Fecal Waste Contaminates our Waterways:
Molecular technologies offer new solutions

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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 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 $1 \times 10^9$ 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

GSA Publ. No. NPS00-0579. Washington, DC: USDA, 2000

RL Kellogg, CH Lander, DC Moffitt, N Gollehon - NRCS and ERS
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)
**SOLUTION**… Method designed to collect, isolate, identify, and measure a *host-associated indicator* from an environmental sample.
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

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

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)

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


Identification of Host-Associated Indicators:
Host distribution with reference samples: NON-target pollution sources

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

<table>
<thead>
<tr>
<th>Reference Sample</th>
<th>HumM2</th>
<th>qHS</th>
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</thead>
<tbody>
<tr>
<td>Antelope</td>
<td></td>
<td></td>
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<tr>
<td>Moose</td>
<td></td>
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<tr>
<td>Mule Deer</td>
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<tr>
<td>Whitetail Deer</td>
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<tr>
<td>Canadian Goose</td>
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<tr>
<td>Duck</td>
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<tr>
<td>Pelican</td>
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<tr>
<td>Racoon</td>
<td></td>
<td></td>
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<tr>
<td>Gull</td>
<td></td>
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<tr>
<td>Elk</td>
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<tr>
<td>Beef Cattle</td>
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<tr>
<td>Dairy Cattle</td>
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<tr>
<td>Goat</td>
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<tr>
<td>Pig</td>
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<td>Turkey</td>
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<tr>
<td>Sheep</td>
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<td>Chicken</td>
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<td>Dog</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dolphin</td>
<td></td>
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<tr>
<td>Sea Lion</td>
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<tr>
<td>Elephant Seal</td>
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</tr>
</tbody>
</table>

Test quantity = 1 ng total DNA/reaction

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

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Concentration Target Host*</th>
<th>Concentration NON-Target Host*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HumM2</td>
<td>3.42</td>
<td>0.18</td>
</tr>
<tr>
<td>qHS</td>
<td>5.07</td>
<td>1.83</td>
</tr>
</tbody>
</table>

* 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

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
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
• 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
• 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

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

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

• Optimization to reagents custom designed for environmental samples

• 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

Phase I: Lab Proficiency

Phase II: Water Matrix Spike Testing

Phase III: Blinded Filter Testing

Phase IV: Data Analysis

Description:

- Confirm each lab is properly implementing the method
- Data used to evaluate performance in freshwater and marine locations
- Blinded filter set
- Data used to evaluate specificity, sensitivity, performance at different concentrations, and reliability of controls
- Establish uniform benchmark performance criteria
- Finalize standard operating procedure (SOP)

Status:

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

**East Fork Little Miami Watershed**

- 1,295 km² 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
  \( (E. \text{ coli} \text{ and enterococci MPN cell counts}) \)

Field Demonstrations:
Identification of Non-Point Pollution Sources

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

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?

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

**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
Outreach and Resources

- Regional and State Workshops


- Using Microbial Source Tracking to Support TMDL Development and Implementation. (April 2011)

- Microbial Source Tracking Guide Document. EPA/600/R-05/064 (June 2005)
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QUESTIONS