Proceedings of the 2009 U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water

MAY 20 - 21, 2009
REGION 3 OFFICES
PHILADELPHIA, PA
The 2009 U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water
May 20 - 21, 2009

EPA Region 3 Offices
Shenandoah Room, #104
1650 Arch Street
Philadelphia, PA

Workshop Objectives

• Provide a forum to discuss proposed solutions to the methodological challenges in the search for better methods of detection and assessment of waterborne microbial contaminants.

• Facilitate collaboration and cooperation among scientists and policy-makers from research entities, EPA, states, local agencies, and stakeholders.

• Assist EPA in identifying what research or technologies are needed to better inform decisions and/or policies associated with the assessment of microorganisms in water.

• Give STAR grantees of the past two solicitations regarding “Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens and Cyanobacteria and Their Toxins in Drinking Water” the opportunity to present their latest findings. Summaries of the grantees’ projects can be found at: http://epa.gov/ncer/rfa/2005/2005_pathogens_drinking_water.html and http://cfpub.epa.gov/ncer_abstracts/index.cfm?fuseaction=recipients.display&rfa_id/456/records_per_page/ALL

Wednesday, May 20, 2009

1:00 p.m. Welcome and Overview of EPA’s Office of Research and Development and the Science To Achieve Results (STAR) Program
Barbara Klieforth, EPA, Office of Research and Development, National Center for Environmental Research

1:25 p.m. OGWDDW Microbial Research Needs from a Regulatory Perspective
Sandhya Parshionikar, Team Leader, Microbiology Technical Support Center Office of Ground Water and Drinking Water

1:55 p.m. Overview Presentation From EPA Region 3
Victoria P. Binetti, EPA, Region 3

2:15 p.m. Crypto and Molecular Methods Work Being Done With EPA Regions 2 and 3
Eric Villegas, EPA, National Exposure Research Laboratory, Microbiological and Chemical Exposure Assessment Research Division
### Wednesday, May 20, 2009 (continued)

<table>
<thead>
<tr>
<th>Time</th>
<th>Presentation</th>
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| 2:35 p.m.| Development of a Universal Microbial Collector (UMC) for Enteric Pathogens in Water and Its Application for the Detection of Contaminant Candidate List Organisms in Water  
Kelly R. Bright, University of Arizona |
| 2:55 p.m.| Break                                                                                           |
| 3:15 p.m.| Development and Evaluation of an Innovative System for the Concentration and Quantitative Detection of CCL Pathogens in Drinking Water  
Saul Tzipori, Tufts University |
| 3:35 p.m.| On-Chip PCR, Nanoparticles, and Virulence/Marker Genes for Simultaneous Detection of 20 Waterborne Pathogens  
Syed Hashsham, Michigan State University |
| 3:55 p.m.| Rapid and Quantitative Detection of *Helicobacter pylori* and *Escherichia coli* O157 in Well Water Using a Nano-Wired Biosensor and QPCR  
Evangelyn C. Alocilja, Michigan State University |
| 4:15 p.m.| Assessment of Microbial Pathogens in Drinking Water Using Molecular Methods Coupled With Solid-Phase Cytometry  
Barry Pyle, Montana State University |
| 4:35 p.m.| Detecting Pathogens in Water by Ultrafiltration and Microarray Analysis  
Anthea K. Lee, Metro Water District of Southern California |
| 5:00 p.m.| Adjourn                                                                                         |

### Thursday, May 21, 2009

<table>
<thead>
<tr>
<th>Time</th>
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| 8:30 – 9:00 a.m. | Robust Piezoelectric-Excited Millimeter-Sized Cantilever Sensors for Detecting Pathogens in Drinking Water at 1 Cell/Liter  
Raj Mutharasan, Drexel University |
| 9:00 – 9:20 a.m. | National Risk Management Research Laboratory (NRMRL) Microbial Research  
Jorge Santo Domingo, EPA, NRMRL, Water Supply and Water Resources Division, Microbial Contaminants Control Branch |
| 9:20 – 9:40 a.m. | Rapid Concentration, Detection, and Quantification of Pathogens in Drinking Water  
Zhiqiang Hu, University of Missouri |
### Thursday, May 21, 2009, (continued)

<table>
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<tr>
<th>Time</th>
<th>Session</th>
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| 9:40 – 10:10 a.m. | Simultaneous Concentration and Real-Time Detection of Multiple Classes of Microbial Pathogens From Drinking Water  
Mark D. Sobsey, University of North Carolina at Chapel Hill |
| 10:10 – 10:30 a.m. | Break                                                                  |
| 10:30 – 10:50 a.m. | Quantitative Assessment of Pathogens in Drinking Water  
Kellogg Schwab, Johns Hopkins University  |
| 10:50 – 11:40 a.m. | Discussion on the Next Generation of Methods and Research Needs |
Donald Anderson, Woods Hole Oceanographic Institute |
| 12:00 – 1:10 p.m. | Lunch                                                                   |
| 1:10 – 1:30 p.m. | Development of High-Throughput and Real-Time Methods for the Detection of Infective Enteric Viruses  
Jason Cantera, University of California at Riverside |
| 1:30 – 1:50 p.m. | New Electropositive Filter for Concentrating Enterovirus and Norovirus From Large Volumes of Water  
Mohammad Karim, Oak Ridge Institute for Science and Education Research Fellow, EPA |
| 1:50 – 2:10 p.m. | Automated Methods for the Quantification and Infectivity of Human Noroviruses in Water  
Timothy Straub, Batelle Pacific Northwest Division |
| 2:10 – 2:30 p.m. | Characterization of Naturally Occurring Amoeba-Resistant Bacteria From Water Samples  
Sharon Berk, Mid-Tennessee State University |
| 2:30 – 2:50 p.m. | Break                                                                   |
| 2:50 – 3:10 p.m. | Analysis of Various Toxins Produced by Cyanobacteria Using Ultraperformance Liquid Chromatography-Tandem Mass Spectrometry (UPLC/MS/MS)  
Stuart Oehrle, Northern Kentucky University |
| 3:10 – 3:20 p.m. | Development of Sensitive Immunoassay Formats for Algal Toxin Detection  
Fernando Rubio, Abraxis LLC |
| 3:20 – 4:00 p.m. | Wrap-up & Adjournment                                                  |
This workshop was intended to facilitate progress on the quantitative assessment of microbial agents in water and bring research scientists together with policy makers. EPA’s success is dependent, in large part, on its ability to make credible environmental decisions based on solid scientific information and technical methodologies. Reliable, sensitive, robust, and versatile detection and monitoring tools are needed to address the risk assessment and management of known and emerging microbial contaminants in source water, treated water, and/or distribution systems. The goal of this workshop was to foster discussion on the development of cost-effective, timely, and innovative technology solutions in assessing and managing environmental risks to human health.

Workshop Objectives

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• Assist EPA in identifying what research or technologies are needed to better inform decisions and/or policies associated with the assessment of microorganisms in water.

• Give Science To Achieve Results (STAR) grantees of the past two solicitations regarding “Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens and Cyanobacteria and Their Toxins in Drinking Water” an opportunity to present their latest findings. Abstracts of the grantees’ projects can be found at: http://epa.gov/ncer/rfa/2005/2005_pathogens_drinking_water.html and http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/recipients.display/rfa_id/456/records_per_page/ALL
The STAR Drinking Water Program

Barbara Klieforth,
National Center for Environmental Research

EPA Organization
Mission: To protect public health and to safeguard and improve the natural environment – air, water, and land – upon which life depends

NCER/STAR

Support for EPA's Mission

OFFICE OF RESEARCH AND DEVELOPMENT

PROGRAM OFFICES
Air, Water, Waste, Pesticides/Toxics

REGIONAL OFFICES

Implementation

National Decisions

Support for EPA's Mission

NCER's Extramural Programs
Science To Achieve Results (STAR)
Targeted Research Grants through RFAs
Exploratory/Futures Grants
Graduate Fellowships
Competed Centers
Greater Research Opportunities

Earmarks
Small Business Innovation Research (SBIR) Contracts
Experimental Program to Stimulate Competitive Research (EPSCoR)

Grantees and fellows are among the top scientists in the country

Science To Achieve Results (STAR) Program

EPA STAR Program

Established in 1995 as part of the overall reorganization of ORD
Mission: include this country's universities and nonprofit groups in EPA's research program and ensure the best possible quality of science in areas of highest risk and greatest importance to the Agency
Issue approximately 20-25 RFAs each year
Each year: receive 2500-3200 grant applications
Award about 250-300 new STAR grants, fellowships & SBIR contracts per year
Manage about 1000 active research grants and fellowships

EPA STAR Research Program

Goal-directed solicitation planning
Significant cross-agency and interagency involvement with solicitation planning, writing, and review
Competitive solicitations
Joint Solicitations with other Agencies
External peer review
Internal relevancy review: program office and regional input
Fund highest priority projects
Grantees and fellows are among the top scientists in the country
Communicate research results through website, ORD laboratories, program office and regional meetings, and publications (www.epa.gov/ncer)
**STAR Results in Action:** Regulations and Voluntary Actions

- Results from the Marshfield Clinic Research Foundation’s research led to major, statewide changes (e.g., UV disinfection) in treatment of water from groundwater sources (Borchardt)
- UNC at Chapel Hill examined impacts of water distribution systems in contributing to GI illness, results are included in considerations for updating the Total Coliform Rule (Tolbert)
- Results used by EPA’s Office of Water in preparing: “Economic, Environmental, and Benefits Analysis of the proposed Metal Products & Machinery Rule” (Henigens)
- University of Iowa findings on mechanisms and kinetics of chlorine loss & byproduct formation in distribution systems used in the Stage 2 Disinfectants and Disinfection Byproducts (DBP) rule published in 2006
- STAR research results on “integrated pest management” used by cities & states to reduce children’s exposures to pest allergens
- STAR research findings led to voluntary industry action – protective clothing and hand-washing facilities for agricultural workers expected to reduce “take home” pesticide exposures
- **University of Maryland’s Center for Marine Biotechnology’s 1st of its kind PCR technique that rapidly detects Helicobacter pylori in environmental samples. H. pylori had previously been extremely difficult to detect because of its ability to transform into a non-culturable form.**
- **STAR researchers developed molecular detection techniques for pfisteria – used by states and CDC for real time monitoring of pfisteria events**
- **STAR research developed promising method for assessing pesticide concentrations in saliva – accurate & less invasive method to quantify exposure & dose**
- **Research played a key role in the preparation of a manual on economic valuation for the British Department of Environment, Regions, and Transport (Carson)**

**STAR & SBIR Results in Action:** Practical Applications

- **Tufts' U. alternative method (portable continuous flow centrifuge) for concentrating low numbers protozoa from large volumes of water approved as an alternative concentration method by EPA (Tzepori)**
- Soybean oil plastics being used to manufacture tractor parts for John Deere (Wool)
- Developed a benign catalyst to replace chlorine in oxidation processes (Collins)
- Developed a substitute for lead solder now used broadly in the electronics industry (Wong)
- STAR-supported grant research has led to new, environmentally friendly packaging manufactured by Cargill-Natureworks and used by the Wal-Mart Corporation Advanced Technology Materials, Inc developed dry scrubber using deposition for semiconductor industry. Business grew from five partners to 1100 employees and sales over $250 million (NASDAQ : ATMI)

**STAR Results in Action:** Education

- New course in green engineering
- Fellows are now professors in many, major universities
- Fellows are working in government agencies
- Fellows elected to 36 scientific panels and/or advisory committees
- Sustainability curricula expanded in many universities as a direct result of P3
- Four new small businesses created because of P3

**Science To Achieve Results (STAR) Program**

- Program begun in FY 1996
- Funding levels historically between $2.5-5.0 M/yr
- NCER has been funded research in a wide variety of areas
- Research completed 3-4 years after award
- Solicitation preparation and Programmatic Reviews have extensive participation from OW, ORD, and Regional Offices

**Drinking Water ( & Water Quality)**

- Current components
  - Identifying and quantifying microbes in water
  - Decision making for water infrastructure sustainability
  - Source water/aquifer protection from potential impacts of geologic sequestration of carbon dioxide
- Recent solicitations
  - Integrated Design, Modeling, and Monitoring of Geologic Sequestration of Anthropogenic Carbon Dioxide to Safeguard Sources of Drinking Water
  - Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens and Cyanobacteria and Their Toxins in Drinking Water
  - Innovative and Integrative Approaches for Advancing Public Health Protection Through Water Infrastructure Sustainability
History of STAR Drinking Water Projects

http://epa.gov/ncer/science/drinkingwater/recipients.html

Science To Achieve Results (STAR) Program

Other Water-related RfAs

Some examples:
- Forecasting Ecosystem Services from Wetland Condition Analyses (2008)
- Enhancing Ecosystem Services from Agricultural Lands (2009)
- EcoHABs
Overview

- The SDWA requirements and regulatory process.
- Research input in Drinking water regulations
- Sources of data used
- Research Needs
  - General
  - Specific issue
  - Total Coliform Rule
    - Revisions
    - Research and Information Collection Partnership
  - Long term

Safe Drinking Water Act

- SDWA requires regulation of contaminants that:
  - May have an adverse health effect
  - Must consider sensitive sub-populations of infants, children, pregnant women, elderly individuals, with history of serious illness
  - Occur or are likely to occur in PWSs (considering frequency and level)
  - Present a meaningful opportunity for health risk reduction
  - Based on best available science and data

Safe Drinking Water Act Requirements

- EPA must publish Maximum Contaminant Level Goals (MCLGs)
  - Must set levels at which no health effects occur and which allows for adequate margin of safety
  - Required EPA to regulate specific microbial contaminants (viruses, Giardia, Legionella, total coliform, heterotrophic bacteria)
- EPA must promulgate MCLs or treatment technique requirement as close to the MCLG as is “feasible” (taking costs into consideration)
  - Required EPA to set treatment technique requirements for surface and ground water systems to protect for pathogens

Generalized Flow of Regulatory Processes

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

- Health Effects
  - Epidemiology data
  - Indecitiveness, meatability, mortality
  - Dose response data
  - Population characteristics
- Control Measures
  - Efficacy data
  - Feasibility of treatment technologies
  - Analysis of potential side-effects
  - Indicators
- Economic Considerations
  - Cost analyses
  - Benefit analyses
  - System impact assessment

MCLG or MCL or Treatment Technique

Sources of Research Data Used

- EPA Office of Research and Development
  - In house research
  - STAR grants
- Regions
- Water Research Foundation (formerly AwwaRF)
- Contracts with Universities and research institutions
- Interagency agreements
- Co-operative agreements
- Other published, peer reviewed literature

Office of Ground Water and Drinking Water

Research Needs: General

- Exposure Data
  - Analytical Methods
  - Innovative approaches to measurement
  - Practical implementable technologies
  - Occurrence data
  - Outbreak analyses
  - Endemic prevalence
  - Epidemiological studies
- Health effects
  - Dose response
  - Subpopulations affected
  - Host factors involved

Research Needs: Examples of Specific Issues

- Methods that detect pathogen infectivity/viability/strain identification
- Exposure to pathogens from drinking water contamination events
- Role of Biofilms in pathogen exposure and their impact on chlorine residuals
- Survival of nucleic acids under various treatments
- Innovative approaches for sampling and detection
- Research in Support of Revised TCR/DS
**Total Coliform Rule (TCR)**

- **2000 - Stage 2 Federal Advisory Committee (FAC) Agreement In Principle (AIP) suggested review** of distribution system issues with the 6-year review of the TCR
- **2003 - Six year review of existing drinking water regulations → TCR should be revised**
- **2007 - Federal Advisory Committee convened to provide recommendations on**
  - how EPA should revise the TCR, and
  - what research and information collection should be conducted to better inform distribution system risk

**Total Coliform Rule Revisions**

- The Advisory Committee developed an AIP to be the foundation for the proposed rule
  - A more proactive approach to public health protection
  - Use of monitoring results shift from informing public notification to informing investigation and corrective action
    - 2010: Propose rule revisions
    - 2012: Final rule
    - 2015: compliance starts
  - Includes recommendations for distribution system research and information collection and the formation of a Research and Information Collection Partnership

**Research and Information Collection Partnership (RICP)**

- Recommended by TCR Federal Advisory Committee to:
  - Inform and support the drinking water community to develop future risk management decisions regarding drinking water distribution systems
  - Partnership formed January 29, 2009 between EPA and Water Research Foundation
  - Steering Committee provides input on research and information collection priorities
    - 3 members from EPA
    - 3 additional members
    - Public health
    - Environmental
    - State Regulator

- Developed a research agenda to identify decision relevant research and information collection needs or priorities
  - Biofilms
  - Contaminant Accumulation
  - Nitrification
  - Main Repair
  - Intrusion
  - Cross Connection Control
  - Storage
  - First Draft Research Agenda – September 2009
    - Initial priorities for research and information collection identified - 2010

**Long Term Research Needs**

- Online monitoring/Rapid results
  - Perturbations in water quality
  - Outbreak analysis
    - Quantitative
    - Genotyping/Strain identification
    - Sensitivity
  - High throughput detection
  - Universal detection of all classes of pathogens
  - Miniaturization of technology
  - Use in field
  - Genomics/Proteomics

**New STAR RFA**

- EPA seeking new and innovative research applications that link opportunities to advance public health protection with improvements in the condition and function of the water infrastructure.
  - The focus on improving the effectiveness of the water infrastructure for protecting public health.
  - Should clearly demonstrate an integrated, multi-disciplinary approach that leads to advances in design, operation, and management of the water infrastructure and should directly tie those advances to public health protection in conjunction with improving water efficiency and reducing energy requirements.
Region 3 Overview

Victoria P. Binetti
US Environmental Protection Agency

Workshop on Innovative Approaches For Detecting Microorganisms And Cyanotoxins in Water
Philadelphia, PA May 20, 2009

We are employing a “Healthy Waters” strategy to restore and protect our waters by

- Protecting four water uses
  - Aquatic life
  - Recreation
  - Fish consumption
  - Drinking water health

- Reducing causes of impairment
  - Nutrients
  - Sediments
  - Toxics
  - Pathogens

....and by

- By addressing contaminant sources
  - Agriculture
  - Developed/Developing lands
  - Mining
  - Transportation

- Using approaches like
  - Wholesale solutions
  - Prevention partnerships
  - Integrated strategies
  - Green solutions

![Number of Systems vs. Population Served graph]

- Community Water Systems
  - Population Served
    - Very Small
    - Small
    - Medium
    - Large
    - Very Large
Some observations on drinking water program implementation in Region 3

- Many public water systems are small, under-resourced, and have limited technical capacity
- Greatest number of violations overall are related to monitoring
- Most frequent health-based violations relate to pathogen regulations: Total Coliform Rule, Surface Water Treatment Rules
- Newer regulations requiring source water sampling are challenging

Implementing a multi-barrier approach to safe drinking water:

- Prevent/Reduce pathogens in source waters
- Eliminate/Inactivate pathogens through treatment
- Assess/Monitor to detect pathogen occurrence in finished water
- Assess exposure, health effects

Needs today from the field include:

- Monitoring & quantification methods – Cryptosporidium, bacteria, viruses
- Low-cost, reliable tools for viability assessment, speciation
- Pathogen indicators
- Real-time E. coli identification
- Efficacy of best management practices for nutrient & sediment control, in prevention of pathogen contamination
- Efficacy of best management practices used for protection of surface waters, in protection of ground water

Issues for the Research Agenda

- Distribution system is the next frontier
- Aging, deteriorating infrastructure increases pathogen exposure risk
- Longer-lived, healthier—but more vulnerable—population?
- Impacts of population growth, climate change and patterns of development on water use and water supply needs will drive treatment and technology – e.g., water efficiency, water reuse, aquifer storage & recovery, etc.
- Climate change will affect pathogen distributions, geographically and seasonally
- Water security concerns will remain—detection, response, recovery
Molecular-Based Detection Systems for Cryptosporidium Oocysts

**Giardia & Cryptosporidium**

Eric N. Villegas, Ph.D.
STAR Grants Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water
US EPA Region 3, Philadelphia, PA
May 20-21, 2009

**Overview**

1. Brief introduction to waterborne Cryptosporidium
   - Biology and diversity of Cryptosporidium species
   - Current detection methodologies

2. US EPA-NERL’s waterborne protozoan research program
   - Building a “Protozoan Detection Toolbox”

3. Perspectives on the future of the “Protozoan Detection Toolbox”
   - Future directions and considerations

**Cryptosporidium species**

- Enteric protozoan parasite
- Chronic diarrhea and death in susceptible groups
- At least 20 species, with many more genotypes
- Waterborne transmission (Milwaukee Outbreak)

**Cryptosporidium Species Infecting Humans and Selected Animals**

<table>
<thead>
<tr>
<th>Host</th>
<th>Major Species</th>
<th>Minor Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td>C. hominis and C. parvum</td>
<td>C. meleagridis, C. felis, C. canis, C. suis, cervine genotype</td>
</tr>
<tr>
<td>Cat</td>
<td>C. felis</td>
<td>C. baileyi</td>
</tr>
<tr>
<td>Cattle</td>
<td>C. parvum, C. bovis, C. andersoni, deer-like genotype</td>
<td>C. suis</td>
</tr>
<tr>
<td>Chickens</td>
<td>C. baileyi</td>
<td>C. meleagridis, C. baileyi</td>
</tr>
<tr>
<td>Deer</td>
<td>Cervine genotype</td>
<td>C. meleagridis, C. baileyi</td>
</tr>
<tr>
<td>Dog</td>
<td>C. canis</td>
<td>C. suis</td>
</tr>
<tr>
<td>Pig</td>
<td>C. suis</td>
<td>Pig genotype 5</td>
</tr>
<tr>
<td>Sheep</td>
<td>Cervine genotype 1-3, bovine genotypes</td>
<td></td>
</tr>
</tbody>
</table>

**Method 1622/1623:**

Detection of Cryptosporidium and Giardia

**Limitations:**
- Does not differentiate human infectious vs. animal forms
- No live vs. dead discrimination

**Challenges for the 21st Century**

“Water Quality Tricorder”

1. Fast and user friendly
2. Sensitive and quantitative
3. Species/genotype specific
4. Live vs. dead

**Protozoan Detection Systems:**

- Method 1622/1623: Detection of Cryptosporidium and Giardia
- Sample Collection
- Elution
- Immunomagnetic Separation
- Immunofluorescence Detection
- Limitations:
  - Does not differentiate human infectious vs. animal forms
  - No live vs. dead discrimination
Question Driven Research

1. What are the total levels of Cryptosporidium in the watershed?
2. How complex is the Cryptosporidium species diversity in the watershed?
3. What are the total levels of pathogenic Cryptosporidium in the watershed?
4. Are the Cryptosporidium oocysts in the watershed viable/infectious?
5. Other questions…

Goals
Tracking Sources of Contamination in a Watershed

- Identify types of Cryptosporidium oocysts present
- Use PCR-RFLP and Method 1623
- Identify potential sources of Cryptosporidium oocysts in the Potomac River

Potential Sources:
- Storm water runoffs
- Wastewater treatment discharges
- Wild animals
- Agricultural/animal operations

Methodology

Collection of 20-L water samples (93 samples)
Filtration of two 10-L samples

Method 1623
One filter to an LT2 certified laboratory
One filter to CDC laboratory

Immunomagnetic separation of oocysts
PCR, DNA sequencing

Microscopy

Species and Genotypes Found

Summary and Impact:

Summary
- A cattle specific species (C. andersoni) was the predominant oocyst detected
- Pathogenic C. hominis and C. parvum were not detected in all 93 samples analyzed
- Only minor species/genotypes infecting humans were detected (10 samples)
- Molecular-based detection technique used in this project proves to be sensitive to detect and genotype oocysts in source waters

Impact
- Helped Utilities and Region 3 understand that oocysts in the surrounding county’s source water are predominantly non-pathogenic
- Utilities are setting out to work with the agricultural community by encouraging and implementing better management practices (BMPs) in the local cattle/dairy industry

What Lies Ahead for the Waterborne Cryptosporidium Research Program?

Multiple Pathogen Detection Systems
Quantitative PCR-Based Detection of Cryptosporidium spp.

- Many species and genotypes found in source water
- Most quantitative PCR published have varying degrees of specificities
- Development of multiplex qPCR assays

<table>
<thead>
<tr>
<th>species</th>
<th>All Cryptosporidium spp.</th>
<th>C. parvum specific</th>
<th>C. hominis specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C. hominis</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C. meleagridis*</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. felis*</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. muris</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T. gondii</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- *Purified genome DNA from CDC

Molecular Detection Technologies: A Perspective

1. Molecular-based detection of Cryptosporidium is in its infancy
2. A better understanding of the differences between zoonotic and human-specific Cryptosporidium/Giardia is possible
3. Advances in the “Protozoan Detection Toolbox” will improve our understanding of these parasites and their relationship to public health

Acknowledgements

Questions?

Eric N. Villegas
(513) 569-7017
villegas.eric@epa.gov
Development of a Universal Microbial Collector (UMC) for Enteric Pathogens in Water

Kelly R. Bright
Charles P. Gerba
Dept. of Soil, Water & Environmental Science

Project Aim

To develop a low cost universal microbial concentrator for application to water.

Universal Microbial Concentrator Requirements

- Simple, easy to operate
- High capacity
- High flow rate
- Low cost
- Concentrates diverse microorganisms
- Elution efficiencies similar to existing methods
- Limit interfering substances

Universal Microbial Concentrator

Universal Microbial Concentrator

Identification:

- Culture methods (bacteria)
- Microscopy (parasites)
- PCR/cell culture (viruses)

Cuno Carbon Nanofiber Filters

- Charge-modified granular carbon nanofibers
  - Highly porous
  - Large surface area
  - Highly positively charged
Adsorption of bacteria onto charge-modified carbon nanofibers

<table>
<thead>
<tr>
<th>Organism</th>
<th>Influent Volume (L)</th>
<th>Influent titer (per liter)</th>
<th>Effluent titer (per liter)</th>
<th>Log10 Reduction</th>
<th>Adsorbed microbes (per gram carbon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella terrigena</td>
<td>10</td>
<td>6.7 x 10^7</td>
<td>&lt; 50</td>
<td>&gt; 7.13</td>
<td>&gt; 1.9 x 10^6</td>
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<tr>
<td></td>
<td>125</td>
<td>4.4 x 10^7</td>
<td>&lt; 50</td>
<td>&gt; 6.94</td>
<td>&gt; 8.8 x 10^7</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>10</td>
<td>9.8 x 10^7</td>
<td>&lt; 50</td>
<td>&gt; 8.29</td>
<td>&gt; 1.6 x 10^7</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>2.1 x 10^7</td>
<td>&lt; 50</td>
<td>&gt; 7.62</td>
<td>&gt; 4.3 x 10^7</td>
</tr>
</tbody>
</table>

Adsorption of viruses onto charge-modified carbon nanofibers

<table>
<thead>
<tr>
<th>Organism</th>
<th>Influent Volume (L)</th>
<th>Influent titer (per liter)</th>
<th>Effluent titer (per liter)</th>
<th>Log10 Reduction</th>
<th>Adsorbed microbes (per gram carbon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-2 phage</td>
<td>10</td>
<td>1.9 x 10^8</td>
<td>&lt; 250</td>
<td>&gt; 5.87</td>
<td>&gt; 3.0 x 10^7</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>1.2 x 10^8</td>
<td>&lt; 250</td>
<td>&gt; 5.66</td>
<td>&gt; 2.3 x 10^7</td>
</tr>
<tr>
<td>Q22 phage</td>
<td>10</td>
<td>3.5 x 10^7</td>
<td>&lt; 250</td>
<td>&gt; 5.51</td>
<td>&gt; 9.0 x 10^7</td>
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<tr>
<td></td>
<td>125</td>
<td>1.6 x 10^7</td>
<td>&lt; 250</td>
<td>&gt; 5.40</td>
<td>&gt; 8.6 x 10^7</td>
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<tr>
<td>T phage</td>
<td>10</td>
<td>2.6 x 10^7</td>
<td>&lt; 250</td>
<td>&gt; 5.99</td>
<td>&gt; 4.0 x 10^7</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>1.4 x 10^7</td>
<td>&lt; 250</td>
<td>&gt; 5.77</td>
<td>&gt; 3.6 x 10^7</td>
</tr>
<tr>
<td>phi-174 phage</td>
<td>10</td>
<td>2.4 x 10^7</td>
<td>&lt; 250</td>
<td>&gt; 5.13</td>
<td>&gt; 6.4 x 10^7</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>5.3 x 10^7</td>
<td>&lt; 250</td>
<td>&gt; 5.34</td>
<td>&gt; 1.1 x 10^8</td>
</tr>
<tr>
<td>Q3 phage</td>
<td>10</td>
<td>4.0 x 10^7</td>
<td>&lt; 250</td>
<td>&gt; 5.60</td>
<td>&gt; 6.5 x 10^7</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>1.1 x 10^8</td>
<td>&lt; 111</td>
<td>&gt; 4.96</td>
<td>&gt; 1.6 x 10^8</td>
</tr>
<tr>
<td>Poliovirus / Rotavirus</td>
<td>10</td>
<td>1.0 x 10^7</td>
<td>&lt; 111</td>
<td>&gt; 4.96</td>
<td>&gt; 1.6 x 10^8</td>
</tr>
<tr>
<td>Adenovirus 40</td>
<td>10</td>
<td>1.0 x 10^7</td>
<td>&lt; 111</td>
<td>&gt; 4.96</td>
<td>&gt; 1.6 x 10^8</td>
</tr>
<tr>
<td>Feline Calicivirus</td>
<td>10</td>
<td>1.0 x 10^7</td>
<td>&lt; 111</td>
<td>&gt; 4.96</td>
<td>&gt; 1.6 x 10^8</td>
</tr>
<tr>
<td>Human Norovirus</td>
<td>10</td>
<td>1.0 x 10^7</td>
<td>&lt; 111</td>
<td>&gt; 4.96</td>
<td>&gt; 1.6 x 10^8</td>
</tr>
<tr>
<td>Hepatitis A Virus</td>
<td>10</td>
<td>1.0 x 10^7</td>
<td>&lt; 111</td>
<td>&gt; 4.96</td>
<td>&gt; 1.6 x 10^8</td>
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Adsorption of parasites onto charge-modified carbon nanofibers

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<th>Adsorbed microbes (per gram carbon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptosporidium parvum oocysts</td>
<td>10</td>
<td>1.0 x 10^7</td>
<td>&lt; 100</td>
<td>&gt; 4.00</td>
<td>&gt; 1.6 x 10^7</td>
</tr>
</tbody>
</table>

Argonide NanoCeram® Virus Sampler Filters

- Inexpensive: $40/filter
  (1MDS: $175/filter)
- High flow rates (up to 19 L/min)

NanoCeram® Virus Filters

- Alumina nanofibers [Al(OH)₃] on microglass fiber matrix
- Electropositive, non-woven, pleated, average pore size = 0.2μm
- Pre-sterilized
- Effective for fresh, brackish, seawater
- pH 5-10; Temps. 4-50°C

Experimental Protocol

- Test organism added to dechlorinated tap water at 2.0x10⁸ pfu / 20 L in a pressure vessel.
- Pressure applied (~ 2 p.s.i.) = flow rate of 2.0 L/min.
- Effluent samples collected to determine capture efficiency.
- 450 ml of eluting solution added to the filter housing (30 min hold).
- Eluting solution back flushed through the filter and collected (pH adjusted to 7.5).
- Eluent back flushed a second time.
- Eluent assayed for virus recovery.
Elution Methods

Hydrophobic interactions:
- Surfactants (Tween 80)
- Chaotropic agents (Cl\textsubscript{3}CCO\textsubscript{2}Na)

Electrostatic interactions:
- High pH (Beef extract, glycine, NaOH)
- Salt solutions
- Sodium polyphosphate (highly negatively charged)

Secondary Concentration Step

Volume reduction - centrifuge tube ultrafiltration (Vivaspin concentrator)

Reduces volume ~1000-fold (from 150 ml to ~150 μl)

Recovery of Microorganisms from NanoCeram\textsuperscript{®} Filters

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>pH</th>
<th>Filter Retention (%)</th>
<th>Elution Efficiency (%)</th>
<th>Method Efficiency After Concentration Step (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2 bacteriophage</td>
<td>9.3</td>
<td>99.95</td>
<td>55.9</td>
<td>54.6</td>
</tr>
<tr>
<td>Poliovirus 1</td>
<td>9.3</td>
<td>99.90</td>
<td>41.6</td>
<td>26.0</td>
</tr>
<tr>
<td>Adenovirus 2</td>
<td>9.3</td>
<td>99.90</td>
<td>36.8</td>
<td>22.2</td>
</tr>
<tr>
<td>Coxsackie B5</td>
<td>9.3</td>
<td>99.89</td>
<td>51.7</td>
<td>37.9</td>
</tr>
<tr>
<td>Echovirus 1</td>
<td>9.3</td>
<td>99.65</td>
<td>107</td>
<td>163.5</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>9.3</td>
<td>99.997</td>
<td>6.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

MS2 Phage Recovery From NanoCeram\textsuperscript{®} Filters

<table>
<thead>
<tr>
<th>Elution Solution</th>
<th>3% (w/v) Beef Extract</th>
<th>0.05 M Glycine</th>
<th>0.01 M Phosphate Buffer</th>
<th>0.3% (v/v) Tween 80</th>
<th>1.0% Sodium polyphosphate</th>
<th>Elution Efficiency (% Recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21.9</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>24.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>55.9</td>
</tr>
</tbody>
</table>

Virus Assay

- Quantify number of viruses recovered using plaque-forming assay or tissue culture infectious dose 50 assay (TCID\textsubscript{50}).

<table>
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<tr>
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<td>9.3</td>
<td>99.997</td>
<td>6.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

Method Advantages

- Much lower cost ($40 for NanoCeram\textsuperscript{®} filters vs. $175 for 1MDS filters)
- No organics used in the elution step
- Reduced volume (~ 200 μl vs. ~ 20 ml)
- Higher efficiencies than those reported for some enteric viruses.
Future Work

- Comparison to existing methods in a field study collecting surface water samples in Arizona, Michigan, and Mexico:
  - 1MDS filters, ultrafiltration
  - Adenoviruses, enteroviruses
  - cell culture, polymerase chain reaction
- Evaluate physical methods for recovery of parasites (Microsporidia) from NanoCeram® filters.

Acknowledgments

Luisa Ikner
Marcela Soto

Questions?
Overview

- Milestones of the Continuous Flow Centrifugation methodology (CFC) developed at Tufts
- Objectives of the current STAR award 2006 – 2009
- Progress: new automated method/equipment for multiple waterborne pathogens
- Future tasks
- Acknowledgements

Tier 2 mean results of the CFC compared to Method 1623 criteria

<table>
<thead>
<tr>
<th>Matrix/Organism</th>
<th>Method 1623 Acceptable Range of Mean Recovery (%)</th>
<th>CFC Study Mean Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent Water</td>
<td>Cryptosporidium 21-100</td>
<td>42.5</td>
</tr>
<tr>
<td>Giardia</td>
<td>Cryptosporidium 17-100</td>
<td>47.2</td>
</tr>
<tr>
<td>Source Water</td>
<td>Cryptosporidium 13-111</td>
<td>37.4</td>
</tr>
<tr>
<td>Giardia</td>
<td>Source Water 15-118</td>
<td>32.6</td>
</tr>
</tbody>
</table>

PCFC Approved by EPA as a Standard Concentration Method

Method 1622: Cryptosporidium in Water by Filtration/IMS/FA

Changes in the December 2005 Version of the Method
The method was revised again in 2005 to support promulgation of EPA’s Long Term 2 Enhanced Surface Water Treatment Rule. Changes incorporated into the June 2003 version include:

- Nationwide approval of the use of variable continuous-flow centrifugation as a modified version of the method. The product met all method acceptance criteria for Cryptosporidium using 50-L source water samples.

Continued...

2005 - the CFC 200 and 625B bowl became commercially available

2007 – Under the second EPA STAR award

1st automated CFC prototype
Objectives for 2006-2009

- Simultaneous concentration of representative microorganisms from each group of the CCL list
- Validation of the concentration methodology
- Detection and quantitative identification of the CCL list using multiplex miniaturized fiber optic bead microarrays coupled with a compact scanner
- Side by side comparison of this detection methodology with EPA standard methods

Expanding the CFC methodology beyond protozoa concentration

- Design of a new multiple pathogens bowl
- Design of a portable computerized concentration/elution equipment
- Design of a disposable tubing kit
- Choosing the programming software
- Testing variable operating protocols

How does it work?

- Filtration components are based on size exclusion which is prone to clogging and the overall procedure is labor intensive and expensive
- The new automated CFC methodology employs centrifugal force to sediment the protozoa and bacteria inside the bowl with minimal clogging problems.
- The modified bowl allows the “particle-free sample” to flow through the positive charged component in the core and the viruses are adsorbed by the positive electrostatic forces
- Elution buffers are injected sequentially where the trapped protozoa/bacteria first, then the viruses second, are dislodged and the concentrates are delivered to two separate sterile bags.

Automated CFC protocol

Water Sample 10 – 50 L

Concentration
- Assemble the modified bowl/tubing harness
- Turn on the CFC, select operation mode
- Protozoa, bacteria and viruses are concentrated inside the bowl

Elution
- Protozoa/bacteria buffer is injected; the bowl goes through shaking cycles, the concentrate (~200ml) is delivered to a sterile infusion bag
- Virus buffer with a neutral charge is then injected, the bowl goes through rinsing cycles, the concentrate (~20ml) is delivered to a 2nd bag

Detection
- The first concentrate is divided into bacteria and protozoa aliquots, and together with the virus concentrate are then processed for detection using standard or rapid methods

New bowl for simultaneous pathogen concentration

Spiking experiments using the automated CFC
Recovery efficiency of the automated CFC with 10 L tap water samples spiked with multiple microorganisms

- C. parvum were spiked and the oocysts detected from the concentrate using method 1623
- MS2 bacteriophages (ATCC 15597-B1) were spiked and detected from the concentrate using the agar overlay method (the host was E. coli 1559)
- B. anthracis spores (kanamycin resistant strain, sterne) detected by MF

Recovered concentrates and detection methods

Virus concentrate
- Agar overlay procedure for MS2 phage

Bacteria/protozoa concentrate
- EPA Method 1623 for C. Parvum
- Vacuum filtration and media growth for B. anthracis

Detection and integration

- We have concentrated on the detection of DNA isolated from E. coli as a model system. We have demonstrated the detection of PCR amplicons from three virulence genes using multiplexed bead-based microarrays.
- We expanding the protocol and microarray to include all bacteria and viruses listed as CCL3 candidates as listed
CCL3 candidates

- Caliciviruses
- Campylobacter jejuni
- Entamoeba histolytica
- Escherichia coli (0157)
- Helicobacter pylori
- Hepatitis A virus
- Legionella pneumophila
- Naegleria fowleri
- Salmonella enterica
- Shigella sonnei
- Vibrio cholerae

Accomplishments

- A prototype automated pathogen concentrator was designed and constructed
- This includes modification of the hardware and of the disposables
- The device weighs 45lb; 110/220VAC/12VDC
- Capable of simultaneous concentration of protozoa (Cryptosporidium), bacterial spores (B. anthracis) and MS2 from volumes of 10-50L
- Computer programmable PLC capable of handling numerous automated protocols

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Spike dose</th>
<th>Recovery (%) for</th>
<th>Recovery (%) for</th>
</tr>
</thead>
<tbody>
<tr>
<td>protozoa</td>
<td>300ml</td>
<td>40.0±6.06</td>
<td>40</td>
</tr>
<tr>
<td>spores</td>
<td>50mg</td>
<td>34±14.14</td>
<td>~30</td>
</tr>
<tr>
<td>MS2</td>
<td>10^9</td>
<td>4±6.3</td>
<td>50</td>
</tr>
</tbody>
</table>

The next phase

- Walt’s lab is currently working on the bioinformatics of the CCL list for the microarray detection: this will be completed over the next 12 months
- Once the detection platform is complete, the automated CFC spiked concentrates will be applied and quantitated
- The detection will be compared with currently approved standard methods
- Ideally this approach should be evaluated by water testing labs – filed testing, as was done for C. parvum and Giardia
- Evaluate the technology as a continuous monitoring system

Acknowledgements

- EPASTAR program (RD 83300301) which is funding this work
- Haemonetics for technical and material support over the past 10 years
- Staff of the Division of Infectious Diseases for technical support
Objectives

1. Reducing the Time to Detect Growth using Dye-doped Nanoparticles
2. On-chip PCR based Detection of 20 Pathogens
3. Enhancement in Sample Concentration by Cross-flow Filtration

Nano-particles for monitoring of growth

Growth curve by plate count, real time PCR, absorbance and dye doped NP assay

Time taken to determine the increase in growth by various methods

Contact the presenters
2. On-chip PCR based Detection of 20 Pathogens

Estimated 5-19.5 million illnesses/yr from water; $20 billion loss

Etiology of 174 waterborne disease outbreaks due to non-chemical agents between 1991 and 2002 (Crump et al., 2006)

List of 20 Pathogens

1. Aeromonas hydrophila
2. Burkholderia pseudomallei, mallei
3. Campylobacter jejuni
4. Clostridium perfringens
5. Enterococcus faecalis, faecium
6. Escherichia coli, Shigella
7. Helicobacter pylori
8. Haemophilus influenzae
9. Legionella pneumophila
10. Lepthospira interrogans
11. Listeria monocytogenes
12. Mycobacterium avium, paratuberculosis, tuberculosis, leprae
13. Pseudomonas aeruginosa
14. Salmonella typhimurium
15. Staphylococcus aureus
16. Vibrio cholerae, mimicus, vulnificus
17. Vibrio parahaemolyticus
18. Yersinia enterocolitica, pestis, pseudotuberculosis
19. Cryptosporidium parvum, hominis
20. Giardia lamblia, intestinalis

DNA Biochip: Multiplex PCR Amplification

Multiplex PCR-amplification followed by DNA chip-based amplicon identification

Without Multiplex Amplification

mixture of amplicons
ing 1 reaction

identification of amplicons using DNA biochip

With Multiplex Amplification

~1% of the population

0.01 to 0.0001%

BioTrove’s On-Chip PCR System

Hydrophilic and hydrophobic coatings enable reagents to load into and stay within the bottomless through-holes via passive capillary action.

FunGene Pipeline

- Harves Functional Genes from GenBank using Hidden Markov Model (HMM)
- Training sequences chosen by experts is input
- Matching sequences are output

Large set of Virulence and Marker Genes

200 VMGs
LOD: 10 copies!

Quantification without standard curves?

High GC Content and Larger Genomes: More Problematic

Predicted Ct versus theoretical Ct based on empirical equation

(gs = genome size of target organism, GC = GC content of target organism’s genome, Tm7 = theoretical melt temperature of last 7 bases on primer 3’ end, al = amplicon length)

Larger difference at lower copy numbers

Hand-held Gene Analyzer

Effect of Exposure Time, Path Length, and DNA Concentration on Signal Intensity
3. Enhancement in Sample Concentration by Cross-flow Filtration

**Goals:** Increasing
1. Rate of concentration ($J_{\text{permeate}}$)
2. Recovery
3. Reproducibility

**Approach:**
- Hydraulic management (Goals 1 & 2)
  - $\Delta P \rightarrow J_{\text{permeate}}$
  - $J_{\text{permeate}} \rightarrow$ recovery
- Preparation of reproducible non-adhesive membrane (Goals 2 and 3):
  - Non adhesive surface $\rightarrow$ recovery
  - Controlled approach to membrane blocking $\rightarrow$ reproducibility

---

**Rate of Sample Concentration**

Amount of water filtered in 30 min normalized to 1 m² of membrane surface area

**Influence of $\Delta P$ and $J_{\text{crossflow}}$ on Bacteriophage Recovery**

Contact the presenters

**Design of non-adhesive surface**

- Protein Blocking of the membrane:
  - May not always be “appropriate or practical due to concern related to the amount of time needed (…) and potential for microbial contamination”
  - Hill et al. 2005

- New approach to membrane blocking
  - Reproducible, non-adhesive coatings based on multilayer polyelectrolyte films
  - Fast and straightforward coating procedure
  - Design flexibility (charge, hydrophilicity)
  - Have been shown to reduce adhesion of bacteria, mammalian cell and proteins
  - Recoverable coating

Source: G. Decher, 1997

**Pump Evaluation: Reduction in Cross-flow over Time**

Influence of $\Delta P$ and $J_{\text{crossflow}}$ on microorganism recovery: Experimental setup

Membrane: PES 30kDa, blocked with 5% calf serum, 44 cm²

---

**Influence of $\Delta P$ and $J_{\text{crossflow}}$ on microorganism recovery:** Experimental setup

- Membrane: PES 30kDa, blocked with 5% calf serum, 44 cm²
- Watson Marlow 621 cc peristaltic pump
- Pressurized feed tank: 1L feed
- Membrane module
- Pressure gauge
- Concentrate
- Permeate
- Balance

**Contact the presenters**
**PEM deposition procedure**

- Source: G. Decher, 1997

**Potential of Polyelectrolytes as Blocking Agents**

- Fibroblast adhesion before and after deposition of PEM (PAA/PAAm)
- Some polyelectrolytes inhibit phage infectivity of bacteria (plaque assay cannot be used)
- Epifluorescence and PCR are being evaluated as alternative methods of quantifying viruses

- Yang et al. 2003
- Patel et al. 2007

**Summary**

1. NP-based assay faster but expect to be busy
2. On-chip PCR: efficient screening tool, for samples that will result in 10 copies
3. Sample concentration speed can be considerably improved with higher pressure (8 fold to 150 L/30 min-m²)
4. Improvements in blocking the filters: ongoing

**Acknowledgements**

Michigan Economic Development Corporation’s 21st Century Jobs Fund

Doctoral candidates:
- Robert Stedtfeld
- Elodie Pasco
- Farhan Ahmad
- Yu Yang

PIs: Syed Hashisham, Volodymyr Tarabara, and James Tiedje
Rapid and Quantitative Detection of Helicobacter pylori and E. coli O157 in Well Water Using a Nano-Wired Biosensor and qPCR

2009 U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water
May 20-21, 2009, Philadelphia, PA

E.C. Alosilla, J.B. Rose, E. Dreelin
Shannon McGraw, Michelle Packard, Jungwon Yoo,
Lauren Bul, and Teresa Brokis
Michigan State University

Outline
- Hypothesis
- Results by objectives
- Summary of results
- Future work

Hypothesis
- A disposable biosensor and qPCR can be combined seamlessly to develop a unique biosensor-qPCR as a tool for near real-time determination of contaminant occurrence in drinking water.

Objectives
- Develop a protocol for processing water samples for the biosensor and qPCR.
- Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for E. coli O157:H7 and H. pylori in groundwater samples from the field.
- Develop a method for detecting and enumerating E. coli O157:H7 and H. pylori by qPCR using bacteria isolated and screened by the biosensor system.
- Validate a method for testing viability of E. coli O157:H7.

Highlight of Results
- Developed a novel target extraction system using an electrically active magnetic nanoparticles.
- Developed a protocol for use of automated DNA extraction and evaluated it in difficult samples.
- Developed a database on CFU vs qPCR units for E.coli and Enterococci, and will be adding in the data from each sample for the O157.
- E. coli O157:H7 biosensor has been tested in pure and seeded water samples.
- Viability test has been developed; sensitivity and specificity were evaluated.

Flowchart of Research Plan
- Membrane filtration of water sample – 8 liters at source
- Extraction/elution of cells at water source
- Biosensor for qualitative field screening of microbial contaminants
- qPCR for quantitative enumeration
- Viability test for E. coli O157:H7
Biosensor

**ANALYTE**
- Antibodies
- Nucleic Acids (Epitopes)
- Aptamers
- Enzymes
- Nanoscale materials

**BIORECEPTOR**
- Whole cells

**TRANSUCER**
- Amplifier

**SIGNAL PROCESSING**
- Data Acquisition

- **Advantages:**
  - Rapid detection time
  - High sensitivity and specificity
  - Compatible with data processing technologies
  - Can be ruggedized

---

Real-Time Quantitative PCR (qPCR)

- Detects PCR product fluorescently in each well plate.
- Fast PCR screening without gels.
- Quantifies amount of PCR product at each cycle.
- Detects presence or quantify fraction of sample made up by particular species using species specific primers.
- Uses threshold detection for relative abundance.

---

Results By Objectives

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---

Membrane Filtration (MF) & Enrichment

- Using EAM Nanoparticles for Target Extraction
  - Electrically active magnetic nanoparticles (EAM) functionalized with antibodies

---

100 ml water sample at the source

- Electrively active magnetic nanoparticles for separation and concentration
- Biosensor for qualitative field screening of microbial contaminants
- Viability test for E. coli O157:H7
Iron oxide-polyaniline core/shell \( \rightarrow \) EAM

- Unique electronic structure and flexible electrical properties of protonated polyaniline
- Magnetic properties from the core
- Simple and low cost preparation
- Excellent environmental stability

**Magnetic Measurement of EAM**

![Graph showing magnetic measurement results](image)

**Magnetic characterization**

<table>
<thead>
<tr>
<th>( \gamma )-Fe(_2)O(_3): Aniline Wt. Ratio</th>
<th>Coercivity (300K) Oe</th>
<th>Retentivity (300K) emu/g</th>
<th>Saturation Magnetization (emu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0.1</td>
<td>180</td>
<td>15.3</td>
<td>61.1</td>
</tr>
<tr>
<td>1:0.4</td>
<td>180</td>
<td>9.57</td>
<td>40.3</td>
</tr>
<tr>
<td>1:0.6</td>
<td>180</td>
<td>9.48</td>
<td>37.7</td>
</tr>
<tr>
<td>1:0.8</td>
<td>180</td>
<td>9.18</td>
<td>33.5</td>
</tr>
</tbody>
</table>

Low coercivity and retentivity values \( \rightarrow \) EAMs are in the paramagnetic regime.

**Electrical conductivity of EAM**

![Graph showing electrical conductivity](image)

**Characterization of EAM**

![Scanning Electron Microscopy Images](image)

**Transmission Electron Microscopy and Electron Diffraction Images**

TEM images of (left) unmodified Fe\(_2\)O\(_3\) NPs and (right) electrically active magnetic NPs.

XRD shows EAM is crystalline.

Magnetic properties from the core

Simple and low cost preparation

Excellent environmental stability

Four point probe measurements in compressed pellets of 2000 microns in thickness.
Energy dispersive spectroscopy

<table>
<thead>
<tr>
<th>Element</th>
<th>Weight%</th>
<th>Atomic%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>28.49</td>
<td>44.34</td>
</tr>
<tr>
<td>NK</td>
<td>67.72</td>
<td>8.20</td>
</tr>
<tr>
<td>F</td>
<td>29.88</td>
<td>2.64</td>
</tr>
<tr>
<td>Ti</td>
<td>71.82</td>
<td>10.37</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td></td>
</tr>
</tbody>
</table>

1:0.6 EAM Nanoparticle

Electrical Characterization of EAM

- EAM follows ohmic behavior.
- Ohm's law: \( I = V/R \)

Experiments for immuno-EAM capture -- for \( 10^6 \) cfu/ml

- Incubation time: 15, 30, 60 min
  - 30 min had most cell capture
- Antibody concentration: 0.1, 0.25, 0.5, 1.0 mg/ml
  - 0.5 mg/ml had most cell capture
- EAM concentration: 10, 20, 25 mg/ml
  - 10 mg/ml had the most cell capture

Ab-EAM for Cell Capture

- Method: Physical adsorption (Muhammad Tahir et al., 2003)
- Combine EAM nanoparticles with monoclonal antibodies to target cell in PBS solution
- Conditions:
  - Room temperature incubation
  - Time: 45 min

Capture efficiency for E. coli O157:H7

- Cell capture was confirmed by plating:
  - Observation: Capture process decreased cell count by less than a factor of 10.
Results By Objectives
- Develop a protocol for processing water samples for the biosensor and qPCR.
- Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for E. coli O157:H7 and H. pylori in groundwater samples from the field.
- qPCR
- Develop a method for detecting and enumerating E. coli O157:H7 and H. pylori by QPCR using bacteria isolated and screened by the biosensor system.
- Validate a method for testing viability of E. coli O157:H7.

Primers and probes for the qPCR assays

<table>
<thead>
<tr>
<th>Organism</th>
<th>Target gene</th>
<th>Primer/Probe</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli O157</td>
<td>uidA</td>
<td>5'CAATGGTGATGTCAGCGTT3'</td>
<td>Developed by this study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'CACTGACACTGGCTTCTG3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6FAM-TTGCAACTGGACAAGGCACT- TAMRA</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>uidA</td>
<td>5'CAATGGTGATGTCAGCGTT3'</td>
<td>Developed by this study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'CACTGACACTGGCTTCTG3'</td>
<td></td>
</tr>
<tr>
<td>Enterococci</td>
<td>23SrDNA</td>
<td>AGA AAT TCC AAA CGA ACT TG</td>
<td>Published by El et al, 2002</td>
</tr>
</tbody>
</table>

Standard curve for E. coli O157 assay

Standard curve for 10-fold serial dilutions of E. coli O157 uidA gene. Linear regression analysis shows an R² of 0.99, a slope of -3.39 and an intercept of 39.121.

Standard curve for E. coli assay

Standard curve for 10-fold serial dilutions of generic E. coli uidA gene. Linear regression analysis shows an R² of 0.995, a slope of -3.22 and an intercept of 38.439.

Standard curve for Enterococci assay

Standard curve for 10-fold serial dilutions of generic enterococci 23SrDNA gene. Linear regression analysis shows an R² of 0.999, a slope of -3.34 and an intercept of 39.574.
**Results By Objectives**

- Develop a protocol for processing water samples for the biosensor and qPCR.
- Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for *E. coli* O157:H7 and *H. pylori* in groundwater samples from the field.
- **Biosensor**
  - Develop a method for detecting and enumerating *E. coli* O157:H7 and *H. pylori* by qPCR using bacteria isolated and screened by the biosensor system.
  - Validate a method for testing viability of *E. coli* O157:H7.

**Performance of biosensor**

![Image of biosensor](image)

**Sample Preparation**

![Image of sample preparation](image)

**Key Results**

- Rapid qPCR methods have been developed for two fecal indicators *E. coli* and Enterococci and two pathogens *Helicobacter* and *E. coli* O157:H7.
- qPCR has been used to detect *Helicobacter* in sewage and detects what is likely the viable non-cultivable state (previous report and publication).
- qPCR is highly correlated to *E. coli* and Enterococci in sewage but this same assay does not detect all of the species present in manure, either due to interferences or more likely due to specificity of the primers.
**Antibodies and Bacterial Isolates**

- Antibodies
  - Purified mouse monoclonal anti-*E. coli* O157:H7 (OEM Concepts)
  - Purified goat polyclonal anti-*E. coli* O157:H7 (Kirkegaard & Perry Laboratories Inc.)
- Bacterial Isolate
  - *E. coli* O157:H7 C3000

**Results in Pure Culture**

- Data can be analyzed as a positive or negative detection based on concentration averages or individual readings
- Negatives seen at 10^8 and 10^7 cfu/ml; has fewer recorded data points

**Seeded Water Samples With Various Cell Concentrations**

- 27 positive samples ranging in concentration of 10^7-10^9 cfu/ml
- 70% cut-off
- 80% true positive; 0% false negative
- Cumulative signal taken 2, 4, 6 min after sample application

**Seeded Water Samples With Various Cell Concentrations**

- 27 positive samples ranging in concentration of 10^7-10^9 cfu/ml
- 70% cut-off
- 80% true positive; 98% false negative
- Detection signal taken 6 min after sample application
Key Results

- Sensitivity studies need to be continued.
- Can not currently quantify the concentration of bacteria in the sample because of observed hook effect due to cell crowding and variances between testing.
- The overall time interval from obtaining a sample to readout with the biosensor is < 20 minutes.
- Biosensor design and parameters need to be modified/improved to minimize false negative.

Results By Objectives

- Develop a protocol for processing water samples for the biosensor and qPCR.
- Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for *E. coli* O157:H7 and *H. pylori* in groundwater samples from the field.
- Develop a method for detecting and enumerating *E. coli* O157:H7 and *H. pylori* by qPCR using bacteria isolated and screened by the biosensor system.
- Validate a method for testing viability of *E. coli* O157:H7.

BacTiter-Glo™ Microbial Cell Viability Assay

- Concentration of *E. coli* C3000 (ATCC #59957) by centrifugation
- Antibody separation
  - Goat-derived, polyclonal, biotinylated antibody (Meridian Life Sciences, Cat# B65109B)
  - Magna-Sphere streptavidin-coated magnetic beads (Promega Cat # Z5481)
- The BacTiter™ Microbial Cell Viability Assay (Promega Cat# CM230)
- Greater numbers of positive results compared to the standard methods
  - Likely due to low specificity

Comparison of Noise Levels as a Result of Diluents

TSB resulted in a loss of detection at a dilution of 10^−1, while *E. coli* in both MH broth and peptone water were significantly positive when compared to blanks.

Comparison of Peptone and MH Broth as Diluents
Comparison of Incubation Times

E. coli C3000 Grown in Mueller Hinton Broth

Introduction of Magnetic Separation to Assay

Use of Portable Centrifuge and Luminometer

Testing of Environmental Samples

Surface Water Samples Week 1: One Week Refrigeration

Sensitivity and specificity were insignificantly affected by time.

The BacTiter-GloTM assay reliably detected live E. coli cells at concentrations as low as 10³ cfu/ml.

Although a slight decrease in sensitivity; a detection as low as 1.37 x 10³ cfu/ml remained possible with magnetic separation.

Results revealed sensitivity levels in the range at the low 10² cfu/ml.

Sixty river surface water samples obtained from Ingham County Health Department.

Gold standard:
- Samples less than 300 cfu/mL considered negative
- Equal to or greater than 300 were positive

Receiver operator curve (ROC) analysis performed using the gold standard as determined by Ingham County Health Department Data.
Surface Water Samples Week 2: One Day Refrigeration

Receiver Operating Characteristic (ROC) Curve Using Cutoff of 300 cfu/mL

Effect of Gold Standard Cutoff on ROC

Adoption of Gold Standard Set to 600 cfu/mL
- Cutoff level of 300 cfu/mL
- 6 sampling sites with contradicting results
- Contradiction between sampling location (left, center or right)
- Cutoff level of 600 cfu/mL
  - Decreased number of contradicting sites to one
  - Previously positive results now negative

Specificity Testing: *E. coli* vs. *Salmonella*

The resulting ROC curve (x axis=FPR; y axis=TPR) shows viability assay has little value since the area under the curve is only 0.36. (FPR=false positive rate; TPR=true positive rate)

The greatest diagnostic value of the assay is noted when the gold standard cutoff is set to approximately 600 cfu/mL; the area under the curve has approximately 0.76.

Comparison of current and proposed cutoff levels on week 2 results: Red star indicates sites with contradicting results using the 300 cfu/mL cutoff; green star indicates contradiction with the 600 cfu/mL cutoff.
Specificity Testing: *E. coli* vs. *Salmonella*, *S. Aureus*, and *Enterococcus*

![Graph showing specificity testing results for different bacteria.](image)

Alternate Approach to Viability Test

**A: Thoma-Harley ATP**

\[ \text{Gluose} + \text{ATP} \rightarrow \text{Gluose 6-Phosphate} + \text{ADP} + \text{H}^+ \]

(Detectable change in sample increase)

**B: Hexokinase-bound E. coli O157:H7**

\[ \text{Gluose} + \text{ATP} \rightarrow \text{Gluose 6-Phosphate} + \text{ADP} + \text{H}^+ \]

(Detectable change in sample increase)

**C: Negative Controls**

\[ \text{Gluose} \rightarrow \text{ATP} \]

(No increase)

Output: Papers and Thesis

**Peer-reviewed Publications:**


**Thesis:**

Arun Nayak, MS 2008; Stability And Quantitative Surveillance Of *Helicobacter pylori* And *Campylobacter jejuni* In Environmental Waters By Real Time qPCR.

Future Work

**qPCR**

- Develop a publication on the qPCR indicator studies.
- Characterize the occurrence of 0157 in sewage and manure along with *E. coli* and *Enterococcus* as an indicator with qPCR.

**Biosensor**

- Do test with seeded environmental water samples; do test using environmental water samples.
- Test alternative design using SPCE biosensor.

**Viability assay**

- Continue investigation into the replacement of currently employed biotinylated antibody with a more effective method of isolating E. coli from other bacterial contaminants prior to viability testing. In order to decrease cross-reactivity of developed assay.
- Optimize the sensitivity and specificity determination of a strain-specific assay to detect viable E. coli O157:H7 in surface water samples.
- Implement alternative design using ATP-hexokinase system.

**Biosensor-qPCR system**

- Integrate biosensor-qPCR-viability assays into a monitoring system.

Acknowledgment

**Funding sources for outputs of this project:**

- US Environmental Protection Agency
- Department of Homeland Security through the National Center for Food Protection and Defense
- Michigan Department of Environmental Quality

**Graduate students working on this project:**

- Shannon McGraw, Michelle Packard, Sangeetha Srinivasan

**Undergraduate students working on this project:**

- Lauren Bul, Teresa Brinks
- Postdoc working on this project:
  - Jongseol Yuk

**Other students who are members of the Alocilja Research Group:**

**Output: Presentations**

- Presentations:
  - Sangeetha Srinivasan, Shannon McGraw, Lauren Bell, Sangeetha Srinivasan, Arto Stell, and Joan R. Rose. Detection of waterborne pathogens using Real Time PCR and Biosensor methods.
  - Presentations for the USEPA workshop on innovative approaches for Detection of Microorganisms in Water. Cincinnati, OH, June 4-6, 2007.
Any Question?
Assessment of Microbial Pathogens in Drinking Water using Molecular Methods Coupled with Solid Phase Cytometry

Barry H. Pyle, Associate Research Professor
Department of Microbiology, Montana State University
U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water, May 20-21 2009
Philadelphia, PA

COLLABORATORS
Anne Camper
Susan Broadaway
Al Parker
Jo-An Lindstrom
Montana State University
Bozeman, MT
Tim Ford
University of New England
Biddeford, ME

Overall Objective

- To develop and evaluate innovative approaches for quantitative assessment of pathogens

Target Microbial Pathogens

- *Escherichia coli* O157:H7
- *Helicobacter pylori*
- *Legionella pneumophila*
- *Mycobacterium avium*
- *Aeromonas hydrophila*
- *Giardia lamblia*
- *Cryptosporidium parvum*

Procedures

- Fluorescent in situ hybridization (FISH)
  - Enhance with tyramide amplification
  - Use polyamide nucleic acid (PNA) probes
- In situ nucleic acid amplification
  - Specific target genes inside individual cells (Hodson et al., 1995)
  - Improved methods, e.g. (Notomi et al., 2000; Maruyama et al., 2003 & 2005)
- Membrane filtration
- Solid Phase Laser Cytometry

Solid phase laser cytometry

- Scan a 25 mm diameter membrane filter in 3-4 minutes
- Detect individual fluorescent particles
- Discriminate between cells & debris
- Locate particles on microscope
- Validate bacteria, eliminate other particles
Solid Phase Laser Cytometer

ChemScan
RDI
(AES-Chemunex)

Range of Cell Labels
- Total Cell Count
  - Sybr Green
- Total Viable Count
  - ChemChrome
  - Enzyme activity
  - Membrane integrity
- Identification Tests
  - Antibodies
  - Specific enzymes
  - Nucleic acid probes
  - FISH
- Dual Labeling
  - Fab-CTC
  - ChemChrome-Fab
  - DVC-FISH (Baudart et al., 2002)

E. coli O157:H7, Immuno-magnetic Beads, CTC, FITC

CHEMCHROME V3-Labeled Bacillus cereus

B. cereus - B183 Antibody With Anti-Mouse TRITC Label

B. cereus - CHEMCHROME WITH B183 Antibody-TRITC

Pyle et al., 1999
Pyle et al., 2000
Pyle et al., 2000
E. coli SYBR Green vs FISH

SYBR Green Stained  FISH with ECO-Alexa

Images captured at same camera settings

Epifluorescent Microscopy

<table>
<thead>
<tr>
<th>SYBR Green</th>
<th>FISH Eco Alexa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log CFU/ml</td>
<td>Log CFU/ml</td>
</tr>
<tr>
<td>5.87</td>
<td>5.78</td>
</tr>
<tr>
<td>6.19</td>
<td>6.38</td>
</tr>
<tr>
<td>6.20</td>
<td>6.01</td>
</tr>
<tr>
<td>6.09</td>
<td>6.06 Mean</td>
</tr>
</tbody>
</table>

Goal Performance Characteristics
- Detection of different target bacteria with specific probes
- Detection of low numbers of pathogens
- Includes VBNC bacteria
- Can include infectivity and/or virulence
- Viable or active cells
- Single cell enumeration
- Sensitivity – 1 cell per filterable volume
- Rapid – Results within 6-8 hours

Scope of Project
- Drinking water and source waters
- Native American students at Little Big Horn College and Montana State University-Bozeman to participate

ACKNOWLEDGMENTS
- U.S. Environmental Protection Agency
  Barbara Klieforth, Project Officer
- NIH Environmental Health Sciences
- NASA
- DoD – U.S. Army
- AES-Chemunex, Inc.
- LigoCyte Pharmaceuticals, Inc., Bozeman
- Montana State University
References


References (continued)


Detecting Pathogens in Water by Ultrafiltration and Microarray Analysis

Anthea K. Lee
Metropolitan Water District of Southern California

Metropolitan Water District of Southern California (MWD)
- Consortium of 26 cities and water districts
- Provide water for >18 million people in Southern California; 5200 square mile service area
- Delivers an average of 1.7 billion gallons of water daily

MWD Facts
- 5 treatment plants
- 5 pumping plants (1,617 feet lift total)
- 9 reservoirs
- 775 miles of pipeline

MWD System

Pathogen Detection Strategy
- Water sample
- Ultrafiltration
- DNA Extraction
- Whole Genome Amplification
- Confirm infectivity
- Microarray detection

Model organisms
- E. coli K12
- Salmonella typhimurium
- Cryptosporidium parvum
- Human Adenovirus type 2
**Ultrafiltration**

Flowchart showing the process of ultrafiltration.

**Ultrafiltration Recovery**

Graph showing recovery percentage over different concentration levels.

**C. parvum survive ultrafiltration**

Graph showing the survival of C. parvum through ultrafiltration.

**Salmonella Replication Assay**

- Cell monolayer
- Infect with *Salmonella*
- Kill extracellular bacteria
- Incubate or not
- Lyse cells and plate intracellular bacteria

**Adenovirus Plaque Assay**

- A549 human lung carcinoma cells
- Infect for 1 hour
- Add agar overlay
- Incubate 7 days
- Stain with crystal violet
- Count plaques

**Pathogen Detection Strategy**

1. Water sample
2. Ultrafiltration
3. DNA Extraction
4. Whole Genome Amplification
5. Confirm infectivity
6. Microarray detection
WGA for 10 ng starting material

<table>
<thead>
<tr>
<th>Kit</th>
<th>expected yield* (μg/mL)</th>
<th>actual yield** (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>REPL-Ig Ultrafast Mini (Qiagen)</td>
<td>350-500</td>
<td>357</td>
</tr>
<tr>
<td>Illustra GenomiPhi V2 (GE Healthcare)</td>
<td>200-300</td>
<td>317</td>
</tr>
<tr>
<td>GenomPlex Complete (Sigma)</td>
<td>40-93</td>
<td>none detected</td>
</tr>
<tr>
<td>DOP-PCR (Roche)</td>
<td>not specified</td>
<td>9</td>
</tr>
</tbody>
</table>

• Need 1-5 μg per microarray

WGA Results post-ultrafiltration

<table>
<thead>
<tr>
<th>Organism (10^6 inoculum)</th>
<th>Extraction Kit</th>
<th>Mini WGA range (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Invitrogen forensic kit</td>
<td>0.13-0.35</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Invitrogen forensic kit</td>
<td>0.634-4.53</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>MoBio ultraclean soil kit</td>
<td>1.95-8.39</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Invitrogen Purelink viral RNA/DNA kit</td>
<td>Not done yet</td>
</tr>
</tbody>
</table>

• 10^6 inoculum
• 17 fg DNA/bacterial cell
• Starting material ~0.01 ng DNA/10,000 cells
• Scaling up using Midi kit

E. coli K12 microarray to test integrity of WGA products

- 40 bp probes
- every 800 bp
- ~5800 probes
- cognate mismatch for each probe
- factory standard positive and negative controls

Target Preparation

- WGA product
- cleave with CsaI P enzyme
- label with biotin
- hybridize to microarray
- add fluorochrome-labeled streptavidin

Need to optimize microarray conditions

Summary of Progress

- Ultrafiltration recoveries for E. coli, Salmonella and Cryptosporidium are satisfactory
- Can use WGA to amplify genomic DNA recovered from ultrafiltration
- Infectivity confirmed for Cryptosporidium
Future Directions

- Optimize UF for Adenovirus
- Optimize larger scale WGA
- Optimize microarray parameters
- Finish infectivity studies
- Design custom microarray

Acknowledgments

Katrin Hanley
Jin Huang
Patty Huang
Anne Johnson
Paul Rochelle
Other colleagues at MWD
Research Objectives

1. Explore and establish experimentally piezoelectric-actuated millimeter-sized cantilever sensors suitable for detecting one pathogen in one liter of water using new cantilever oscillation and measurement modalities

2. Develop flow cell-PEMC sensor detection assembly for large sample volume

3. PEMC sensor for confirming pathogen identity by DNA signature

PPE

Motivation

- Waterborne parasites (Cryptosporidium, Giardia) have low infective dose (~10).
- Conventional methods require several days and need trained personnel (EPA method 1622 and 1623)
- Large-sample processes within a few hours without preparation is beneficial for environmental monitoring

Model parasites: Cryptosporidium parvum oocysts, Giardia lamblia cysts
Surrogates: E. coli O157:H7, E. coli JM101

Progress

1. Sensitive mode established; flow cell (version 4 designed & tested) model experiments with E. coli O157:H7, Crypto and Giardia show detection limit ~ 10 – 50

2. Successful 1 liter samples completed using modified flow cell; 1 cell/mL completed

3. DNA-based detection of E. coli O157:H7 (strX2 gene) at ~700 cells without amplification demonstrated in buffer

In Progress

1. Version-5 flow cell design and fabrication; river water Crypto at 10 and 100 liters

2. DNA-based detection of Crypto and Giardia

Cantilever dynamics

Resonant frequency of Cantilever in air:

\[ f_r = \frac{K}{\sqrt{M}} \]  

(1)

In liquid:

\[ f_a = \frac{K}{\sqrt{M + m_L}} \]  

(2)

When analyte of mass \( \Delta m \) binds:

\[ f_a = \frac{K}{\sqrt{M + m_L + \Delta m}} \]  

(3)

A virus weighs ~ 10^{-15} g (1 fg)
E. coli O157:H7 bacterium weighs ~ 10^{-12} g (1 pg)
A 30 kDa toxin weighs ~ 5 x 10^{-20} g (0.05 ag)
A 100-nt ssDNA weighs 5 x 10^{-20} g (0.05 ag)
Dominant Higher modes = PEMC innovation

Higher frequency modes are more sensitive

\[ f_r = \frac{v^2}{2 \pi \rho \mu L} = \frac{k}{2 \pi \sqrt{M}} \frac{1}{\sqrt{f_0}} \]

Sensitivity \[ \Delta f_r \propto \frac{k}{2 \pi M} \]

Experimental Apparatus

Mass change sensitivity

1-Hexadecanethiol 256 Da
1 fM = 256 ag/mL
\~ 602,000 molecules/mL

Self-Assembly of 1-Hexadecanethiol

16S-rRNA sequence

Pic from: http://www.ifm.liu.se/applphys/ftir/sams.html

Interface Chemistry

1. Amin-Linked Immobilization

Glass \( \rightarrow \) -NH\(_2\) group \( \rightarrow \) Covalently immobilized Ab

2. Cysteamine + glutaraldehyde + antibody

3. Protein G + IgG antibody


E. coli O157:H7 –
Confirmation, Repeatability and quantitation

\[ \Delta f = A \log(C_{\text{target}}) + B \]

- control: E. coli absent, Ab present
+ control: E. coli present, Ab absent

**Quantification of C. parvum in milk and in PBS**

\[ \Delta f = A \log(C_{\text{oocysts}}) + B \]

- Both in PBS and in milk gave good semi-log correlation.
- Detection in milk is 40-50% less sensitive than in PBS

**Flow rate influences sensor response**

\[ \Delta f = A \log(C_{\text{oocysts}}) + B \]

- Different sensors with variations (n=11) gave good correlation
- High flow rate gave larger response

16S-rRNA - human serum

**E. coli O157:H7 in water [APTES-Immobilization]**

Repeatability

One Liter Sample

**Cryptosporidium oocyst in tap water**

IgM immobilized via APTES

Sensor response is proportional to concentration

**Flow rate influences sensor response**

Cyst.+ Glutaraldehyde + mono IgG

- Protein G + Goat IgG

\[ \Delta f = A \log(C_{\text{oocysts}}) + B \]

- Both in PBS and in milk gave good semi-log correlation.
- Detection in milk is 40-50% less sensitive than in PBS

**Quantification of C. parvum in milk and in PBS**

**Flow rate influences sensor response**

Cyst.+ Glutaraldehyde + mono IgG

- Different sensors with variations (n=11) gave good correlation
- High flow rate gave larger response
Confirmations w/ release & SEM imaging

G. lambia cysts

Cysts + Glutaraldehyde + mono IgG

Sample preparation

1.5 mL PBS + 50,000 E. coli O157:H7 or JM101
Centrifuge at 10,000 g for 5 min
Pellet + 50 μL 1% Triton X

Probe:

HS-C6H12-5'-CCA CTC TGA CAC CAT CCT C-3'

One genomic copy per cell
Stx2 codes for shiga-like toxin2. Probe design - Primer 3

Pellet + 50 μL 1% Triton X
10 mins in boiling water, cool at 2-3 °C for 15 mins

Centrifuge at 10,000 g for 3 mins
Dilute supernatant in 10 mL TE buffer

Stx2-probe based detection– Prep I (buffer)

Detection of Microcystin LR in batch mode

Rijal and Mutharasan, unpublished

Stock 1.5 ng/mL genomic DNA (measured)
Diluted to 23 pg/mL

30-min pre

Probe Complementary to 876-894 on Stx2 (=1241 nt)

Conclusions

• Cantilever sensor mass change sensitivity = 1 ag/Hz
• E. coli in buffer – Detection limit – 10 (in theory one cell)
• One liter sample detection shown
• Crypto and Giardia in buffer & proteinous environment – ~ 10
• Stx2-gene based detection – ~700 cell detection. 100 appears to be feasible
MST research

Jorge Santo Domingo  
US EPA  
NRMRL/WSWRD/MCCB  
Cincinnati, OH

Monitoring Fecal Pollution

Microbial “Fecal Indicators”
- Indicate presence of fecal pollution and potential health risks, not when it happened or what is the source

Microbial Source Tracking or Fecal Microbial Forensics

Use of detectable molecular variations between related fecal microbial strains to infer the origin of pollution sources in a fecally contaminated watershed (or food supply).

Many methods and more to come!!!

Adapted from Cindy Nakatsu; EPA’s MST guide

Library-dependent methods

From this ...  

To this

Adapted from Dombek and others, 2000

NOT Source Trackers

BEST FRIEND!!!
**Host-Specific PCR Assays**

- Culture-independent
- Library-independent
- Rapid detection
- Sensitive
- Defined target
- Automated analysis
- Potential for multiple assays
- Potential for really cheap assays

**Comparative Sequence Analysis of 16S rDNA**

<table>
<thead>
<tr>
<th>Class of group</th>
<th>Genus</th>
<th>No. of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td>Corynebacterium</td>
<td>8/18</td>
</tr>
<tr>
<td>Bacilli</td>
<td>Catellicoccus</td>
<td>64/66</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Bacteroides</td>
<td>1/3</td>
</tr>
<tr>
<td>Clostridia</td>
<td>Clostridium</td>
<td>45/48</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Enterococcus</td>
<td>2</td>
</tr>
<tr>
<td>Methylotrophi</td>
<td>Unknown genus</td>
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<tr>
<td>Alphaproteobacteria</td>
<td>Paracoccus</td>
<td>8/9</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>Helicobacter</td>
<td>8/12</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>Eubacteriaceae</td>
<td>10/13</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>Unknown genus</td>
<td>1/1</td>
</tr>
<tr>
<td>Epsilonproteobacteria</td>
<td>Campylobacter</td>
<td>1/1</td>
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<tr>
<td>Flavobacteria</td>
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<td>13</td>
</tr>
<tr>
<td>Nitrospirae</td>
<td>Unknown genus</td>
<td>1/1</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>Synechococcus</td>
<td>1/1</td>
</tr>
<tr>
<td>Archaea</td>
<td>Unknown genus</td>
<td>1/1</td>
</tr>
</tbody>
</table>

**Development of gull assays**

**Phylogenetic affiliation of 16S rRNA genes from gull fecal clone library**

- DNA extract from feces
- PCR amplification w/ 16 rDNA primers
- Cloning, sequencing, blast, and phylogenetic analysis
- Rare groups used for assay development
Host-specificity tests of gull assay (Gull2) against feces from various animals

<table>
<thead>
<tr>
<th>Animal</th>
<th>Location</th>
<th>Samples tested</th>
<th>Gull2 PCR – No. of positive samples</th>
<th>qPCR – No. of positive samples</th>
<th>Average copy no. per ng DNA ± std dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>DE</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>23.41 ± 11.24</td>
</tr>
<tr>
<td>Cow</td>
<td>WV</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>12.4 ± 6.2</td>
</tr>
<tr>
<td>Human</td>
<td>WV</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>4.7 ± 2.3</td>
</tr>
<tr>
<td>Goat</td>
<td>DE</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>11.3 ± 6.0</td>
</tr>
<tr>
<td>Sheep</td>
<td>DE</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>9.2 ± 5.1</td>
</tr>
<tr>
<td>Horse</td>
<td>WV</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>2.8 ± 1.4</td>
</tr>
<tr>
<td>House cat</td>
<td>WV</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>2.3 ± 1.2</td>
</tr>
<tr>
<td>Domestic Dog</td>
<td>WV</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>Coyote</td>
<td>TXT</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Deer</td>
<td>WV</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>California Sea lion</td>
<td>CA</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Black Vulture</td>
<td>TX</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3.2 ± 1.6</td>
</tr>
<tr>
<td>Canadian goose</td>
<td>WV</td>
<td>62</td>
<td>-</td>
<td>-</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Turkey</td>
<td>DE</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Pigeon</td>
<td>WV</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>2.9 ± 1.5</td>
</tr>
<tr>
<td>Duck</td>
<td>GA</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>1.5 ± 0.8</td>
</tr>
<tr>
<td>Chicken</td>
<td>WV</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>Penguin</td>
<td>OH</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td>Parrot</td>
<td>OH</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td>Dove</td>
<td>OH</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td>Pelican</td>
<td>OH</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td>Ibis</td>
<td>OH</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>4.0 ± 2.1</td>
</tr>
<tr>
<td>Gull</td>
<td>WV</td>
<td>8</td>
<td>+</td>
<td>23.41</td>
<td>23.41 ± 11.24</td>
</tr>
</tbody>
</table>

Detection of gull feces in environmental water samples using gull-specific (Gull2) assay

<table>
<thead>
<tr>
<th>Sampling locations</th>
<th>Sample type</th>
<th>Time of collection</th>
<th>Water samples</th>
<th>No. of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grant Park Beach, WI</td>
<td>Freshwater</td>
<td>September-October, 2007</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Maumee Bay, Oregon, OH</td>
<td>Freshwater</td>
<td>October, 2007</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Bluffers Park Beach, Ontario, Canada</td>
<td>Freshwater</td>
<td>May-August, 2007</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sunnyside Beach, Ontario, Canada</td>
<td>Freshwater</td>
<td>May-August, 2007</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Doheny State Beach, CA</td>
<td>Pond</td>
<td>June-Sept, 2007</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

Feces productions in the U.S.

- Beef cattle: 44%
- Chickens: 22%
- Pork: 29%
- Dairy: 10%
- Eggs: 1.0%
- Seafood: 1.0%
- Poultry: 1.0%
- Wildlife: 1.0%
- Human: 0.7%

Butchered livestock produces 1 x 10^12 kg/year.
**Next steps**

- Fragments are cloned and sequenced
- Sequences are classified by function and potential bacterial host
- Sequences associated with host-microbial interactions are used to develop PCR assays
- Assays are tested for host-specificity, host-distribution, and detection limits (both in fecal sources and water samples)

---

**Description of primers tested for host specificity**

<table>
<thead>
<tr>
<th>Source</th>
<th>Primer set</th>
<th>Sequence length (bp)</th>
<th>AOCT</th>
<th>Primers</th>
<th>E. coli</th>
<th>Feces</th>
<th>Data</th>
<th>Precp.</th>
<th>Host</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.03</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>Site 1</td>
<td>Site 2</td>
<td>Site 3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.03</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>Site 1</td>
<td>Site 2</td>
<td>Site 3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.03</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>Site 1</td>
<td>Site 2</td>
<td>Site 3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.03</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>Site 1</td>
<td>Site 2</td>
<td>Site 3</td>
<td></td>
</tr>
</tbody>
</table>

**Enterococci Density in AH**

- Bird 1 marker was detected

**Lessons learned**

- Detection limits can vary dramatically per fecal sample, host, water sample
- Host distribution can also vary considerably
- Preferential distribution and secondary habitats issues like *E. coli*
- Different markers for different sources of the same fecal sources
- Combination of assays best approach to enhance confidence levels

**Lessons learned**

- The more (markers) the merrier; you never know which marker will work
- Survival of the targeted population is rather important
- Feces might not always be the best starting point for assay development
- There is unknown bacterial groups that might be used for assay development
- Abundance of host-specific populations can vary
Regional projects

RARE Project – Evaluate MST assays in tropical inland waters

Regional Methods Program – Comparison of MST and PST assays

Acknowledgements

USEPA Computational Toxicology Grants
USEPA WSWRD

Jingrang Lu – NRC Award
Regina Lamendella, Daniel Oerther – UC
Rod Mackie, Tony Yanarell – UIUC
George DiGiovanni – UT El Paso
Stephen Hill, Tom Edge – Environment Canada
Rapid Concentration, Detection, and Quantification of Pathogens in Drinking Water

Zhiqiang Hu, Department of Civil and Environmental Engineering
Lela K. Riley, Department of Veterinary Pathology
Mengshi Lin, Department of Food Systems & Bioengineering
University of Missouri, Columbia MO 65211

Outline

- Lanthanum-Based Concentration and Microrespirometric Detection of Microbes in Water
  - Turbidity-based and Fluorescence-based microrespirometry to enumerate microbes and determine microbial activity in water
  - Lanthanum-based microbial concentration
- Rapid detection and quantification of water-borne pathogens by SERS coupled with nanosubstrates

Introduction

- Rapid detection of potential pathogens in water is crucial to drinking water supplies.
  - The numbers of microorganisms in water samples are often too low to be detected.
- Coagulation/floculation coupled with filtration is an attractive method for concentration.
- LaCl₃ is a flocculant that can concentrate microbes by strong electrostatic interaction.
  - Compared with traditional flocculants (e.g., alum and ferric salts), LaCl₃ only hydrolyzes slightly in the water so that it minimize the impact on microbial properties.

Part I. Lanthanum-Based Concentration and Detection

Introduction

- Traditional assays enumerate microbes by measuring the turbidity of the organisms.
- Oxygen-based microrespirometry, however, can enumerate the live microbes by measuring oxygen consumption and determine microbial activities at the same time.
- Lanthanum chloride was used to concentrate the microbes in water before they were detected and quantified by microrespirometry.

Materials and Methods

- Bacterial Strain used: *E. coli* (ATCC 47076)
- Flocculants/Coagulants: LaCl₃, FeCl₃, and Al₂(SO₄)₃ (final concentrations = 0.2 mM).
- Concentration procedures
  - Mixed at 200 rpm for 1 min, followed by slowly mixing at 30 rpm for 20 min.
  - The samples were allowed to settle for 1 hour.
  - The supernatant fluids (75mL) were carefully removed without disturbing the flocs.
**Microrespirometric Detection**

- **Composition in microwells**
  - For every floculant treatment, aliquots (20 µL) of supernatant or sediment samples were taken and added to the microplate wells followed by the addition of 180µL BBL medium.

- **Turbidimetric assay**
  - The microtiter plate was read at 600 nm.

- **Microrespirometric detection**
  - Oxygen probe and mineral oil were added.
  - Time-resolved fluorescence measurements were recorded with 340 nm excitation and 642 nm emission.

---

**Time profiles of E.coli growth at different initial cell concentrations**

- Turbidimetric assay
- Microrespirometric assay

---

**Strong Correlation between Bacterial Concentration and Time to Threshold**

**Concentration study Using turbidimetric assay**

- Time profiles of absorbance of different samples

---

**Concentration Study based on microrespirometric assay**

- Time profiles of oxygen probe signals of different samples
- Concentration efficiencies and recovery rates of different treatments

---

**Bacterial Distribution Using Different Flocculants**

- Turbidimetric assay
- Microrespirometric assay
Effect of flocculants (LaCl₃, FeCl₃ and Al₂(SO₄)₃) on E. coli bacterial growth

Microscopic (ESEM) Examination of Flocs with Different Chemical Treatment

LaCl₃ treatment (a)  
FeCl₃ treatment (b)  
Al₂(SO₄)₃ treatment (c)

Summary

- Compared with traditional flocculants, LaCl₃ has the highest relative concentration and recovery efficiencies. The lanthanum-based method coupled with ultrafiltration provides a promising pathogen concentration method for water utilities.

Part II. Rapid detection and quantification of water-borne pathogens by SERS coupled with nanosubstrates

Surface enhanced Raman spectroscopy (SERS)

- When analyte molecules are adsorbed on metal surface with nanoscale roughness, Raman signal can be tremendously enhanced due to spatially localized surface plasmon resonance (SPR) from the “hot spots” where huge local enhancements of electromagnetic field are obtained.
- The enhancement factor can be more than 10⁶. Limit of detection can reach the parts per billion (ppb) level or possibly a single molecule.

Surface enhanced Raman spectroscopy (SERS) and nanosubstrates

- A Renishaw RM1000 Raman spectrometer system with 785 nm near-infrared diode laser source;  
- Gold substrate (Klarite): fabricated on silicon wafers coated with gold, nanotextured pyramidal subunits.
Objective

- To develop and validate SERS-based method for pathogen detection and quantification.
- Several species representing the major categories of pathogens in drinking water were chosen for SERS testing:
  - Enterococcus faecalis
  - Helicobacter pylori
  - Human adenovirus
  - Calicivirus
  - Encephalitozoon cuniculi
  - E. coli O157:H7
  - Cryptosporidium parvum

SERS spectra of four virus strains show “fingerprint-like” spectral patterns that can be used to classify and identify these strains; gold nanosubstrates were used in measurement.

Distinctive SERS spectral patterns were observed between three waterborne bacteria.

We also collected SERS spectra of bacteriophage MS2 on gold nanosubstrates.

Three bacterial pathogens exhibit different SERS spectra that can be used to identify them.

Clear data segregations were obtained between C. muris and C. parvum; and viable and non-viable C. parvum.

These results indicate that SERS can be used to identify and discriminate between different Cryptosporidium oocysts as well as viable or not based on their unique and distinct vibrational spectral information.
Summary

SERS coupled with nanosubstrates and statistical tools shows great potential to rapidly detect and identify different water-borne pathogens.

Acknowledgements

Funded by EPA STAR Program (#83384001)

The effect of flocculation on pH

<table>
<thead>
<tr>
<th>Before flocculation</th>
<th>7.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>After flocculation</td>
<td></td>
</tr>
<tr>
<td>La^{3+}</td>
<td></td>
</tr>
<tr>
<td>Fe^{2+}</td>
<td></td>
</tr>
<tr>
<td>Al^{3+}</td>
<td></td>
</tr>
<tr>
<td>0.2mM</td>
<td>7.03</td>
</tr>
<tr>
<td>1mM</td>
<td>6.62</td>
</tr>
<tr>
<td>5mM</td>
<td>5.43</td>
</tr>
</tbody>
</table>

Band assignment of Raman peaks in the range of 300 – 2200 cm\(^{-1}\)

<table>
<thead>
<tr>
<th>Range shift (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>~ 340</td>
<td>COO (carboxyl stretching)</td>
</tr>
<tr>
<td>~ 600</td>
<td>Amide (N-H)</td>
</tr>
<tr>
<td>~ 800</td>
<td>NH (amide)</td>
</tr>
<tr>
<td>~ 750</td>
<td>N-H bend</td>
</tr>
<tr>
<td>~ 850</td>
<td>N-H bend</td>
</tr>
<tr>
<td>~ 950</td>
<td>Threonine</td>
</tr>
<tr>
<td>~ 977</td>
<td>Lipid (C-C deformation)</td>
</tr>
<tr>
<td>~ 1005</td>
<td>Phenylamine</td>
</tr>
<tr>
<td>~ 1031</td>
<td>Carbohydrates (C-C deformation)</td>
</tr>
<tr>
<td>~ 1138</td>
<td>DNA (N-G) bending</td>
</tr>
<tr>
<td>~ 1175</td>
<td>O-DH bending</td>
</tr>
<tr>
<td>~ 1220</td>
<td>Phosphate</td>
</tr>
<tr>
<td>~ 1233</td>
<td>Lipid (C-N(_2) deformation)</td>
</tr>
<tr>
<td>~ 1377</td>
<td>Nucleic acids (A, G)</td>
</tr>
<tr>
<td>~ 1665</td>
<td>Arndt L</td>
</tr>
<tr>
<td>~ 1751</td>
<td>(=\text{C}=\text{O}) stretching</td>
</tr>
</tbody>
</table>

(Maquelin and others 2002)

PCA of GD-7 (Picornavirus) and MNV-4 (Norovirus)

- PCA was able to classify two virus stains: GD-7 (Picornavirus) and MNV-4 (Norovirus)
Simultaneous Concentration and Real-time Detection of Multiple Classes of Microbial Pathogens from Drinking Water

Prof. Mark D. Sobsey
Department of Environmental Sciences and Engineering
Gillings School of Global Public Health
University of North Carolina
Chapel Hill, NC  27599-7341

Objective 1

• Refine and validate new and improved, rapid **hollow fiber ultrafiltration methods** to concentrate viruses and cellular pathogens (bacteria and protozoan parasites) from waters of variable quality
  – Particles
  – Dissolved organic matter
• Compare to existing virus concentration methods (1MDS VIRADEL)

Objective 2

• Fabricate (or identify) and evaluate improved and cost-effective **electropositive filters** to rapidly and efficiently concentrate enteric viruses from waters of different quality by adsorption to and elution
  – Nanoceram cartridge filter (Argonide)
• Compare to existing virus concentration methods (1MDS VIRADEL)

Objective 3

• Improve and evaluate post primary concentration sample preparation techniques:
  – Rapid **PEG precipitation**
  – Post PEG precipitation treatments to improve virus detection by quantitative real-time (RT-)PCR
  – Large volume **nucleic acid extraction**
• Further concentrate viruses
• Remove inhibitors
• Facilitate efficient, specific, and sensitive real-time, molecular detection of viral nucleic acids
  – Human adenoviruses
  – Human enteroviruses
  – Human noroviruses

Objective 4

• Improve and optimize direct detection of viral RNA/DNA by real-time molecular methods for rapid and efficient detection of low numbers of target viruses
  – Sample volume per (RT-)PCR reaction
  – Additives to (RT-)PCR mixtures

Objective 5

– develop complete protocols of the methods and provide them to a select number of other water virology laboratories to conduct a collaborative (round-robin) test of the methods that characterizes their performance; and
Concentration of Adenoviruses, Noroviruses and Echoviruses from Water

- **Primary concentration**
  - Recirculating flow hollow fiber ultrafiltration
  - 2 brands of filters
  - Modified endcaps to increase flow rate/flux
  - Alternative beef extract elution solutions
  - Performance in waters of different quality (source and treated)
  - Once-through, gravity-flow hollow fiber ultrafiltration
  - Nanoceram electropositive adsorbent filter
    - Nano alumina (AlOOH) fibers
    - Virus concentration from seawater

- **Secondary concentration**
  - Polyethylene glycol precipitation
  - Effect of PEG and NaCl concentrations

Recirculating HFUF Methods and Materials

- **Hollow-fiber ultrafilters (HFUF):**
  - Fresenius F80A
    - (Fresenius Medical Care, Lexington, MA)
  - Hemocor HPH
    - (Minntech Corporation, Minneapolis, MN)

**HFUF flow modifications:**
- Modified end caps with larger diameter openings
- Increased flux for more rapid sample processing

- **Test water:** ≥10-liter volumes of untreated source and de-chlorinated finished waters (SFPUC: San Francisco Public Utility Commission)
- **HFUF units:** ca. 75,000 MWCO, designed for kidney dialysis
- Peristaltic (flexible tubing roller) pump to re-circulate water through the unit
- As water re-circulates, permeate is separated from retained particles, concentrating particles, including microorganisms, to <300 ml volume

HFUF Recovery of Adenovirus 2

- **Hollow Fiber Ultrafiltration**
  - Virus assay by cell culture infectivity

Recycling HFUF Recovery of Adenovirus 41

- **Eluting solution comparison for Ad41 recovery from HFUF primary concentrates**

<table>
<thead>
<tr>
<th>Eluting Solution 1 (Standard)</th>
<th>Eluting Solution 2</th>
<th>Eluting Solution 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 L Phosphate-buffered Saline (PBS)</td>
<td>10 g laureth-12</td>
<td>50 µL antifoam-A</td>
</tr>
<tr>
<td>10 g NaPP</td>
<td>10 g laureth-12</td>
<td>50 µL antifoam-A</td>
</tr>
<tr>
<td>50 µL antifoam-A</td>
<td>50 µL antifoam-A</td>
<td>50 µL antifoam-A</td>
</tr>
</tbody>
</table>

HFUF Recovery of Adenovirus 41

- **Lower spike virus concentration (10⁵/10L) (Left)**
- **Recovery from large volume (100L) (Right)**
**HFUF Recovery of Pathogenic Microbe Suite**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Spike Conc'n. (cfu, pfu/L)</th>
<th>Source Water</th>
<th>Drinking Water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trials (N)</td>
<td>Average Recov. (%)</td>
<td>Trials (N)</td>
</tr>
<tr>
<td>E. coli O157</td>
<td>500</td>
<td>3</td>
<td>52±6</td>
</tr>
<tr>
<td>Salmonella</td>
<td>500</td>
<td>3</td>
<td>85±13</td>
</tr>
<tr>
<td>Aeromonas</td>
<td>500</td>
<td>3</td>
<td>11±3</td>
</tr>
<tr>
<td>Echovirus-12</td>
<td>2000</td>
<td>3</td>
<td>49±45</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>20</td>
<td>3</td>
<td>29±11</td>
</tr>
<tr>
<td>Giardia</td>
<td>20</td>
<td>3</td>
<td>9±3</td>
</tr>
</tbody>
</table>

**Bacteria, Virus and Spore Recovery from Treated OWASA Water (10L) by Conventional & Modified Fresenius F200A HFUFs**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Flowrate (L/min)</th>
<th>Trials (N)</th>
<th>Average Recovery (%)</th>
<th>Flowrate (L/min)</th>
<th>Trials (N)</th>
<th>Average Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K011</td>
<td>0.17±0.02</td>
<td>6</td>
<td>112±35</td>
<td>13</td>
<td>56±21</td>
<td></td>
</tr>
<tr>
<td>Coliphage MS-2</td>
<td>0.46±0.04</td>
<td>6</td>
<td>109±13</td>
<td>13</td>
<td>83±12</td>
<td></td>
</tr>
<tr>
<td>Bacillus atrophius</td>
<td>5</td>
<td>71±19</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Flow rate was significantly greater for HFUFs with modified endcaps (Mann Whitney Test; p value <.0001)

**Microbe Recovery from Water using Once-through Gravity HFUF**

- Gravity flow HFUF, ca. 30 cm long, 2 cm diameter, 20 nm pore size filter
- 10 L volumes of dechlorinated drinking water
- Spike with high concentrations of E. coli K011 (bacterium), coliphage PRD-1 (indicator virus), and spores of Bacillus atrophius (protozoan surrogate)
- Filter by gravity flow (1 meter head) or with a peristaltic pump
- Recover test microbes from filter by backflushing with buffered elution solution
- Used two successive flushes of ca. 250 mL each

**Microbial Recoveries from 10L Volumes of Water by Once-through HFUF**

- Average recoveries by gravity flow:
  - E. coli K011 = 90%,
  - PRD-1 ~100%
  - Bacillus atrophius spores = 74%
- Recoveries using a peristaltic pump:
  - E. coli K011 = 48%,
  - PRD-1 = ~100%
  - Bacillus atrophius spores = 52%

**PEG (Polyethylene Glycol) Precipitation of Viruses in HFUF Retentates**

- Widely used for virus concentration
  - Protein precipitation
- Minimal virus inactivation; no extreme pH changes
- Secondary virus concentration methods need to be compatible with detection by both molecular and infectivity methods
- PEG precipitation has not been adequately evaluated or optimized for Adenoviruses, Noroviruses and Echoviruses
- Evaluate effects of PEG and NaCl concentrations on method recovery of these viruses from HFUF retentates and adsorbent filter eluates

**Effects of PEG & NaCl Concentrations on Adenovirus Recovery from Treated and Source Water Retentates**

<table>
<thead>
<tr>
<th>PEG</th>
<th>NaCl</th>
<th>Ad 1</th>
<th>Ad 2</th>
<th>Treated Water</th>
<th>Source Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0%</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0%</td>
<td>0.2%</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0%</td>
<td>0.4%</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0%</td>
<td>0.6%</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0%</td>
<td>0.8%</td>
<td>2</td>
<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>0%</td>
<td>1%</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0%</td>
<td>1.2%</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0%</td>
<td>1.4%</td>
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<tr>
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<td>1.6%</td>
<td>2</td>
<td>2</td>
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<tr>
<td>0%</td>
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<tr>
<td>0%</td>
<td>2%</td>
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<tr>
<td>0.2%</td>
<td>0.4%</td>
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<tr>
<td>0.2%</td>
<td>0.6%</td>
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<tr>
<td>0.2%</td>
<td>0.8%</td>
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<tr>
<td>0.2%</td>
<td>1%</td>
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<tr>
<td>0.2%</td>
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<tr>
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<td>1.6%</td>
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<tr>
<td>0.2%</td>
<td>1.8%</td>
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</tr>
<tr>
<td>0.2%</td>
<td>2%</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
### Echovirus 12 and MS2 Recovery (%) by Different PEG Precipitation Conditions

- **Echovirus 12**
- **MS2**

(Duplicate trials each)

### Conclusions for PEG Precipitation from HFUF Retentates

- Effective for secondary virus concentration
- Higher virus concentrations in PEG pellets than in supernatants after centrifugation
- PEG-concentrated PEG samples were compatible with virus detection by both molecular and cell culture infectivity methods
- Overall, 9% or 12% PEG with either 0.1 or 0.3 M NaCl are effective conditions; – 0.3 M NaCl better than 0.1 M for Ad 41 in source water
- Virus recoveries by PEG precipitation were more variable from source water retentates compared to those from drinking water retentates

### Argonide Nanoceram Electropositive Filter

- Nanoceram filter (Argonide Corporation, Sanford, FL)
- Recently developed electropositive filter
- Reportedly unaffected by pH and salinity of water
- Made from nano alumina (AlOOH) fibers, 2 nm diam. & 0.3 μm long; grafted to microglass fibers; made like paper; 5” pleated cartridge
- External surface area about 500 m² per gram of material to provides a large area for adsorption of electronegative particles

### Filter and Water Sources

- Nanoceram filter and filter housing
- Challenge with 40 L of viruses-seeded water
- 10⁶ PCR units of adenovirus
- 10⁶ RT-PCR units of coliphage Qβ
- 10⁶ RT-PCR units of Norovirus GII.4
- 10⁶ murine norovirus
- Source and finished water from drinking water treatment plant in Carrboro, NC.
- Finished water dechlorinated with sodium thiosulfate
- Filter at 25 L/min

### Beef Extract Elution of Adsorbed Viruses

- Elution medium: 3% BE (Powder, Becton-Dickinson and Company, Sparks, MD), 0.1 M glycine and with the pH adjusted to 9.5.
- A 500 mL volume was recirculated through the cartridge filter using a peristaltic pump at a flow rate of 1.25-2.75 L per minute
- Flow direction changed every 5 min
- pH monitored
- Final eluent adjusted to pH 7.3

### Viral Nucleic Acid Extraction

- Chemical extraction from 100 μL sample volumes
- Guanidinium thiocyanate (GuSCN) extraction via Boom et al. (1990).
- Extract applied to a HiBind RNA minicolumn (OMEGA Bio-Tek, Doraville, GA) and centrifuged at 16,000 x g for 1 minute.
- Columns with nucleic acid washed 2X with 75% ethanol
- Nucleic acids eluted from column with nuclease free water
- Stored at -80°C until analysis.
Virus Quantification by Real-Time PCR

- Previously described real-time PCR quantification:
  - adenovirus 41 (Jothikumar et al, 2005)
  - norovirus (Jothikumar et al, 2005)
  - murine norovirus (Bae and Schwab, 2008)
  - coliphage Qβ (Kirs and Smith, 2001)
- Quantitech probe PCR & RT-PCR kits (Qiagen, Valencia, CA)
  - Reaction volume = 25 μL; 2 μL of extracted viral nucleic acid.
- Smart Cycler thermocycler (v. 2.0c, Cepheid, Sunnyvale, CA).
- Calibration curve used to calculate virus particles (VP) based on cycle threshold value (Ct) created from ten-fold serial dilutions of viral stocks
  - Adenovirus: VP/2 μL = 10(-0.2814 * Ct value + 12.256) (R² = 0.9986)
  - Norovirus: VP/2 μL = 10(-0.239 x Ct value + 10.41) (R² = 0.990)
  - Qβ: VP/2 μL = 10 (-0.306 x Ct value + 13.266) (R² = 0.996)
- Total VP calculation: Total VP = VP/2 μL x 250 x vol. of spike, filtrate or BE solution (in mL)
- Adsorption efficiency: \[1-(\text{total VP in the filtrate}/\text{total VP in the spike})\] x 100
- Elution recovery: (total VP in eluent/total VP in spike) x 100

Virus Recovery from Source Water using Nanoceram Filter

<table>
<thead>
<tr>
<th>Virus</th>
<th>% Ads. (±)</th>
<th>% Recovery (±)</th>
<th># Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus 41</td>
<td>81% (± 2.4%)</td>
<td>2.4% (± 0.48%)</td>
<td>4</td>
</tr>
<tr>
<td>Qβ Coliphage</td>
<td>53% (± 29%)</td>
<td>10% (± 2.8%)</td>
<td>4</td>
</tr>
<tr>
<td>Murine Norovirus</td>
<td>74% (± 18%)</td>
<td>9.8% (± 3.3%)</td>
<td>3</td>
</tr>
</tbody>
</table>

Virus Recovery from Finished Water using Nanoceram Filter

<table>
<thead>
<tr>
<th>Virus</th>
<th>% Ads. (±)</th>
<th>% Rec. (±)</th>
<th># Trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad 41</td>
<td>97% (± 2.1%)</td>
<td>1.4% (± 0.59%)</td>
<td>8</td>
</tr>
<tr>
<td>Qβ coliphage</td>
<td>95% (± 0.86%)</td>
<td>36% (± 20%)</td>
<td>8</td>
</tr>
<tr>
<td>Norovirus</td>
<td>ND</td>
<td>26.8%</td>
<td>2</td>
</tr>
</tbody>
</table>

Ad41 and Norovirus GII.4 Recovery by PEG Precipitation from Nanoceram Filter Eluates

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean % Recovery (±)</th>
<th>0.1 M NaCl</th>
<th>0.3 M NaCl</th>
<th>0.5 M NaCl</th>
<th>0.5 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus 41</td>
<td>1.7% (± 0.14%)</td>
<td>2.9% (± 1.0%)</td>
<td>36% (± 2.3%)</td>
<td>39% (± 6.4%)</td>
<td></td>
</tr>
<tr>
<td>Norovirus GII.4</td>
<td>5.6% (± 1.1%)</td>
<td>5.4% (± 0.46%)</td>
<td>52% (± 7.5%)</td>
<td>59% (± 4.8%)</td>
<td></td>
</tr>
</tbody>
</table>

- Higher mean % recoveries of both viruses using 9% instead of 6% PEG (unpaired t-test, p < 0.05)
- Mean % recoveries not significantly different between 0.1 M and 0.3 M NaCl for Ad41 (unpaired t-test, p = 0.078) or Noro GII.4 (unpaired t-test, p = 0.122)
Sample Processing Steps at which to Remove/Separate/Block Inhibitors

- Prior to nucleic acid extraction
- During nucleic acid extraction
- After nucleic acid extraction
- During nucleic acid (RT-)PCR amplification

PEG Samples and Viruses

- PEG concentrates from 40-L water samples processed by Nanoceram filter adsorption-elution (beef extract)
- 3 mL of composite concentrate, added 10 µL of adenovirus, norovirus, and MS-2 stocks
  - virus levels: 9.2x10^8, 2.8x10^4 and 5.2x10^8 PCR units
- Viruses also spiked into 3 mL of PCR grade deionized (DI) water, (Dracor) as an inhibitor-free control sample
- Both PEC concentrate and DI control processed
- qPCR CT values of PEG and DI control samples were compared to calculate ΔCt values
  - ΔCt = CT_Sample - CT_DIcontrol
  - Smaller ΔCt: less inhibition
  - Larger ΔCt: more inhibition

Treatments before NA Extraction with GuSCN

- Sephadex G-200 column chromatography
  - High salt TE buffer to prepare columns
  - Biospin polypropylene columns
    - Bio-Rad Cat. #732-6204, 3 cm, 0.8 ml capacity
    - 1 mL polypropylene syringe column (BD) with sterile glass wool (Supelco)
- Chelex 100 + Sephadex G-200 columns
  - Chelex in bottom half; G-200 in top half

Modifications during nucleic acid extraction

- GuSCN extraction of different sample volumes
  - 400, 300, 200, 100, and 50 µl samples
- Chloroform extraction of 300 µL & 100 µL sample volumes
  - 1:1 volume ratio
- Polyvinylpyrrolidone (PVP)-GuSCN extraction
  - 1% final concentration of PVP in sample-GuSCN mix

Post-extraction Modifications

- Isopropanol precipitation of NA Extract
  - Sample NA extract supplemented with Na acetate and isopropanol; centrifuged; NA ppt. washed with 70% EtOH; centrifuged; NA ppt. dried, then resuspended in water

qPCR Methods

Adenovirus: JTVXF primer, JTVXR primer, JTVXP probe
- Norovirus: JGIII primer, COG2R primer, Ring2-TP probe
  - Jothikumar and Lowther (2005)
- MS-2: ms2ks2 primer, ms2ks1 primer, ms2ks3 probe
  - Bae and Schwab (2008)
- Smart Cycler (Cepheid)
Modifications to qPCR Mix

- Add PVP
- Add PVP and glycerol
- Add Bovine Serum Albumin (BSA)

Fluorescence Spectrophotometry:

- Fluorescent excitation emissions matrix (EEM) to quantify dissolved organic matter
- Detects and differentiates humic acids, fulvic acids, tryptophan and other potential organic inhibitors
- Sample run included quinine hemisulfate stock solutions for calibration and reagent grade water for comparison and background subtraction

EEM Peak Regions, Based on Excitation (Y-axis) and Emission Wavelengths (X-axis)

Treatments for qPCR Inhibitors

- No treatment before, during, or after extraction of viruses concentrated from water samples improved viral detection by qPCR with the same effectiveness for adenovirus, norovirus, and MS-2 in PEG concentrates of surface water samples
- Different methods or treatments may be needed for each type of water sample and virus.
- Specific treatments were more effective in lowering delta ct values for qPCR detection of viruses in many of the samples.

qPCR detection of three viruses in different water sample volumes subjected to chloroform extraction relative to detection in reagent water

<table>
<thead>
<tr>
<th>Sample Volume</th>
<th>Adenovirus</th>
<th>Norovirus</th>
<th>MS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>∆Ct value</td>
<td>∆Ct std dev</td>
<td>p</td>
</tr>
<tr>
<td>CHCl3, 300 µL</td>
<td>7.23</td>
<td>0.539 &lt;0.01</td>
<td>8.62</td>
</tr>
<tr>
<td>CHCl3, 100 µL</td>
<td>2.96</td>
<td>0.309 &lt;0.01</td>
<td>3.24</td>
</tr>
</tbody>
</table>

Comparison of different surface water sample volumes subjected to GuSCN extraction for differences in qPCR detection of adenovirus and norovirus relative to detection in reagent water

<table>
<thead>
<tr>
<th>Sample Volume</th>
<th>Adenovirus</th>
<th>Norovirus</th>
<th>Adenovirus</th>
<th>Norovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L.O.D. value</td>
<td>L.O.D. std dev</td>
<td>p</td>
<td>L.O.D. value</td>
</tr>
<tr>
<td>400 µL</td>
<td>7.44</td>
<td>0.08 &lt;0.01</td>
<td>7.57</td>
<td>0.228 &lt;0.01</td>
</tr>
<tr>
<td>300 µL</td>
<td>4.96</td>
<td>0.02 &lt;0.01</td>
<td>7.39</td>
<td>0.208 &lt;0.01</td>
</tr>
<tr>
<td>200 µL</td>
<td>5.33</td>
<td>0.08 &lt;0.01</td>
<td>7.52</td>
<td>0.208 &lt;0.01</td>
</tr>
<tr>
<td>100 µL</td>
<td>4.37</td>
<td>0.02 &lt;0.01</td>
<td>7.52</td>
<td>0.208 &lt;0.01</td>
</tr>
<tr>
<td>50 µL</td>
<td>2.47</td>
<td>0.02 &lt;0.01</td>
<td>7.52</td>
<td>0.208 &lt;0.01</td>
</tr>
</tbody>
</table>
Most Effective Sample Treatments

- Sephadex G-200 followed by chloroform extraction
  - Best for adenovirus in NPS water sample
  - Best for MS-2 in quasi-PS water sample
- Chloroform extraction alone
  - Good for norovirus in NPS water sample.
  - Best for MS-2 in NPS water sample
- GuSCN extraction of smaller sample volume
  - Best for norovirus in both samples
- Sephadex G-200 and Chelex 100 treatment
  - Best for adenovirus in quasi-PS water sample

Overall Summary

- Primary virus concentration by improved recirculating UFUF is effective and rapid
- Primary virus concentration by once-through HFUF shows promise
- Primary virus concentration by Nanoceram filters is effective and very rapid but less effective than desired for adenoviruses
- PEG precipitation is effective for 2nd step virus concentration
- PEG sample treatments prior to nucleic acid extraction reduce sample inhibition and improve virus detection by qPCR

Thank-you!
Questions? Comments? Suggestions?

Collaborators:
Erik Andersen
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Christopher Gibbons
Hee Suk Lee
David Love
Roberto Rodriguez
O.D. Chip Simmons III
Lauren Thie
Jan Vinjé
Jianyong Wu
Ming Jing Wu

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NWRI
SCCWRP
UNC Sea Grant
Quantitative Assessment of Pathogens in Drinking Water

Kellogg J. Schwab Ph.D.
Johns Hopkins University
Bloomberg School of Public Health
Department of Environmental Health Sciences

Microorganisms in Source and Finished Water

Microbial contaminants can be divided into 3 categories:

1. Parasites
2. Viruses
3. Bacteria

KEY concepts to keep in mind

1. Size of the microorganism
   - Parasites > Bacteria >> Viruses
2. Resistance to environmental degradation and chemical inactivation
   - Parasites > Viruses >> Bacteria

Waterborne Pathogens and Gastroenteritis

- On average, between 1991 and 2002, 17 waterborne disease outbreaks (WBDOs) were reported annually.
- 38% of outbreaks had an unidentified etiology
- WBDOs were primarily associated with inadequately treated water systems and contamination issues related to aging distribution systems
- In some instances, the water systems were in compliance with current water quality standards

Waterborne Pathogens and Gastroenteritis

- Multiple Factors Influence Reporting of AGI
  - Public awareness of waterborne illnesses
  - Local requirements for reporting cases of particular diseases
  - The surveillance and investigative activities of state and local public health and environmental agencies
  - Availability of and extent of laboratory facilities
- Current waterborne disease surveillance system is passive
  - Waterborne disease outbreaks are likely to be under reported
  - Endemic waterborne disease risk in the United States is not well understood

Why is all of this of interest?

One of the major limiting factors in assessing microbial loads in source and treated drinking water has been the lack of an effective microbial collection method capable of efficiently and simultaneously recovering low levels of bacteria, viruses and protozoa, which then can be identified and quantified rapidly with or without cultivation.
Research Objective

Develop rapid, sensitive recovery and detection methods for the quantitative assessment of pathogenic microorganisms present in drinking water.

Microbial Recovery

Develop and optimize sensitive concentration and isolation methods utilizing filtration technology capable of simultaneously recovering low levels of protozoa, viruses, and bacteria from large volumes of water.

• Demonstrate ability of tangential flow filtration (TFF) to efficiently recover/concentrate intact microorganisms from water

• Determine lower limit of detection for each class of microorganism

Steps for Processing 1 to 1,000 L Water Samples

1. Add Blocking Reagent
2. Add Microbial Surrogates
3. Process Water Sample
4. Determine lower limit of detection for each class of microorganism

Microbial Surrogates Utilized in Method Evaluation

• Vegetative Bacteria
  – Escherichia coli CN-13
  – Enterococcus faecalis
• Spore-forming bacteria
  – Clostridium perfringens
• Bacteriophage
  – MS2
  – PRD1
• Viruses
  – Murine norovirus (MNV-1)

Pathogen Detection

Develop rapid, quantitative molecular detection techniques for the identification of target pathogens including direct comparison with existing traditional culture methods.

• Optimized FISH methods for the identification of protozoa.
• Developed mass spectrometry (MS) methods for the identification of select microorganisms.
• Refined qPCR and qRT-PCR assays for the detection of select microorganisms.
• Developed loop-mediated isothermal amplification (LAMP and RT-LAMP) for the detection of select microorganisms.
• Employed the use of internal standard controls for the detection of PCR inhibition caused by molecular inhibitors present in water samples.

Fluorescent In Situ Hybridization (FISH)

• Employs a fluorescently labeled oligonucleotide probe targeting species-specific sequences of 16S rRNA
• rRNA
  – Exists in multiple copies
    • Present in high copy numbers in viable cells
  – Single-stranded regions allow easy access for the probe and natural signal amplification
• Hybridization
  – Probes recognized by fluorescent antibodies
• Observed under epifluorescence microscope
Detection using Mass Spectrometry (MS)

- Detect capsid protein
  - Multi-copy
  - Uniquely identifiable
- Purify protein
- Digest with proteases (trypsin)
  - Spike peptide standards
- Chromatographically separate peptides and then determine amino acid sequence
- Search masses against genome databases (e.g. NCBI)
- Assess confidence-based score
- Quantification

MS Key Findings – Norovirus

- The NV capsid protein is detectable in the clinical range using MALDI-TOF MS
- Clinical sample complexity requires a more nuanced approach (ESI-MS/MS)
- Using additional sample processing, MS/MS methods can improve sensitivity by 2-3 orders of magnitude
- AQUA peptides allow for the quantification of peptides from capsid protein of norovirus

Molecular Methods: Real Time PCR

- DNA is amplified by a cycling of steps:
  - Denaturation
  - Primer annealing
  - Primer extension
- TaqManTM probe technology allows for real time quantification of target RNA/DNA
  - Fluorescent probe is cleaved during extension
  - Target is quantified in the form of a cycle threshold (Ct) value

Molecular Methods: LAMP

- Loop-mediated isothermal amplification (LAMP) is a novel detection method which relies on auto-cycling strand displacement DNA synthesis.
  - RT may be used in conjunction for detection of RNA viruses
- Increased sensitivity and specificity compared with conventional PCR
  - Multiple primers must recognize several distinct regions on the target RNA/DNA
- Products can be analyzed in real time by measuring the increase in turbidity during DNA amplification.
  - Allows for real time quantification

Field Application

Apply tangential flow ultrafiltration and quantitative molecular detection to large-volume, water samples for the analysis of microorganisms.

- Spike environmental water samples with microbial surrogates to evaluate the efficiency of recovery and detection methods.
- Apply complete concentration, recovery, and detection process to a variety of water samples including ground water, surface water, and finished drinking water.
- Compare newly developed technologies for the recovery and detection of microorganisms in water to existing US EPA methods.
- Identify viruses that are endemic and stable in the environment and investigate their use as traceable markers of fecal contamination

Collection of Environmental Samples

- Water sampling in Lower Yakima Valley, WA
  - Sampled surface water and ground water impacted by surrounding dairy industry
  - Application of optimized TFU method for concentration of 100L water samples
Water Sampling in Yakima Valley, WA

- Processing
  - Applied optimized TFU in the field
  - Seeded each sample with known concentration of MNV-1 to evaluate recovery efficiency
- Parameters
  - Utilized a Multiparameter Water Quality Sonde
    - Temperature, turbidity, pH, conductivity, dissolved oxygen, nitrate, and nitrite
- Analysis
  - IDEXX Most Probable Number (MPN) method
  - Total coliforms, E. coli, and Enterococci
  - FISH/IFA, qPCR/RT-PCR
  - Ongoing for select protozoa and viruses

Surface Water (n=11)
Groundwater (n=10)

Public Health Implications

- Developing a universal method for the recovery of microorganisms will enable water utilities and regulatory agencies to better address problems within source waters and public water systems.
- The utilization of molecular detection techniques will provide increased confidence in the sensitivity, specificity, and inhibition detection/control critical for estimating levels of risk.
- A more comprehensive understanding of the microbial contamination of water sources will allow for exposure risk assessments to be generated for individual microorganisms
- Future applications of this method:
  - Further the development of the usefulness of host-specific viruses in microbial source tracking efforts
  - Currently limited by lacking concentration and detection methods
  - Assist in the formulation of effective control measures for the reduction of water-related transmission of pathogenic microorganisms

Acknowledgements

Collaborators
- Drs Rolf Halden, Thaddeus Graczyk
- Students – Kristen Gibson, David Colquhoun

Funding – EPA STAR R833002
Development and application of a fiber optic array system for detection and enumeration of potentially toxic cyanobacteria

Donald M. Anderson
Woods Hole Oceanographic Institution, Woods Hole, MA

The problems:

- Many cyanobacteria produce potent toxins that threaten human health.
- Cyanobacterial blooms can take multiple forms, ranging from dense surface scums to dilute suspensions that can still cause harm.
- Many different species and strains co-occur, and strains of the same species can be toxic or non-toxic, or can vary dramatically in the amount of toxin produced under different conditions.
- Distinguishing characteristics can be difficult to discern under the light microscope, yet such fine levels of discrimination are not feasible in monitoring programs that generate large numbers of samples.

The overall project goal is to adapt and validate a rapid and accurate optical fiber-based technology for cyanobacterial cell detection and enumeration in both laboratory and field settings.

Specific objectives are to:
1) Design rRNA signal and capture probes for the three most important toxic cyanobacteria (Microcystis aeruginosa, Cylindrospermopsis raciborskii, and Anabaena flos-aquae);
2) Design and test a second probe pair for each species, to incorporate redundancy into the array;
3) Test these probes in the fiber-optic array format and determine detection limits, specificity, and dynamic range;
4) Refine hybridization conditions to reduce processing time;
5) Develop procedures to analyze multiple cyanobacterial species simultaneously using a single fiber bundle in a multiplexed format.
Individual cladded optical fibers

Silica jacket


Image of Fiber Bundle

Microsphere Arrays

Fluorescently-labeled signal probe

Target cyanohAB rRNA

Capture probe immobilized to microsphere


Wet etching with HCl


Microspheres in Etched Wells

HAB rRNA Sandwich Assay
Optical Fiber-based Microsphere Array

1) Different DNA probes are first attached to beads
2) Probes are combined into a library
3) Probe bead library is distributed randomly into wells on fiber array surface

DNA Hybridization

1) Encoding image
2) Background Image
3) Specific Hybridization of Complement to Probe C
4) Increased Signal for Probe C Sensors

rRNA sample preparation

13 mm, 0.5 mm-pore-size filter membrane
DNA, RNA dispensed in solution by cell lysis
Lysis buffer (200 mL)
85°C 5 min
Cell lysate filtered through 0.45 mm filter membrane

Fiber optics instrumentation

Excitation Filter Wheel
Emission Filter Wheel
Dichroic Housing
Microscope Objectives

Imaging System

Computar Image Processing
Optical Imaging Fiber
Encoding strategies for multi-species detection: an optical bar code

Internal Encoding

- Same dye, different concentrations
- Combination of different dyes or different color dyes

Number of dyes: \( M \)

Number of concentration levels of each dye: \( n \)

Number of "optical bar code options": \( M^n \)

e.g. 4 colors, 5 concentration levels of each, could detect \( 4^5 = 1024 \) species.

Theoretically, one sensor array can be designed to detect and enumerate all HAB species in a given region.

Images from DNA Sensor Array

- Encoding image
- Background
- Hybridization with BA2 target, 10 min

Detection limit for *Alexandrium fundyense*

![Graph showing net fluorescence signal vs. cell number](image)

- Lower number of cells need longer hybridization time to reach saturation

Optimization of hybridization time (no stirring)

- Method of hybridization time with target

Multiplexed array with 3 sensors

- Multiplexed array was tested with cultured cells of *A. fundyense, A. ostenfeldii, and Pseudo-nitzschia australis*
- Each sensor showed positive signal only with its target organism - no significant cross-reactivity

Detection of *A. fundyense* cells in natural seawater

![Bar chart showing net fluorescence signal vs. volume of seawater](image)
Application to cyanoHABs

Methods

- Signal and capture probe design
  - 16S rRNA gene sequences compiled from GenBank for target cyanoHAB taxa: *Cylindrospermopsis raciborskii*, *Microcystis aeruginosa*, and *Anabaena flos-aquae*
  - Probe identification performed using sequence alignments of target/non-target species
  - Included published probes for *Microcystis, Anabaena/Aphanizomenon*, and “Nostoc group” (*Nostoc, Anabaena/Aphanizomenon*)

- Probes tested against target and non-target species using fluorescent in situ hybridization (FISH) to determine efficacy and assess cross-reactivity; probes that exhibit cross-reactivity require re-design

- Probes successfully tested for cross-reactivity are then transitioned to fiber-optic microarray format and tested against synthetic target and cell lysates from target species

Probe design

**Capture Probes for Target Species**

<table>
<thead>
<tr>
<th>Species</th>
<th>Probe 1</th>
<th>Probe 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Microcystis</em></td>
<td></td>
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<tr>
<td><em>S. elongates</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. raciborskii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. flos-aquae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. cylindrica</em></td>
<td></td>
<td></td>
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<tr>
<td><em>M. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. flos-aquae</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Signal Probes**

<table>
<thead>
<tr>
<th>Species</th>
<th>Signal 1</th>
<th>Signal 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. raciborskii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. flos-aquae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. cylindrica</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Example Probes**

- **Cylindrospermopsis raciborskii** LB 2897
  - (a) No probe, Cy3 filter
  - (b) No probe, FITC Band pass filter
  - (c) Probe 2, Cy3 filter
  - (d) Probe 2 (6-FAM), FITC Band pass filter

- **M. aeruginosa** LE-3
  - (a) No probe, Cy3 filter
  - (b) No probe, FITC Band pass filter
  - (d) Probe 1, Cy3 filter
  - (e) Probe 1, FITC Band pass filter

- **Anabaena flos-aquae** LB 2557
  - (a) No probe, Cy3 filter
  - (b) No probe, FITC Band pass filter
  - (c) Probe 3, Cy3 filter
  - (d) Probe 3, FITC Band pass filter
Cross-reactivity testing (in progress)

<table>
<thead>
<tr>
<th>Cross-reactivity testing (in progress)</th>
<th>Microarray testing</th>
<th>Future directions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twelve probes tested for cross-reactivity (in progress)</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– Micrurus probes (3)</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– Tested against 18 cultures (in progress)</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– All designed (3) and published (2) probes exhibit cross-reactivity with Oscillatoria; redesign in progress</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– Cylindrospermopsis probes (2)</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– Tested against 18 cultures</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– One probe transitioned to fiber optic microarray format</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– Second probe exhibited cross-reactivity with Anabaenopsis; redesign in progress</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– Anabaena probes (5)</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– All designed (3) and published (2) probes either exhibited cross-reactivity or failed to detect target species</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
<tr>
<td>– Taxonomy of Anabaenopsis problematic (not monophyletic); redesign efforts needed to develop probe for Anabaenopsis/Aphanizomenon or “Nostoc group”</td>
<td>Microarray testing</td>
<td>Future directions</td>
</tr>
</tbody>
</table>

Microarray testing

- Capture probe performance tested using Cylindrospermopsis probe #1 (CYL1) coupled to activated microbeads and against a synthetic target
  - Single bead array exposed to Cy5-labeled synthetic targets with sequences complementary to the capture probe
  - Hybridization was performed at room temperature using 100 μl of synthetic target solution (100 μM) and a hybridization time of 10 minutes

Future directions

- Probe redesign and testing
- Transition additional probes to microarray format (single bead arrays) and assess performance using synthetic targets and cell lysates (assess detection limits, specificity, and dynamic range)
- Assess performance of multiplexed array using single and multiple species
  - single species and mixed cultures
  - spiked/unspiked field samples (2009 field sample collections include lakes in OR, MA, MD, CA, FL, and Great Lakes)
- Explore application of the microarray technique on a portable instrument
- Explore remote deployment of the microarray technique on a robotic, in situ instrument
The Environmental Sample Processor (ESP)

Acknowledgements

Woods Hole Oceanographic Institution
Mindy Richlen
Dave Kulis
Rob Arnold

Tufts University
David Walt
Ryan Hayman
Shonda Gaylord

RD-8332801-0

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DNA Base Pairing

http://upload.wikimedia.org/wikipedia/en/f/f0/DNA_Overview.png
Human Enteric Viruses

<table>
<thead>
<tr>
<th>Genus</th>
<th>Popular name</th>
<th>Disease caused</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterovirus</td>
<td>Poliovirus</td>
<td>Paralysis, meningitis, fever</td>
</tr>
<tr>
<td></td>
<td>Coxsackievirus A, B</td>
<td>Meningitis, fever, respiratory disease, hand-foot-and-mouth disease, heart anomalies, rash</td>
</tr>
<tr>
<td></td>
<td>Echovirus</td>
<td>Meningitis, fever, respiratory disease, rash, gastroenteritis</td>
</tr>
<tr>
<td>Hepatovirus</td>
<td>Hepatitis A</td>
<td>Hepatitis</td>
</tr>
<tr>
<td>Reovirus</td>
<td>Human reovirus</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Mastadenovirus</td>
<td>Human adenovirus</td>
<td>Gastroenteritis, respiratory disease, conjunctivitis</td>
</tr>
<tr>
<td>Calicivirus</td>
<td>Human calicivirus</td>
<td>Gastroenteritis, respiratory disease, conjunctivitis</td>
</tr>
<tr>
<td></td>
<td>Norwalk virus</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td></td>
<td>SARV</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td></td>
<td>Hepatitis E</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>Human astrovirus</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Parvovirus</td>
<td>Human parvovirus</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>Coronavirus</td>
<td>Human coronavirus</td>
<td>Gastroenteritis, respiratory disease</td>
</tr>
<tr>
<td>Torovirus</td>
<td>Human torovirus</td>
<td>Gastroenteritis</td>
</tr>
</tbody>
</table>

Detection Methods

<table>
<thead>
<tr>
<th>Principle of the assay</th>
<th>Example</th>
<th>Infectivity test</th>
<th>Detection limit (particles/ml)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM</td>
<td></td>
<td>No</td>
<td>$10^1$ to $10^5$</td>
<td>&lt; 24 hr</td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
<td>No</td>
<td>$10^3$</td>
<td>&lt; 2 hr</td>
</tr>
<tr>
<td>Probe hybridization</td>
<td></td>
<td>No</td>
<td>$10^4$</td>
<td>&lt; 2 hr</td>
</tr>
<tr>
<td>RT-PCR</td>
<td></td>
<td>No</td>
<td>$10^3$ to $10^3$</td>
<td>&lt; 8 hr</td>
</tr>
</tbody>
</table>

Main Aim

To develop methods for high-throughput and real-time detection of enteric enteric viruses

Part 1: Genetically engineered reporter cells
- Viral protease-sensitive fluorescent substrate
- Detects viral protease
- Flow cytometry-based assay for detection of PV in wastewater

Part 2: Nuclease-resistant molecular beacons (MBs)
- Detects viral genome
- Modified MB for visualizing the dynamics of viral replication in living cells

Polyovirus: Life Cycle

- Receptor binding
- Entry into the cell
- Nuclear transport
- Host cell genome
- RNA packaging
- Nucleocapsid formation
- Nucleocapsid egress and assembly
Poliovirus: Genome

(-) RNA

(+) RNA

5' UTR

3' UTR

VPg

RNA-dependent RNA pol.

Non-structure

VPK

polypeptide

2Apro

3Cpro

VPI

VPI

VPI

VPI

VPI

VPI

STKDLTTY↓FGHQKNA*

YFP

FRET

CFP

430 nm

460 nm

530 nm

520 nm

480 nm

Increase in CFP intensity in PV-infected cells vs uninfected cells

FACS can distinguish infected cells from non-infected cells

FACS can detect quantitative differences in the number of infected cells in the sample

An increasing number of infected cells was detected as PV1 infection progressed

FACS assay is suitable for following the kinetics of poliovirus infection

FACS can detect infected cells as early as 5 hpi!
Summary

- Using FACS on fluorescent reporter cells:
  - distinguished infected from uninfected cells
  - detected PV-infected cells as early as 5 hpi (at high infective dose)
  - detected 1 PFU of PV after 12 hpi
  - Good correlation between FACS-based and plaque assays when tested on wastewater spiked with PV1

Molecular Beacons: Structure and principle

MBs: Design and Modifications

MBs: Intracellular delivery

- FACS detected cells infected with 50 PFU of PV1 after 8 hpi, positive signals from cells infected with 1 PFU of PV1 were detected after 12 hpi
- FACS is a sensitive and rapid method for PV1 detection

- No significant difference between FACS and plaque assay results
- FACS is a reliable method for PV1 detection in waste water

- Using FACS on fluorescent reporter cells:
  - distinguished infected from uninfected cells
  - detected PV-infected cells as early as 5 hpi (at high infective dose)
  - detected 1 PFU of PV after 12 hpi
  - Good correlation between FACS-based and plaque assays when tested on wastewater spiked with PV1
Summary

- Modified molecular beacon
  - Nuclease-resistant MB with TAT peptide was designed
  - Detected as few as 1 PFU during the early stage of viral replication
  - Fluorescence assay was comparable with the plaque assay
  - Used to monitor the dynamics of viral replication during a 12-h infection period

Conclusion & Future Perspectives

- Developed methods for detecting infective viruses
- Sensitivity could reach 1 PFU at shorter incubation time than conventional plaque assay
- Reliable for viral quantitation
  - Detection of other epidemiologically important viruses
    - e.g., hepatitis virus, adenovirus, norovirus
  - High throughput screening for viral protease inhibitors
  - Development of other FRET-based sensors

Acknowledgment

- Drs. Marylynn Yates and Wilfred Chen
- Members of Yates and Chen Laboratories
- B. Walters (UCR Institute for Integrative Genome Biology)
- U.S. Environmental Protection Agency

-The End-
New Electropositive Filter for Concentrating Enterovirus and Norovirus from Large Volumes of Water

Mohammad R. Karim, Eric R. Rhodes, Nichole Brinkman, Larry Wymer, and G. Shay Fout

Presentation Outline
- Human enteric viruses
- Why should we be concerned about viruses in water
- General method for virus detection
- Research need for virus sample collection
- Evaluation of a new filter for concentrating viruses from water
- Conclusions

Human Enteric Viruses

<table>
<thead>
<tr>
<th>Genus</th>
<th>Popular Name/Species</th>
<th>Disease caused</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterovirus</td>
<td>Human Enterovirus A</td>
<td>Paralysis, aseptic meningitis, encephalitis, myocarditis, fever, respiratory disease, gastroenteritis, etc.</td>
</tr>
<tr>
<td></td>
<td>Human Enterovirus B</td>
<td>Paralysis, aseptic meningitis, encephalitis, myocarditis, fever, respiratory disease, gastroenteritis, etc.</td>
</tr>
<tr>
<td></td>
<td>Human Enterovirus C</td>
<td>Conjugate, enterovirus 111, enterovirus 112, enterovirus 113, enterovirus 114, enterovirus 115, enterovirus 116</td>
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<tr>
<td></td>
<td>Human Enterovirus D</td>
<td>EV68, EV70</td>
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<tr>
<td>Reovirus</td>
<td>Human reovirus</td>
<td>Unknown</td>
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<tr>
<td>Rotavirus</td>
<td>Human rotavirus</td>
<td>Gastroenteritis</td>
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<td>Mastadenovirus</td>
<td>Human adenovirus</td>
<td>Gastroenteritis, respiratory disease, conjunctivitis</td>
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<tr>
<td>Norovirus</td>
<td>Norovirus A</td>
<td>Gastroenteritis</td>
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<tr>
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<td>Norovirus B</td>
<td>Gastroenteritis</td>
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<td>Norovirus C</td>
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</tr>
<tr>
<td></td>
<td>Norovirus D</td>
<td>Gastroenteritis, respiratory disease</td>
</tr>
</tbody>
</table>

Number of Waterborne Disease Outbreaks Associated with Drinking Water, by Year and Etiologic Agent — United States, 1971–2006

Total 814 outbreaks and 575,819 cases of illness were reported
Overall, 8% of outbreaks were caused by viruses

Percentage of Waterborne-disease Outbreaks Associated with Drinking water, by Illness and Etiology – United States, 2005 - 2006

- Overall, 8% of outbreaks were caused by viruses
- The most common etiologic agents were viruses, followed by bacteria and algae
- No outbreaks were reported due to parasites
**Routes of Enteric Viruses Transmission**


**Drinking Water Contaminant Candidate List 2 (CCL 2)**

- Caliciviruses
- Coxsackieviruses
- Echoviruses
- Adenoviruses

http://www.epa.gov/OGWDW/ccl/ccl2.html

**How do we detect viruses in water?**

**Virus Methods in General**

- Sample collection
- Elution
  - Beef Extract
- Reconcentration
  - Organic Flocculation
  - Celite Concentration
  - Polyethylene Glycol
- Virus assay by cell culture or molecular methods (RT-PCR, qRT-PCR)

**Virus Methods: sample collection apparatus**

**Pore Size of Filter Medium and Size of Microbial Particles**

<table>
<thead>
<tr>
<th>Size (m) (in log scale)</th>
<th>MS2 bacteriophage</th>
<th>Coliform bacteria</th>
<th>Cryptosporidium oocysts</th>
<th>Giardia cysts</th>
<th>Rotavirus</th>
<th>PRD1 bacteriophage</th>
<th>M. avium Complex</th>
<th>Yersinia</th>
<th>B. coli cysts</th>
</tr>
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<tbody>
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</tr>
</tbody>
</table>

RO1: reverse osmosis; NF1: nanofiltration; UF1: ultrafiltration; MF1: microfiltration; DE1: diatomaceous earth filtration.

2 Including slow sand filtration. Slow sand filter has a lower pore size than rapid-rate filter.

3 These bacteria are rod-shape. The sizes shown represent the smallest dimension.
### Types of Filters Commonly Used in Virus Concentration Procedures

- **Negatively charged Filters**
  - Requires conditioning the water prior to filtration
  - pH adjustment to 3.5
  - Addition of multivalent cations

- **Positively charged filters**
  - 1MDS electropositive filters (Cuno, Meriden, CT) are commonly used for environmental water sampling.
  - Does not require conditioning the water.
  - However, requires pH adjustment for waters with pH values exceeding 8.0

### Research need for virus sample collection

Virus Sample Collection

- **Virosorb® 1MDS Filter**
  - Recommended by ICR Method
  - Charge-modified, glass and cellulose medium
  - Pore size 5-8 micron
  - Available in 25.4 cm cartridge
  - Cost >$200

  These filters are not cost-effective for routine viral monitoring

- **NanoCeram® Filter**
  - The active ingredient of the filter media is nano alumina (BOCDOI) fiber.
  - The fibers are only 2 nanometers in diameter, and 0.2 µm long and have a surface area of 500-600 m²/g.
  - The nano fibers contain naturally occurring electropositive charge.
  - In NanoCeram cartridge filters, these fibers are dispersed throughout a microglass fiber matrix resulting in a non-woven media.
  - Size: 12.7 cm X 6.35 cm; total surface area 240 cm²
  - Average pore size is 2 micron
  - Cost approximately $40

Retention of Poliovirus 1 by NanoCeram® Filters

<table>
<thead>
<tr>
<th>No. of replication</th>
<th>Seed titer* (PFU)</th>
<th>Titer in the filtrate (PFU)</th>
<th>Virus Retention (%)</th>
</tr>
</thead>
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<tr>
<td>8</td>
<td>5.0 x 10^5</td>
<td>1.0 x 10^5</td>
<td>80</td>
</tr>
<tr>
<td>9</td>
<td>4.0 x 10^5</td>
<td>&lt;DL b</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>6.0 x 10^5</td>
<td>1.0 x 10^5</td>
<td>83</td>
</tr>
</tbody>
</table>

*Total virus PFU in 100 liters of deionized water
b Detection limit

Poliovirus was seeded in 100 L of deionized water and filtered through NanoCeram® filters.

Virus methods: elution

- Single elution
- Double elution with 1 min contact times
- Double elution with 1 min, then overnight, contact times
- Triple elution

* USEPA ICR method

Poliovirus Recovery by NanoCeram® Filters Using Six Different Elution Procedures.

<table>
<thead>
<tr>
<th>Method</th>
<th>Elutions</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1st elution</td>
<td>50</td>
<td>38</td>
<td>35</td>
<td>41 (± 8)</td>
<td>56-71</td>
</tr>
<tr>
<td></td>
<td>2nd elution for 1 min</td>
<td>8</td>
<td>36</td>
<td>18</td>
<td>21 (±14)</td>
<td>11-33</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>58</td>
<td>74</td>
<td>53</td>
<td>62 (±11)</td>
<td>47-77</td>
</tr>
<tr>
<td>2</td>
<td>1st elution</td>
<td>53</td>
<td>62</td>
<td>31</td>
<td>49 (±16)</td>
<td>32-64</td>
</tr>
<tr>
<td></td>
<td>2nd elution for 15 min</td>
<td>9</td>
<td>32</td>
<td>44</td>
<td>28 (±17)</td>
<td>15-44</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>62</td>
<td>93</td>
<td>74</td>
<td>77 (±16)</td>
<td>47-93</td>
</tr>
<tr>
<td>3</td>
<td>1st elution</td>
<td>97</td>
<td>88</td>
<td>32</td>
<td>66 (±12)</td>
<td>55-88</td>
</tr>
<tr>
<td></td>
<td>2nd elution for 15 min</td>
<td>9</td>
<td>32</td>
<td>44</td>
<td>28 (±17)</td>
<td>15-44</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>107</td>
<td>120</td>
<td>77</td>
<td>94 (±17)</td>
<td>67-120</td>
</tr>
<tr>
<td>4</td>
<td>1st elution</td>
<td>42</td>
<td>57</td>
<td>67</td>
<td>69 (±13)</td>
<td>53-84</td>
</tr>
<tr>
<td></td>
<td>2nd elution for 45 min</td>
<td>9</td>
<td>32</td>
<td>44</td>
<td>28 (±17)</td>
<td>15-44</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>51</td>
<td>93</td>
<td>74</td>
<td>77 (±16)</td>
<td>47-93</td>
</tr>
<tr>
<td>5</td>
<td>1st elution</td>
<td>33</td>
<td>61</td>
<td>31</td>
<td>41 (±11)</td>
<td>20-61</td>
</tr>
<tr>
<td></td>
<td>2nd elution for 120 min</td>
<td>9</td>
<td>32</td>
<td>44</td>
<td>28 (±17)</td>
<td>15-44</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>42</td>
<td>93</td>
<td>74</td>
<td>77 (±16)</td>
<td>47-93</td>
</tr>
<tr>
<td>6</td>
<td>1st elution</td>
<td>33</td>
<td>27</td>
<td>37</td>
<td>32 (±12)</td>
<td>20-50</td>
</tr>
<tr>
<td></td>
<td>2nd elution after overnight contact</td>
<td>9</td>
<td>15</td>
<td>76</td>
<td>71 (±3)</td>
<td>20-96</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>42</td>
<td>40</td>
<td>47</td>
<td>43 (±4)</td>
<td>20-60</td>
</tr>
</tbody>
</table>

Poliovirus was seeded in 100 L of deionized water and filtered through NanoCeram® filters.

Poliovirus Recovery at Different pH of Water

Poliovirus Recovery at Different Flow Rate

Water pH = 7; P=0.08

Poliovirus Recovery at Different Flow Rate from Tap Water Using NanoCeram® Filter

<table>
<thead>
<tr>
<th>Virus</th>
<th>Elutions</th>
<th>Mean percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poliovirus 1</td>
<td>1st elution</td>
<td>35 (± 9)</td>
</tr>
<tr>
<td></td>
<td>2nd elution for 15 min</td>
<td>19 (± 5)</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>54 (± 8)</td>
</tr>
<tr>
<td>Coxsackievirus B5</td>
<td>1st elution</td>
<td>18 (± 12)</td>
</tr>
<tr>
<td></td>
<td>2nd elution for 15 min</td>
<td>9 (± 6)</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>27 (± 17)</td>
</tr>
<tr>
<td>Echovirus 7</td>
<td>1st elution</td>
<td>14 (± 6)</td>
</tr>
<tr>
<td></td>
<td>2nd elution for 15 min</td>
<td>18 (± 8)</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>32 (± 5)</td>
</tr>
</tbody>
</table>

P=0.36

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National Exposure Research Laboratory | Microbiological and Chemical Exposure Assessment Research Division |
Biohazard Assessment Research Branch

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Biohazard Assessment Research Branch
Comparison of Poliovirus Recovery by NanoCeram® and 1MDS Filters From Seeded Tap and River Water

<table>
<thead>
<tr>
<th>Type of filter</th>
<th>Elution</th>
<th>Mean virus recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 L sample</td>
<td>10 L sample</td>
</tr>
<tr>
<td></td>
<td>Tap water</td>
<td>River water</td>
</tr>
<tr>
<td></td>
<td>Tap water</td>
<td>River water</td>
</tr>
<tr>
<td>NanoCeram®</td>
<td>1st elution</td>
<td>23 ±14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21 ±18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>182 ±42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 ±16</td>
</tr>
<tr>
<td></td>
<td>2nd elution</td>
<td>28 ±13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 ±15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 ±64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 ±15</td>
</tr>
<tr>
<td></td>
<td>Combined percent recovery</td>
<td>51 ±26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38 ±35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>277 ±32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65 ±32</td>
</tr>
<tr>
<td>1MDS</td>
<td>1st elution</td>
<td>39 ±4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 ±40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31 ±14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 ±4</td>
</tr>
<tr>
<td></td>
<td>2nd elution</td>
<td>28 ±6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11 ±4</td>
</tr>
<tr>
<td></td>
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<td>13 ±13</td>
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<td></td>
<td></td>
<td>17 ±9</td>
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<tr>
<td></td>
<td>Combined percent recovery</td>
<td>67±6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36 ±27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44 ±19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 ±11</td>
</tr>
</tbody>
</table>

For 100 L samples p=>0.05; For 10 L samples, tap water p=<0.001, river water p=0.015

Comparison of Norovirus Recovery by NanoCeram® and 1MDS Filters From Seeded Tap and River Water

<table>
<thead>
<tr>
<th>Type of filter</th>
<th>Mean virus recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tap water</td>
</tr>
<tr>
<td>NanoCeram®</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>1MDS</td>
<td>1.2 ± 1.4</td>
</tr>
</tbody>
</table>

Norwalk virus was seeded in 10 L of dechlorinated tap water or river water and filtered through NanoCeram® or 1MDS filters.

RT-PCR Detection of Poliovirus From Seeded Tap and River Water Samples

<table>
<thead>
<tr>
<th>Type of filter</th>
<th>Elution</th>
<th>RT-PCR detection of poliovirus in 100 L seeded water samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tap water</td>
<td>River Water</td>
</tr>
<tr>
<td>NanoCeram®</td>
<td>1st elution</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2nd elution</td>
<td>+</td>
</tr>
<tr>
<td>1MDS</td>
<td>1st elution</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2nd elution</td>
<td>+</td>
</tr>
</tbody>
</table>

* First elution was done for one minute and 2nd elution was done for 15 minutes.
* `+` indicates RT-PCR positive and `-` indicates RT-PCR negative

Conclusions

- The mean retention of poliovirus by NanoCeram® filters was 84 percent.
- The highest virus recovery (77%) was obtained by immersing the filters in beef extract for 1 min during the first elution and 15 minutes during the second elution.
- The recovery efficiencies of poliovirus, coxsackie B5, and echovirus 7 were 54%, 27%, and 32%, respectively.
- There was no significant difference in poliovirus recovery at tap water pH range of 6 to 9.5.
- There was no significant difference in virus recovery over a water flow rates of 5.5 L/min to 20 L/min.
Conclusions

- NanoCeram® filters were comparable or better than the 1MDS filters.
- Cost approximately one-sixth of 1MDS filter, thus can be used for routine viral monitoring of water.

Published in Applied and Environmental Microbiology.

Future Directions

- This work has paved the way toward a validation project aimed at replacing the 1 MDS filter with the Nanoceram® filter in an enterovirus detection method.
- If this validation goes as expected, this new method will be considered for UCMR 3.

Thank-you!
Questions?
Comments?
Suggestions?
Automated Methods for the Quantification and Infectivity of Human Noroviruses in Water

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Richard Ozanich, Co-PI, Richard.Ozanich@pnl.gov.
Rachel Bartholomew, Co-PI, Rachel.Bartholomew@pnl.gov.
Cindy Bruckner-Lea, Co-PI, Cindy.Bruckner-Lea@pnl.gov

Methods for Capturing Pathogens from Large Volumes of Water – Aim 1

- Need: Ability to efficiently capture and concentrate viruses, bacteria, and protozoa from large volumes of water
  - Pathogen concentrations in water are often very low (<1/100 mL for bacteria to <1/1,000 L for viruses)
- Methods we are investigating are mostly off the shelf technology
  - Hollow fiber filtration: Large volumes require large columns, high flow rates can be problematic
  - Sodiocalceic glass wool: Very cheap, and may have great potential for viruses – investigating this summer DOE FaST team
- Modified system (next slide) may allow flow rates up to 15 L per minute

Secondary Concentration: The Major Bottleneck – Aim 2

- For water we get to a primary filtrate and then:
  - Centrifugation will concentrate bacteria and protozoa, but it is a manual process.
  - Viruses are left in the supernatant and still need to be concentrated.
  - Or we use single-plex immunomagnetic separation: e.g. the “disease of the day” approach, and we lose information about other pathogens.

Project Overall Aims

Large Scale System, Adapted from Vince Hill

Challenge: Automate to deliver concentrated samples for further processing

BEADS: Bridging the Gap between Large Volume Concentration and Detection

Sample: Large volume, matrix high, pathogens low)
Detector: Clean, tiny volume (μL-mL)

BEADS = Biodetection enabling analyte delivery system
For secondary concentration and purification

Biochip
Culture
Immunochip
Biodetection Enabling Analyte Delivery System (BEADS)

Guiding principles
1. Analytical separations can be performed on an interactive surface like a derivatized bead
2. Analytes of interest (cells, DNA, proteins, etc) are perfused over a column of beads and captured
3. Matrix materials are washed away, leaving purified analytes

Perfuse and capture
Wash away matrix

General Approach: Renewable Surfaces
- Interactive surface on beads is delivered fresh for each sample
- Compatible with users’ pathogen detection requirements
  - Nucleic acid techniques
  - Cell culture
  - Immunodiagnostic “sandwich” assays
- Operates within a scaleable fluidics architecture
  - From µL to 10 L volumes
  - Architecture allows us to handle samples that are high in particulate matter and/or soluble inhibitors

Beads are flushed to waste or sent downstream for further analysis

Renewable Separation Columns (RSC) used in BEADS

Renewable separation columns are the defining feature of the BEADS platform. Depending on the user’s needs, any one of these columns, and any type of separation media can be used.

Parallel Research Tracks Include Automation and Reagent Development
- BEADS Scale up
  - Need to process large volumes (1-10 L or greater)
  - From a primary concentrate.
  - Baseline experiments need to show that we can capture and release pathogens as efficiently as our small systems.
  - Possible secondary concentration issues to achieve overall 10^4-10^5 concentration factor.
- Multi-agent capture
  - Default: Use multiplexed preparations of commercially (and custom) available IMS antibodies
  - New generation methods: broad spectrum capture reagents for protozoa, bacteria, and viruses.
  - How will either cell capture approach fare when challenged with low target organisms and high background flora?

Batch Trials with Lectins: Reagent Development for BEADS
- Combinations of biotin labeled lectins were first mixed with bacteria, and then captured on streptavidin magnetic beads (indirect capture)
  - Loss of CFU indicates better capture results
  - Demonstrated capture of vegetative cells and spores
- Challenge: direct capture.
  - Lectins conjugated to the beads do not work as well.
- Viral capture has not shown as much promise

Reverse transcription real-time PCR – Aim 3
- For human noroviruses, there is not much choice for the development of better primers and probes
  - Variations within the ORF1-ORF2 junction – most conserved to detect the most known strains.
- “Fast” vs. Slow real-time PCR
  - Never real-time platforms allow PCR to be completed within 40 minutes. HOWEVER
  - Still need to perform reverse transcription, and that is still relatively slow
  - Your assay must be optimized for this platform...ORF1-ORF2 is not a good place to do this (secondary structure).
- For the purposes of this project, we are using the standard thermal cycling conditions.
Infectivity Assays for Human Noroviruses – Aim 4

Our original work investigated the INT407 cell line grown as 3-D cell cultures. While we see evidence of infectivity, we are not observing significant viral replication. Investigations with 3-D Caco-2 cells has revealed interesting results.

Observational differences between Uninfected and hNoV Infected 3-D Caco-2 cells

Real-time PCR observations indicate viral RNA replication in Caco-2 and INT407 Cells

Possible investigation of the role of STAT-1 in controlling viral replication

Research Summary

- Fluidic architecture is currently being constructed to process large volumes of water.
- Secondary capture reagents being investigated at the bench
  - Testing this summer: DOE Faculty and Student Team (FaST) will allow us to test both the large volume systems and perform batch capture experiments for secondary concentration – No charge to EPA STAR
- Further investigation of Caco-2 cell line for hNoV infectivity.
  - Results have been very promising, and if there is an underlying genetic mechanism inhibiting viral replication, this could provide new insights to develop better infectivity assays.

Acknowledgements

A portion of this research was performed using EMSL, a national scientific user facility sponsored by the Department of Energy’s Office of Biological and Environmental Research located at Pacific Northwest National Laboratory. Funding for this work is provided by the United States Environmental Protection Agency STAR Grant Program (Grant # R833831010). The norovirus infectivity assay is jointly provided by NIAID under the Food and Waterborne Integrated Research Network Program (Contract number NO1-AI-30055) and the STAR Grant Program R833831010 Food and Waterborne Diseases Integrated Research Network.
Amoebae Harbor Novel Pathogens That Slip "Under the Radar Screen"

Recent EPA Study
Examined 40 natural water samples: (lakes, rivers, ponds, wetlands, etc.)
Examined 40 cooling tower samples
Also examined 20 other industrial: chillers, hot tubs, hot water taps/tanks, etc.
Designed a protocol to screen for infected amoebae

Environmental Parameters
Temperature, pH, dissolved organic carbon (DOC), total nitrogen (N) and total bacteria per ml
Logistic regression analyses were performed to find any parameter or set of parameters that were good predictors of the occurrence of infected amoebae

Modified from T. Rowbotham, 1986.
Coccoid Cooling Tower Isolate in HeLa Cell Nuclei (48h)

Coccoid Cooling Tower Isolate in U937 Cell Nuclei (48h)

Hot Tub Amoeba Pathogen in A. polyphaga
“Hot tub” infection of A. polyphaga 18 h

“Hot tub” infection of A. polyphaga 24 h

“Hot tub” infection of A. polyphaga 48 h

Phylogenetic Tree with Novel LLAPs

Phylogenetic tree with “Hot tub”

<table>
<thead>
<tr>
<th>Percent Identity</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94.4</td>
<td>86.6</td>
<td>89.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.8</td>
<td>86.3</td>
<td>90.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13.8</td>
<td>14.2</td>
<td>85.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>11.1</td>
<td>10.5</td>
<td>14.3</td>
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</table>

<table>
<thead>
<tr>
<th>Percent Divergence</th>
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<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CC99</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>HT99</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>L. pneumophila X73402</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C. burnetii AY342037</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

22 of 40 cooling tower samples were positive
3 of 40 natural samples were positive
2 of 20 other industrial samples were positive (hot tubs)

Odds ratio of finding infected amoebae in cooling towers vs natural environments is 16, i.e., 16 times more likely to find them in CTs (based on the way we look for them)

5 novel strains were identified, related to *Legionella*

Only 2 of the 22 infections were from *L. pneumophila*
And 1 of the hot tub infections was from *L. pneumophila*

Several have not yet been isolated or identified

Of those that are culturable, at least 3 tested so far appear to infect human macrophages
Two non-culturable strains also infect macrophages

No environmental parameter was a significant predictor of occurrence of infected amoebae when cooling tower data were used alone

When data from 90 combined samples were used, pH and DOC were significant predictors

BUT cooling towers have higher pH values than almost all natural samples, and also have a higher range of DOC

Therefore it appears to be pH and DOC, but it may be something else specific to CTs that were not measured in this study

Summary/Conclusion

Occurrence of infected amoebae was significantly higher in cooling towers than in nature (16:1 odds ratio)

Non-Legionella were more common than Legionella, and half or more of these were not culturable

7 novel sequences were found, with several yet to be sequenced

Environmental parameters?? Possibly pH and DOC

Update

Several other infected amoeba specimens have been observed in the past year—

- Meat industry (3)
- Eyewash station (TTU)
- Fish tank in public pet store
- Distribution pipes (MTSU)
Table 1. Comparison of infectivity durations of LLAPs in buffered saline vs. LLAPs in a desiccated state

<table>
<thead>
<tr>
<th>Organism/Sample</th>
<th>Origin</th>
<th>Duration of Infectivity (in aqueous state) (days)</th>
<th>Duration of Infectivity (in desiccated state) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STPA1</td>
<td>77% cooling pipe</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>SR1</td>
<td>water pipe</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>FDE1</td>
<td>meat renderies</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>LS1</td>
<td>meat renderies</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>RT7</td>
<td>tree sap</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>LD1</td>
<td>outdoor lake</td>
<td>50</td>
<td>37</td>
</tr>
<tr>
<td>ND1</td>
<td>77% rendering</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>LS2</td>
<td>meat renderies</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>LD1</td>
<td>outdoor lake</td>
<td>50</td>
<td>37</td>
</tr>
</tbody>
</table>

*These organisms still under study.

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Detection of Various Freshwater Cyanobacterial Toxins using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

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Liquid Chromatography/Tandem-Mass Spectrometry (LC/MS/MS) is a powerful tool for the analysis of various analytes in a wide variety of matrices. What is especially attractive about LC/MS/MS is its sensitivity and selectivity. Microcystins, in particular, represent an emerging class of algal toxins of concern to the drinking water industry. Recognizing the potential health risk, the World Health Organization, Australia, and Brazil have established guidelines for the amount of microcystins permissible in drinking water (specifically microcystin LR). Recently, the United States has begun to evaluate the occurrence, health effects, and susceptibility of water treatment of algal toxins. The Environmental Protection Agency (EPA) named freshwater algal toxins to its Contaminant Candidate List (CCL).

In this paper we investigate the use of newer technologies in smaller column packings (sub 2 μm particles) to both improve the selectivity, speed, sensitivity and resolution to screen for many of these toxins (microcystins, anatoxin-a, and cylindrospermopsin) using Ultra-Performance Liquid Chromatography (UPLC®) combined with tandem mass spectrometry. Specific examples, including data from the recent Ohio river algae bloom in August, will be presented.
Various Microcystins and others

Factors affecting cyanobacterial bloom formation:
- Moderate to high levels of essential inorganic nutrients (nitrogen and phosphorus)
- Some are nitrogen-fixing
- Water temperature 10° to 30°C
- pH levels between 6 and 9
- Low flow and low turbidity
- Light is not a large factor - phycobilin

Pathway/Route of Exposure
- Recreational waters - dermal, inhalation, and ingestion
- Drinking water - ingestion, dermal, ingestion.
- Dietary Supplements - ingestion
- Vegetables and Fruits - ingestion

Microcystin Detection Assay
- Enzyme-linked Immunosorbent Assay (ELISA)
  - Uses polyclonal antibodies against different microcystin variants.
  - Samples are read spectrophotometrically to determine microcystin concentration.
  - Detection limit in low ppb
  - Cloudy or Murky samples pose a challenge
- High-Performance LC
  - Powerful separation capability
  - UV detection (not sensitive w/o SPE)
- LC and Mass Spectrometry
  - Offers specificity and sensitivity

Microcystin LR Mass Spectrum
- This is a "Global Challenge"
**Going the Next Step...UltraPerfomance LC/MS/MS**

- Small Particle (sub 2μm)
- Higher separation power
- Higher tensile strength

**UPLC™/MS/MS Separation 8.5 minutes**

**Possible Internal Standards**

**Water Samples—Filter Only**

**Detection Limits**

**Water Samples—Filter Only (spiked at 4ppb)**

**Compound name:** Anatoxin-a, Cylindrospermopsin, Anatoxin-a, Cyclo (Arg-Ala-Asp-D-Phe-Val) (IStd), [Leu5]-Enkephalin (IStd), Microcystin RR, Microcystin YR, Microcystin LR, Microcystin LW, and Microcystin LF
Current methodology exist for common microcystins using C18 based SPE. Anatoxin and cylindrospermopsin add challenges to existing SPE protocols. VERY Preliminary work has begun on using a multimodal SPE protocol* (2 multimodal cartridges for different analytes from a single water sample):

- Load water onto 2 cartridges in series, then separate and process each separately for the different analyte sets.
- Run 2 injections per sample (one for Cylindro, the other for anatoxin and microcystins).

*Patent applied for.
Conclusions

- Separation of all main Microcystins, Anatoxin-a, and cylindrospermopsin is possible in under 10 minutes using UPLC as the separation device (versus 40 Minutes by HPLC)
- MS/MS offers enhanced selectivity and sensitivity
- Combined with new SPE method, one can easily go to sub ppb levels

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Questions?