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Coliphage Analyses for Wastewater and Recreational Waters: Update on Methods and Regulations

Thursday May 9, 2019
1:00 – 2:30 PM ET

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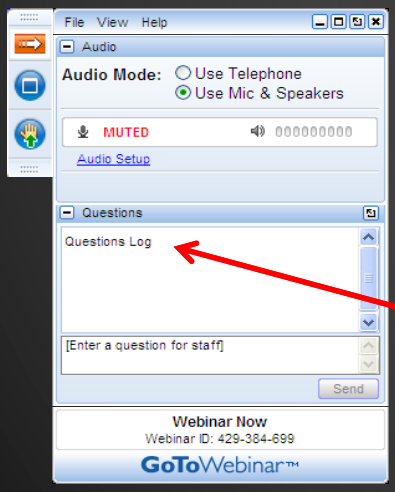
How to Participate Today



- **Audio Modes**
  - Listen using Mic & Speakers
  - Or, select "Use Telephone" and dial the conference (please remember long distance phone charges apply).
- **Submit your questions using the Questions pane.**
- **A recording will be available for replay shortly after this webcast.**

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Today's Moderator

Akin Babatola

Laboratory and Environmental Compliance Manager

CITY OF SANTA CRUZ

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Today's Speakers

- Kari Brisolara
  - Coliphage and Indicator Efficacy
- Kaedra Jones
  - Recreational Water Quality Criteria Development: Coliphage (a viral indicator)
- Robert Salter
  - Simplification of Coliphage Test Methods

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Coliphage and Indicator Efficacy

Examining the Matrices

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Outline

- Overview of Indicators
- The need for Alternative Indicators
- Acceptable Level of Risk and Regulations
  - SDWA, CWA (Rec Water, Wastewater, Biosolids), Water Reuse, ISSC/NSSP (Shellfish)
- Treatment and Response: Virus vs. Coliphage
  - Concerns and Methods
- Additional Considerations and Future Directions

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Issue:

- Based on the data from 2000 to 2012, bacterial pathogens were responsible for 31% of the outbreaks,
- Parasite (protozoa and other higher organisms) were responsible for 18% of the outbreaks, and
- Viruses were responsible for 16% outbreak (Graciaa, et al. 2018).
- The CDC also reported an undefined etiology for 26% of the outbreaks highlighting the need for additional work with pathogen identification.

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Issues in the Quest for Pathogen Indicators of Treatment

There is no such thing as an ideal indicator

No single indicator predicts the responses to survival and health risks of all pathogenic form all sources and for all scenarios of exposure

- Pathogens (and indicators) are diverse
  - Taxonomy
  - Physical, Chemical and biological properties
  - Response to physical, chemical and biological agents and processes
- Diverse Treatment to reduce pathogens and indicators
  - Physical processes
  - Chemical processes for water, wastewater and residuals
  - Biological processes
- Diverse means of pathogen exposure from reclaimed water, WW & biosolids
  - Drinking water
  - Recreational water
  - Irrigation, aquaculture, shell fishing and food processing waters
  - Reclaimed and recycled biosolids and manures

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Treatment Indicators

Indicator of what….?

Pathogens

Treatment

Sources and natural history

Exposures pathways and health risks

- **Things are not always as they seem at first glance**
- **The devil is in the details**

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Ideal Indicator Organism SHOULD:

- Be present when pathogens are present and absent in clean, uncontaminated waters but present in fecal matter
- Be responsive to natural environmental and to treatment processes in a similar matter to pathogens
- Be easily detected by simply, inexpensive laboratory testing in the shortest time with accurate results
- Have a high indicator/pathogen ratio
- Be stable and nonpathogenic
- Be suitable for all types of waste and sludge

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Concerns with the Spiking and Assay Monitoring

- Labor intensive
- Time consuming
- Reliability of accuracy and precision
- Potential danger public health and the environment
- Very Expensive

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There are three organisms which have showed promise as indicators

Clostridium perfringens, Somatic bacteriophage, Aerobic endospores

- They have a density in municipal sludge of 10^4 to 10^6 organisms per gram of dry solids.
- These assays requires days instead of a week to over a month.
- By using these organisms, the performance data can be increased and the process performance can be quickly determined.
- This can lower the cost of disinfection analysis by an order of magnitude.
- **Recent work has recommended utilizing the spore forming organisms and bacteriophages.**

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Classification of Biosolids Matrix: *Proposed Surrogates*

Target	Heat Drying	Anaerobic Digestion	Aerobic Digestion	Advanced Alkaline Stabilization*	Acid Treatment*
Helminth	Plant and fungal surrogates **	Somatic bacteriophage	Plant and fungal surrogates **	Aerobic endospores	*C. perfringens*
Viruses	Reoviruses Somatic bacteriophages	Reoviruses Somatic bacteriophages	Reoviruses Somatic bacteriophages	Reoviruses Somatic bacteriophages	Reoviruses Somatic bacteriophages
Bacteria	Fecal coliform Enterococci *E. coli*	Fecal coliform Enterococci *E. coli*	Fecal coliform Enterococci *E. coli*	Fecal coliform Enterococci *E. coli*	Fecal coliform Enterococci *E. coli*

*High concentration of non-charged biocidal agent

**Vascular and nonvascular plant seeds, and fungal spores and propagules are also included in Advanced Alkaline Stabilization and Acid treatment

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The Necessary Properties of a Good Microbial Indicator

- Safe for personnel to work with and handle.
- More resistant than the pathogens, yet correlate to pathogen inactivation.
- Easy and rapid to analyze for.
- Easy to assay by typical staff at municipal wastewater laboratories.
- Inexpensive and sufficiently reliable with respect to precision and accuracy.

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What can work for viruses?

- Coliphage can be an indicator of viruses in ambient waters (Wu et al., 2011) and predict GI illnesses.
- EPA's Review of "Coliphages as Possible Indicators of Fecal Contamination for Ambient Water Quality" summarized eight epidemiological studies that evaluated the relationship of coliphages and GI illness resulting from exposure to recreational water. Four of the eight studies found a statistically significant relationship between male-specific coliphage and illness levels (Lee et al., 1997; Colford et al., 2005, 2007; Wade et al., 2010; Griffith, personal communication, 2015).
- Thus, 304(a) coliphage-based RWQC also have the potential to be used for beach notification in recreational waters impacted by viral sources (i.e., wastewater effluent).

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Table 2.2. Microbial parameter applicability and suitability

GUIDELINES FOR DRINKING-WATER QUALITY

Parameter	Sanitary survey (catchment)	Source water characterisation	Groundwater characterisation	Treatment efficiency (removal)	Treatment efficiency (disinfection)	Treated water	Distribution system (ingress)	Distribution system (regrowth)	Outbreak investigation
Total coliforms	NR	NR	NR	NR	SA	S	SA*	S	S
Thermotolerant coliforms	SA	SA	SA	NR	SA	SA	SA*	S	S
Escherichia coli	S	S	S	S	S	SA	S*		S
Faecal streptococci (enterococci)	SA	SA							S
Ratio between counts (any parameter)	NR	NR	NR						
Total bacteria (microscopic)				SA	SA		SA	S	S
Viable bacteria (microscopic)				SA	SA		SA	S	S
Heterotrophic bacteria				S	S	NR	S	S	S
Aerobic spore-forming bacteria				S	S	NR			S
Somatic coliphages	SA	SA	SA		SA				S
F specific RNA phages	SA	SA	SA		SA				S
Bacteroides *phages*	SA	SA	SA		SA				S
Sulphite-reducing clostridia	NR	NR	NR						S
Clostridium perfringens	SA	SA	SA	SA					S
Pseudomonas, Aeromonas								S	
Enteric viruses	S	S	S	NR	NR				S
Giardia cysts, *Cryptosporidium* oocysts	S	S	SA	S	NR				S

Key:
S: suitable. *In distribution systems without residual disinfection. SA: suitable alternative.
NR: not recommended. ISD: insufficient data. ■: not applicable.

USE OF SOMATIC COLIPHAGES IN OTHER REGULATIONS

Source: Anicet Blanch, PhD, Dept. Microbiology, University of Barcelona, Spain, WEFTEC 2015 Workshop on Pathogens and Indicators

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USE OF SOMATIC COLIPHAGES IN OTHER REGULATIONS

Government of Quebec, Montreal, Quebec, Canada. 2001. Loi sur la qualité de l'environnement: règlement sur la qualité de l'eau potable c. Q.-2, r. 18.1.1. Gazette Officielle du Québec 24, 3561. Government of Quebec, Montreal, Quebec, Canada.

U.S. Environmental Protection Agency. 2006. National primary drinking water regulations: ground water rule; final rule; 40 CFR parts 9, 141, and 142. Fed. Regist. **71:**65574-65660.

U.S. Environmental Protection Agency. 2015. Review of Coliphages as Possible Indicators of Fecal Contamination for Ambient Water Quality. Evaluating to develop coliphage-based ambient water quality criteria for the protection of swimmers. …….. Stakeholders may send additional data for EPA to consider in the development of future coliphage-based ambient water quality criteria by June 15, 2015.

Source: Anicet Blanch, PhD, Dept. Microbiology, University of Barcelona, Spain, WEFTEC 2015 Workshop on Pathogens and Indicators

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AVAILABLE METHODS

Sludge, treated biowaste and soil. Extraction and enumeration of bacteriophages	**ISO/CEN WI 308099**
Water quality - Detection and enumeration of bacteriophages. Part 2: Enumeration of somatic coliphages	**ISO 10705-2**
Standard methods for the examination of water and wastewater	**APHA, 2005 9224B**
Methods for the validation of biotechnological, thermal and chemical processes for the treatment of animal by-products, sewage sludge and biowastes in order to determine the hygienic safety of the resulting fertilizers or comparable products by exposition of test organisms or test viruses – Part 2 : Validation with test viruses	**ISO/CEN/TC308 working document**

Source: Anicet Blanch, PhD, Dept. Microbiology, University of Barcelona, Spain, WEFTEC 2015 Workshop on Pathogens and Indicators

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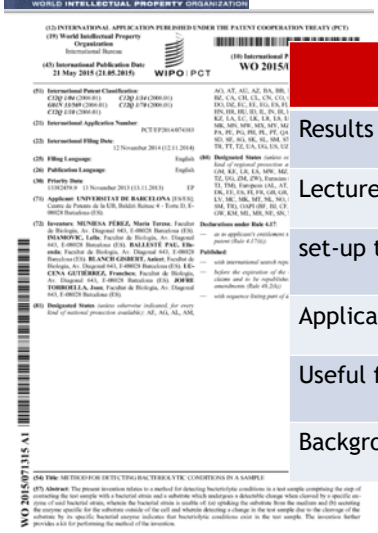
Routine analytical methods



	UB Patent WO2015071315
Results in	**< 3 hours**
Lecture	colorimetric
set-up time	**< 15 min. No enrichment**
Applicable for field use	Yes
Useful for	Water, water supplies, food, sludges, etc...
Background	After O/N incubation

Source: Anicet Blanch, PhD, Dept. Microbiology, University of Barcelona, Spain, WEFTEC 2015 Workshop on Pathogens and Indicators

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Additional Considerations and Future Directions

- Modeling
  - Difficult to assess risk using deterministic beach study epidemiology models
  - Probabilistic models (QMRA) provide better understanding of risk
- What about protozoa? Indicators?

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Recreational Water Quality Criteria Development: Coliphage (a viral indicator)

Kaedra Jones, MPH
ICF
On behalf of Sharon Nappier, PhD, MSPH
U.S. EPA, Office of Water

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Disclaimer

The views expressed in this presentation are those of the author and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

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Outline

- Introduction and the need for a new (viral) indicator
- Recreational Water Quality Criteria
- Application of coliphage as an indicator
- Recent and ongoing work
  - Enumeration methods
  - Criteria derivation

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Bacteria vs. Viruses

Historically, bacteria thought to cause majority of illnesses (e.g., *Vibrio cholera* and *Salmonella typhi*).

- Bacterial pathogens targeted through bacterial indicators (*E. coli* and enterococci).
- Wastewater treatment improvements and **permits based on bacterial indicators effectively control bacterial pathogens.**
- Effective at predicting bacterial impairments of water quality.

However, Quantitative Microbial Risk Assessment (QMRA), epidemiological, and microbial water quality studies indicate **viruses cause majority of swimming-associated illnesses** in human-impacted waters.

- Current treatments, indicators, and permits do not specifically target viruses.
- Thus, viruses enter surface waters from treated and untreated human sources.
- Epidemiological studies indicate bacteria may not always be predictive of viral illnesses.

Thus, EPA has been working to develop viral-based Recreational Water Quality Criteria.

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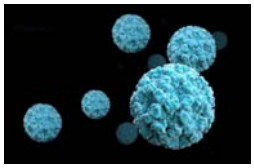
Need for a Viral Indicator

Viruses have:

- Higher environmental persistence;
- Higher resistance to water treatments; and
- Very low infectious doses.

Why not just measure viruses or other pathogens directly in environment?

- Time consuming;
- Requires highly trained staff working in specialized laboratories;
- Technologically infeasible; and
- Large volume of water is required for sample concentration.



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Recreational Water Quality Criteria

Clean Water Act

- Goal: Restore and maintain oceans, watersheds, and their aquatic ecosystems to protect human health, support economic and recreational activities, and provide healthy habitat for fish, plants and wildlife.
- Establishes the basic structure for state water quality standards, including regulation of pollutant discharge into the waters of the United States.

Recreational Water Quality Criteria (RWQC)

Intended to be used by states adopting water quality standards to protect the designated use of primary contact recreation.

- Prevent illness
  - By preventing fecal contamination and/or pathogens from entering surface waters
    - Point source permits (NPDES permits)
- Identify impaired waters
    - 303(d) Listing, Total Maximum Daily Loads (TMDLs)
- Identify potentially hazardous conditions
    - Beach notifications

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2012 RWQC: Current Status

The 2012 RWQC for primary contact recreation are associated with bacterial indicators of fecal contamination.

Highlights

- Indicators: enterococci (for marine and freshwater) and *E.coli* (for freshwater).
- Specified magnitude, duration (30 day), and frequency.
  - Two sets of recommended criteria, each corresponds to a different illness rate.
- Includes supplemental tools:
  - qPCR method for same-day notification.
  - Beach action values for precautionary notification.

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Why Use Coliphages as a Viral Indicator?

- Of fecal origin/highly concentrated in sewage.
- Physically similar to enteric viruses of concern.
- Similar persistence patterns to enteric viruses.
  - To treatment and to environmental insults.
- No appreciable re-growth in ambient waters.
- Non-pathogenic.

Coliphages, under evaluation since the 1970s, are currently applied to groundwater, shellfish harvesting water, and potable reuse applications.

Two coliphage types under consideration: male-specific and somatic.

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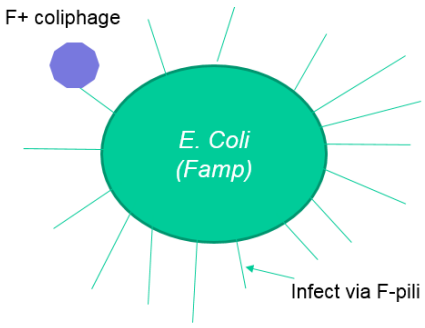
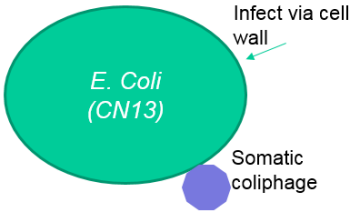
Male-Specific & Somatic Coliphages



Male-Specific (F+)
Coliphage and Host



Somatic
Coliphage and Host

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Current Applications of Coliphage as an Indicator

Country	Biosolids	Groundwater	Recreational water	Drinking water	Reclaimed water	Direct potable reuse	Aquaculture
Australia	2012 (Western Australia); MSC or somatic coliphages < 10 pfu/10 grams			2011 (Australia); should not be present in any 100 mL sample	2012 (Western Australia); MSC and somatic coliphages; Class A+: 6.5 log reduction; for augmentation of drinking water: 9.5 log reduction 2018 (Queensland); Class A: MSC and somatic coliphages < 1 pfu/100 mL		
Canada				2011 (Quebec); MSC should not be present			
United States		2006; MSC and somatic coliphages should not be present	Under development; MSC and somatic coliphages		2011 (North Carolina - Reclaimed Water Effluent Stds) must not exceed monthly GM of 5 pfu/100 mL or daily max of 25 pfu/100 mL (type not specified)		2015; MSC; <50 pfu/100 grams
Colombia	2014; somatic coliphages <5.00 cfu/gram						
European Union				2018; somatic coliphages < 0 pfu/100 mL	2017; total/MSC/somatic coliphages performance target for treatment train is ≥ 6.0 log_{10} reduction		
Italy	Under development						
WHO				2018; coliphage used as indicators for effectiveness of disinfection and physical removal processes for viruses.		2017; in 95% of samples collected daily after >2 treatment stages, total coliphages should be absent from 100 mL samples	

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Criteria Derivation – Progress to Date

Date	Milestone
2015	*Review of Coliphages as Possible Viral Indicators of Fecal Contamination for Ambient Water Quality*
2015	Stakeholder Webinar
2016/2017	Coliphage Expert Workshop; fact sheet (summer 2016) and proceedings (2017)
2016/2017	Listening sessions/webinars; conferences; states; other stakeholders (industry/environmental groups)
2017/2018	Analytical method multi-lab validation (2017) and publication (2018)
2019	Continued research to better understand coliphage distributions
2020	Draft coliphage criteria; send for external peer-review

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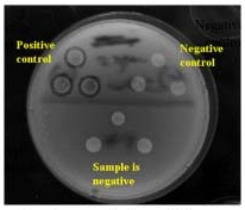
Coliphage Enumeration Methods

EPA Method 1601 (two-step enrichment process) and **EPA Method 1602** (single agar layer procedure) are multi-step processes for quantifying MSC and somatic coliphage.

- Culture-based methods used for enumeration of coliphages present in groundwater and "other waters" (though only validated for groundwater) in support of monitoring programs under the Safe Drinking Water Act and the Clean Water Act.



Positive results for coliphage appear as a clear halo around the spot in the surrounding E. coli lawn.

Image from U.S. EPA, 2001. Method 1601: Male-specific (F+) and somatic coliphage in water by two-step enrichment procedure: Office of Water, Washington, D.C., EPA-821-R-01-030.

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Coliphage Enumeration Methods

Draft **EPA Method 1643** is a single agar layer procedure to detect and enumerate male-specific and somatic coliphages.

- Culture-based method used for monitoring secondary (no disinfection) wastewater matrices under the Clean Water Act.
- Reflects the results of a multi-laboratory validation study of EPA Method 1602 for 100 mL secondary (no disinfection) wastewater samples.

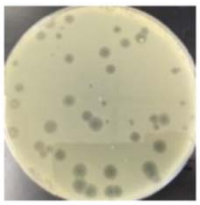

Figure 1: Somatic Coliphage Plaques (CN-13)

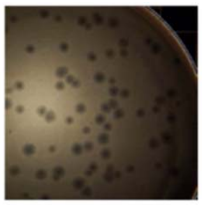

Figure 2: Male-specific Coliphage Plaques (F_{amp})

Image from U.S. EPA, 2018. Method 1643: Male-specific (F+) and Somatic Coliphage in Secondary (No Disinfection) Wastewater by the Single Agar Layer (SAL) Procedure. Office of Water, Washington, D.C., EPA 820-R-18-003.

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Coliphage Enumeration Methods

- Draft **EPA Method 1642** is a procedure to concentrate, detect, and enumerate male-specific and somatic coliphages in recreational waters (fresh and marine) and advanced treatment (secondary with disinfection, tertiary) wastewater effluents.
  - Modified EPA Method 1602 to include dead-end hollow-fiber ultrafiltration to concentrate larger sample volumes (2 L) required for recreational water monitoring.
  - Final sample volume is ~200 mL; can then be assayed for both male-specific and somatic coliphages using single agar layer procedure.

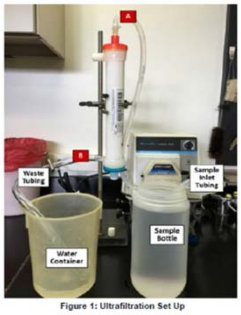

Figure 1: Ultrafiltration Set Up

Image from U.S. EPA, 2018. Method 1642: Male-specific (F+) and Somatic Coliphage in Recreational Waters and Wastewater by Ultrafiltration (UF) and Single Agar Layer (SAL) Procedure. Office of Water, Washington, D.C., EPA 820-R-18-001.

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Criteria Derivation: Progress to Date

- Over the past 4 years, data have been collected, synthesized, and analyzed in order to better understand the concentrations and distributions of norovirus and coliphages in ambient water and raw sewage. Results have been peer-reviewed and published.
- These analyses will be integrated into the QMRA and inform future coliphage criteria.


Water Research

Occurrence of norovirus in raw sewage — A systematic literature review and meta-analysis


Water Research

Occurrence of coliphage in raw wastewater and in ambient water: A meta-analysis

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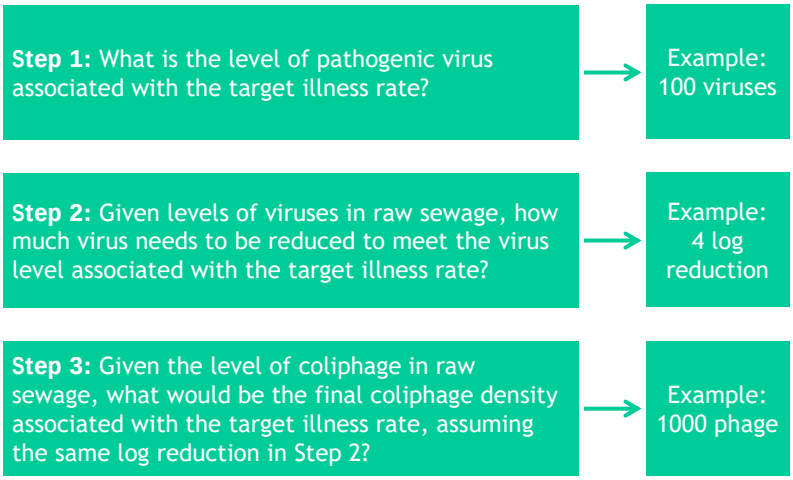
Criteria Derivation: Risk Assessment Approach



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Thank you!

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Robert Salter - VP of Regulatory and Industry Affairs
MS Life Science Engineering Tufts University CHARM SCIENCES INC

- Patents EP13837509.2 US9376704

Fast Phage™

Water Research Activity

- Alternative Test Procedure Fast Phage Equivalent to 1601 for ground water
- MPN adaptations Fast Phage
- Alternative Test Procedure Approval of Ecolite for Detection of Coliform and E.coli in Potable Water

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Simplification of Coliphage Test Methods

Fast Phage Method Equivalent to EPA Method 1601

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EPA Method 1601- Detection of Coliphage after Preenrichment

- Detects 1 PFU coliphage/sample
- Presence/Absence
- Steps
  - Add medium and E.coli host to water-
  - Incubate 37C
  - Place loop of growth (SPOT) on host bacterial lawn
  - Incubate overnight- Detect clear zones – Positive

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Commercial Kit for Coliphage Method 1601

- Single Dose Bacterial Host in Tablet Form
- Single Dose Medium in Dissolvable Film
- Rapid 6-8 Hour Detection (fluorescence)
- Confirmatory plaque assay

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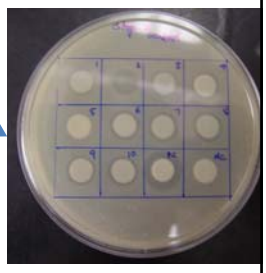
Fast Phage Procedure Overview

- STEP-1 ENRICHMENT- Add Pouch and Tablet to water sample
- 5 hour incubation

Split Transfer



STEP-2 FLOURESCENCE

3 hour (total time <8 hrs)

PLAQUE ASSAY

11 hours (total Time 16 hrs)

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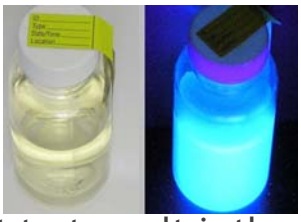
Spot test is the final test result same as 1601

Fluorescence is a fast same day prediction of result



Spot test result is the final confirmation

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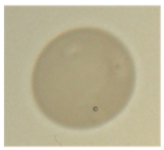
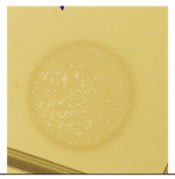
Different coliphage plaque morphologies

The table below are photographs of some plaque morphologies that may be seen on spot plates :

 Clear inhibitory zone	 Zone of inhibition surrounding amplified host growing on agar surface
 Microplaques in amplified host growing on agar surface with inhibition of edge of amplified host and *E. coli* seeded in agar	 Microplaques on edge of amplified host and seeded *E. coli* growing in ager
 Inhibitory zone with inner faint circular growth of amplified host	 Microplaques creating a speckled amplified host growth with a very rough surface

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There is a kit for each Coliphage type. Similar protocol

- Somatic Coliphage- More numerous in sewerage. DNA type viruses. May stem from non-mammalian sources. Correlated to distribution system intrusion.
  HOST=E.coli CN-13, PC=øX174, antibiotic=Naladixic Acid

- Male Specific Coliphage- (infect through F+ pilus) considered mammalian origin. Includes RNA type viruses structurally similar to enteric virus. Used in source tracking.
  Beach study correlation to enteric disease.
  Host Ecoli F_{amp} PC=MS2 Antibiotics- Ampicillin/Stremtomycin

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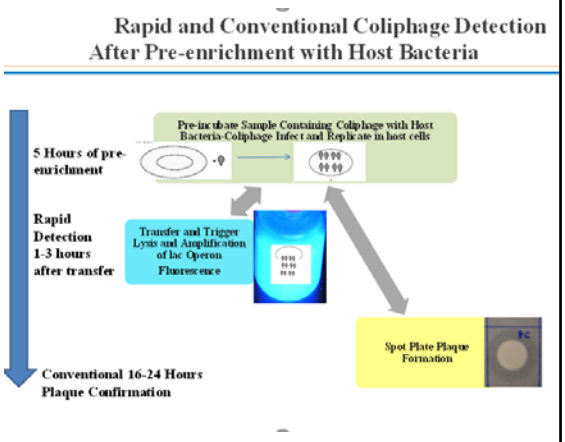
Goals achieved

- Multilab Verification of Detection of Somatic and Male Specific Coliphages at 1.5 and 1.3 PFU/100mL equivalent to 1601
- Open- Set up and perform test with result in a working shift and confirmation within 24 hours
- Meet criteria for detection of coliphage in Ground Water Rule
- EPA Recognized Alternative Test Method



https://doi.org/10.5942/jawwa.2012.104.0115

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Other applications – Research

- MPN application
- Larger volume- using hydrostatic filter enrichment 10L volumes
- Single Step MPN methods (not using enrichment)

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MPN Modification

- Add host and STEP 1 media to 100mL sample dissolve
- Divide into 3 x 30mL, 3 x 3mL and 3 x 0.3mL tubes. Incubate 6 hours
- Spot each tube to plates and incubate overnight.
- Determine positives and read from 3 x 3 log MPN table.

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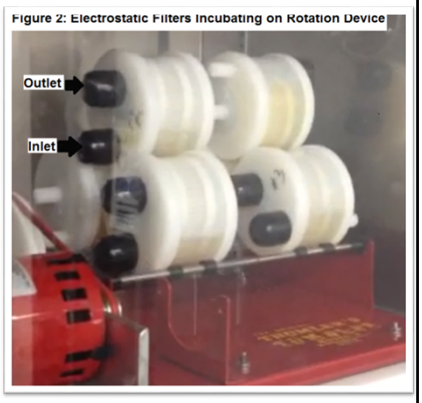
Method Modification Using High Volume Samples – ASM Poster 2014

- Spike 10L water with diluted waste water containing 1 to 2 plaque forming units
- Filter through electrostatic filter housing
- Conduct STEP 1 of Fast Phage in filter housing-
- Complete Fast Phage per instructions



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Somatic MPN method

- Add host tablet and STEP 2 fluorescent media directly to water sample
- Divide sample into multiple tubes or MPN Devices
- Detect fluorescence in 6 hours and use MPN to quantitate PFU/mL

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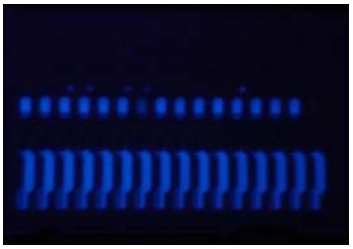
Examples of MPN Devices with Somatic MPN Rapid Detection



TEMPO® 4mL Volume

Quantitray ® 100mL

Food Environmental Virology 2016 Sep;8(3):221-6. doi:10.1007/s12560-016-x.

Appl Environ Microbiol. 2017 Jun 1; 83(11): e02984-16 doi: 10.1128/AEM.02984-16

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Limitations of single step MPN method

- Detects lytic but not lysogenic coliphages
  - Good method for DNA type (Somatic) and classic coliphage markers X175 and MS2
  - Not good for male specific coliphages except MS2
- Underdetects coliphage in biosolids and disinfected samples relative to plaque assay like Method 1602. But might be an OK process effectiveness indicator.

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Applications for simplified coliphage assays

- Waste water
- Irrigation water/produce rinse
- Storm water
- Shell fish
- Drinking water (if concentration step)
- Beach water (if concentration step)
- Filter validation

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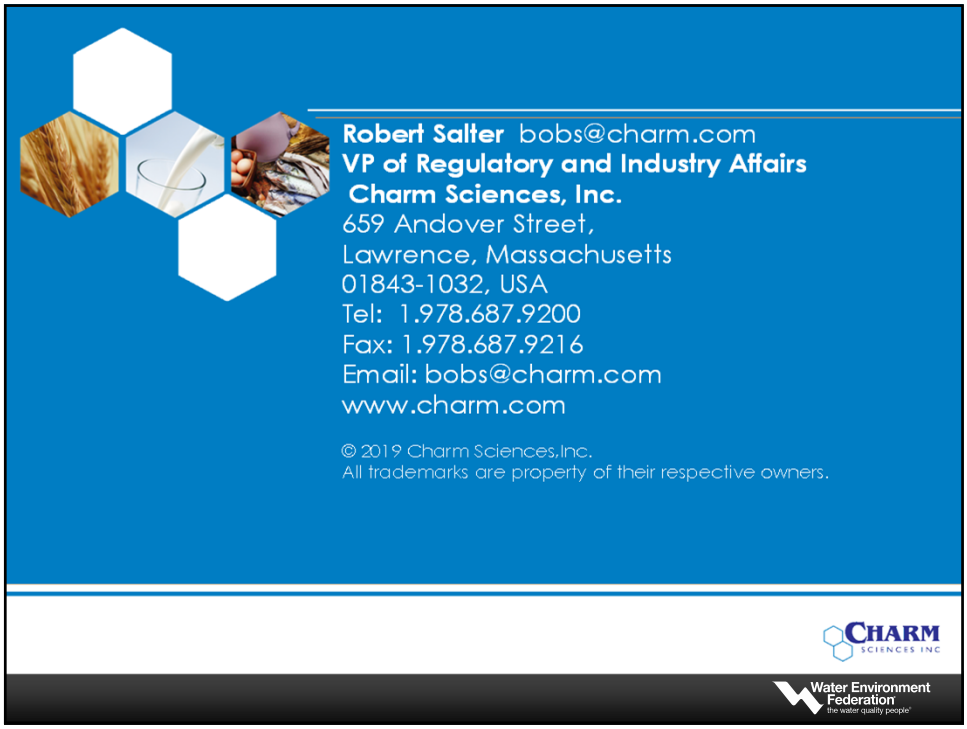
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Questions?
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