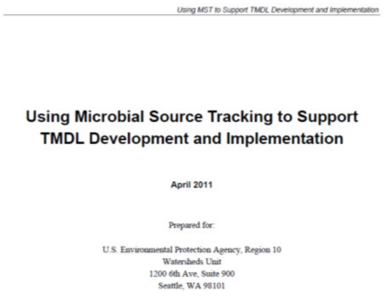
Alexandria B. Boehm
Patricia A. Holden
Jennifer A. Jay
Charles Hagedorn
Charles D. McGee
Stephen B. Weisberg

- Using Microbial Source Tracking to Support TMDL Development and Implementation. (April 2011)
http://www.epa.gov/region10/pdf/tmdl/mst_for_tmdls_guide_04_22_11.pdf


Using MST to Support TMDL Development and Implementation

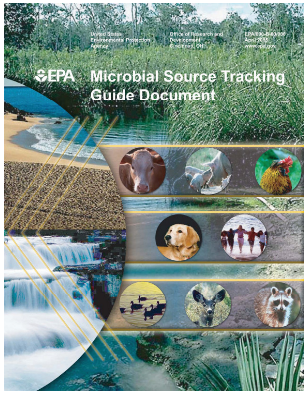
Using Microbial Source Tracking to Support
TMDL Development and Implementation

April 2011

Prepared for:

U.S. Environmental Protection Agency, Region 10
Watersheds Unit
1200 6th Ave, Suite 900
Seattle, WA 98101

- Microbial Source Tracking Guide Document. EPA/600/R-05/064 (June 2005)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Office of Research and
Development
Cincinnati, OH

EPA/000-B-00/000
April 2005
www.epa.gov

Microbial Source Tracking
Guide Document

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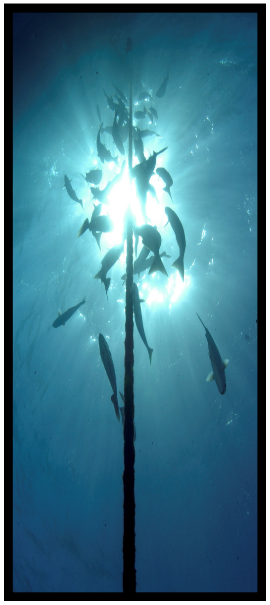

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QUESTIONS