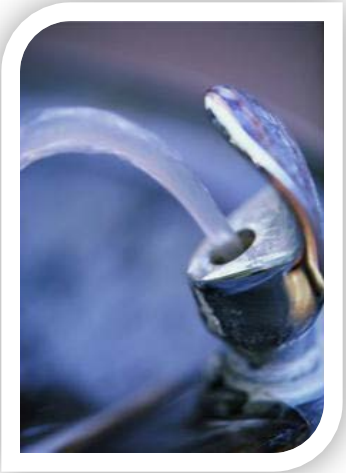
Overview of the Contaminant Candidate List and the Unregulated Contaminant Monitoring Rule

Steven C. Wendelken, Ph.D.



Office of Groundwater and Drinking Water

Standards and Risk Management Division

Technical Support Center

How Are Drinking Water Standards Developed?

- 1996 SDWA amendments changed the process of developing and reviewing NPDWS
  - Contaminant Candidate List (CCL)
  - Unregulated Contaminant Monitoring Rule (UCMR)
  - Regulatory Determination
  - Six-Year Review

Contaminant Candidate List (CCL)

- SDWA requires EPA to list unregulated contaminants that may require a national drinking water regulation in the future
- Every five years CCL defines unregulated contaminants for which EPA needs
  - Occurrence data
  - Analytical methods
  - Potential health effects
  - Evaluation of treatment techniques

UCMR Objective

- Collect occurrence data for suspected drinking water contaminants that do not have health-based standards set under SDWA
- Occurrence information is used to support future regulatory decision-making
  - Supports the Administrator's determination of whether (or not) to regulate a contaminant under the drinking water program

Three Regulatory Determination Criteria

SDWA requires EPA to consider the following criteria in evaluating whether to regulate a contaminant:

1) The contaminant may have an adverse effect on the health of persons;

2) The contaminant is known to occur or there is substantial likelihood that the contaminant will occur in public water systems with a frequency and at levels of public health concern; and

3) In the sole judgment of the Administrator, regulation of such contaminant presents a meaningful opportunity for health risk reduction for persons served by public water systems

***SDWA Section 1412(b)(1)**

Status and Next Steps for Regulatory Determinations 3 (RD 3)

- Published Third Contaminant Candidate List (CCL 3) in Oct 2009, which listed 116 contaminants:
  - 12 microbes (e.g., viruses, bacteria)
  - 104 chemicals (pesticides, industrial chemicals, pharmaceuticals, inorganics)

- Evaluating the health and occurrence information to identify which CCL 3 contaminants have sufficient information to make to the preliminary regulatory determinations

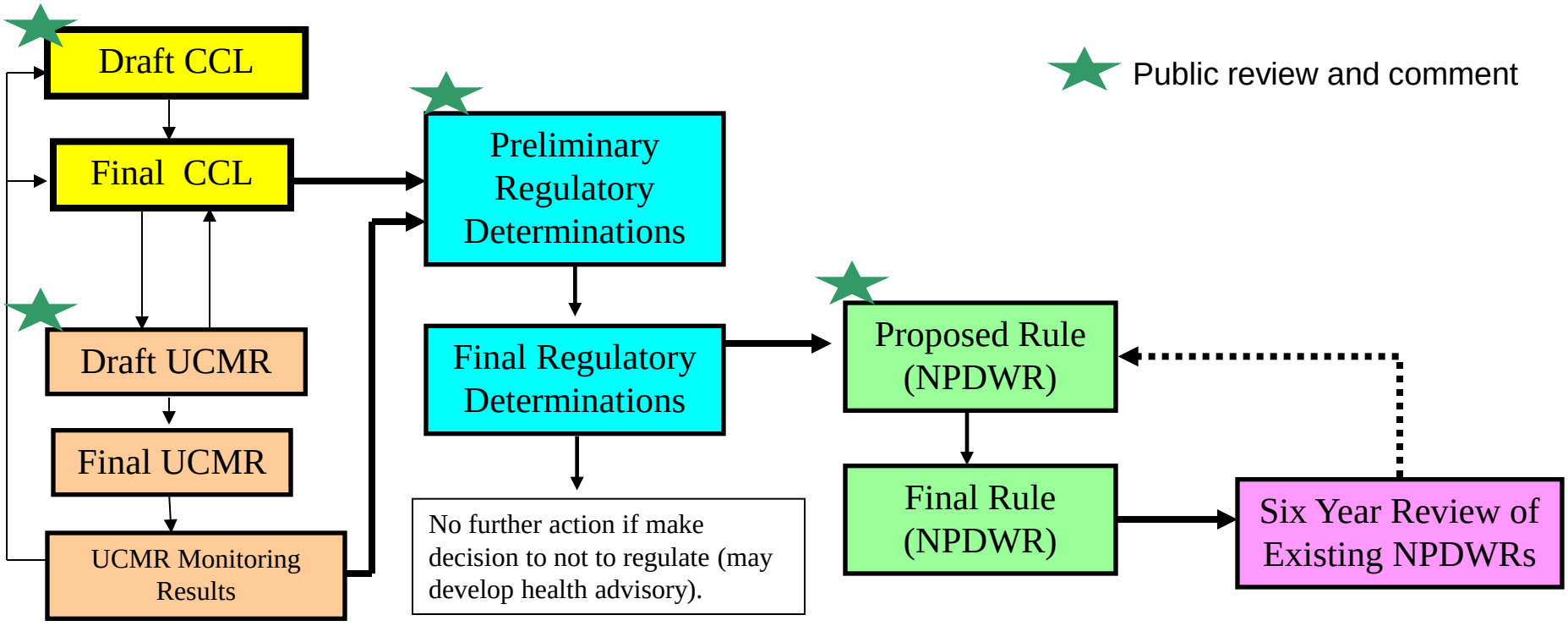
- Expect to publish preliminary RD 3 for public comment in 2013

- After considering public comments, expect to publish final RD3 in 2014

Six Year Review 3

- 1996 SDWA Amendments require EPA to review and, if appropriate, revise existing National Primary Drinking Water Regulations (NPDWRs) every six years
  - In 2003, EPA completed the 1st Six Year Review of 69 NPDWRs; made decision to revise TCR
  - In 2010, EPA completed the 2nd Six Year Review of 71 NPDWRs and made decisions to revise tetrachloroethylene (PCE), trichloroethylene (TCE), acrylamide and epichlorohydrin
- Occurrence analysis is a key component in the Six Year Review process
- Expect to complete Six Year Review 3 by 2016

General Flow of SDWA Regulatory Processes



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

Generation of CCL 3

- EPA considered approximately 7,500 potential chemical and microbial contaminants
- Screening process based on a contaminant's potential to occur in public water systems (PWSs) and the potential for public health concerns
- Further detailed evaluations, public input, and expert judgment and review are the final contaminant selection tools
- Final CCL 3 published September 2009
  - 104 chemicals or chemical groups and 12 microbiological contaminants

Unregulated CCL 3 Contaminants
104 Chemicals and 12 Microbes

1,1,1,2-Tetrachloroethane
1,1-Dichloroethane
1,2,3-Trichloropropane
1,3-Butadiene
1,3-Dinitrobenzene
1,4-Dioxane
17 alpha-Estradiol
1-Butanol
2-Methoxyethanol
2-Propen-1-ol
3-Hydroxycarbofuran (degradate)
4,4'-Methylenedianiline
Acephate
Acetaldehyde
Acetamide
Acetochlor
Acetochlor ethanesulfonic acid (ESA)
Acetochlor oxanilic acid (OA)
Acrolein
Alachlor ethanesulfonic acid (ESA)
Alachlor oxanilic acid (OA)
alpha-Hexachlorocyclohexane (former)
Aniline
Bensulide
Benzyl chloride
Butylated hydroxyanisole
Captan
Chlorate (also D-DBP)
Chloromethane (Methyl chloride)
Clethodim
Cobalt
Cumene hydroperoxide
Cyanotoxins (3)
Dicrotophos
Dimethipin
Dimethoate
Disulfoton
Diuron
Equilenin
Equilin
Erythromycin
Estradiol (17-beta)
Estriol
Estrone
Ethinyl Estradiol (17-alpha)
Ethoprop
Ethylene glycol
Ethylene oxide
Ethylene thiourea
Fenamiphos
Formaldehyde (formerly)
Germanium
Halon 1011 (Bromochloromethane)
HCFC-22
Hexane
Hydrazine
Mestranol
Methamidophos
Methyl bromide (Bromomethane)
Methyl tert-butyl ether
Metolachlor
Metolachlor ethanesulfonic acid (ESA)
Metolachlor oxanilic acid (OA)
Molinate
Molybdenum
Nitrobenzene
Nitroglycerin
N-Methyl-2-pyrrolidone
N-Nitrosodiethylamine (NDEA)
N-nitrosodimethylamine (NDMA)
N-Nitroso-di-n-propylamine (NDPA)
N-Nitrosodiphenylamine
N-nitrosopyrrolidine (NPYR)
Norethindrone (19-Norethisterone)
n-Propylbenzene
o-Toluidine
Oxirane, methyl-
Oxydemeton-methyl
Oxyfluorfen
Perchlorate
Perfluorooctane sulfonic acid (PFOS)
Perfluorooctanoic acid (PFOA)
Permethrin
Profenofos
Quinoline
RDX
sec-Butylbenzene
Strontium
Tebuconazole
Tebufenozide
Tellurium
Terbufos
Terbufos sulfone
Thiodicarb
Thiophanate-methyl
Toluene diisocyanate
Tribufos
Triethylamine
Triphenyltin hydroxide (TPTH)
Urethane
Vanadium
Vinclozolin
Ziram

Adenovirus
Caliciviruses
Campylobacter jejuni
Enterovirus
Escherichia coli (0157)
Helicobacter pylori
Hepatitis A virus
Legionella pneumophila
Mycobacterium avium
Naegleria fowleri
Salmonella enterica
Shigella sonnei

UCMR 3

- Final rule published May 2, 2012
- *http://water.epa.gov/lawsregs/rulesregs/sdwa/ucmr/ucmr3/index.cfm*
- Monitoring will occur from 2013-15
- 28 chemicals and 2 viruses
- Contaminants include hormones, perfluorinated compounds (e.g., PFOS/PFOA), VOCs, metals (including Cr-6 and total Cr), 1,4-dioxane, chlorate and pathogens

UCMR 3 – Monitoring Required

- Assessment monitoring (List 1)
  - All systems serving >10,000 people
  - 800 representative systems serving ≤10,000 people
- Screening Survey (List 2)
  - All systems serving >100,000 people
  - 320 representative systems serving 10,001 to 100,000 people
  - 480 representative systems serving ≤10,000 people

UCMR 3 – Monitoring Required (cont.)

- Pre-Screen Testing (List 3)
  - Selected 800 systems serving ≤1000 people that do not disinfect. Systems with wells that are located in areas of karst or fractured bedrock
- EPA pays for analysis of all samples from systems serving ≤10,000 and arranges for collection of List 3 samples

UCMR 3 – Contaminants:
Assessment Monitoring ("List 1")

- Perfluorinated Chemicals (EPA Method 537)
  - perfluorooctanonic acid (PFOA)
  - perfluorooctane sulfonic acid (PFOS)
  - perfluoroheptanoic acid (PFHpA)
  - perfluorononanoic acid (PFNA)
  - perfluorobutane sulfonic acid (PFBS)
  - perfluorohexane sulfonic acid (PFHxS)

- Metals (EPA Method 200.8)
  - cobalt
  - molybdenum
  - strontium
  - vanadium
  - (total) chromium

•EPA Method 218.7
-hexavalent chromium

• Volatile Organic Compounds (EPA Method 524.3)
- 1,1-dichloroethane
- 1,2,3-trichloropropane
- 1,3-butadiene
- bromochloromethane
- chlorodifluoromethane
- chloromethane
- methyl bromide

• EPA Method 522
-1,4-dioxane

• EPA Method 300.1
-chlorate

UCMR 3 – Contaminants:
Screening Survey ("List 2") and Pre-Screen Testing ("List 3")

Hormones (EPA Method 539) – *List 2*

-17-α-ethynylestradiol
- 17-β-estradiol
- equilin
- estriol
- estrone
- testosterone
- 4-androstene-3,17-dion

Viruses – *List 3*

- enterovirus (qPCR & cell culture)
- norovirus (qPCR)
- "Indicator organisms"
  - Total coliform
  - *E. coli*
  - enterococci
  - coliphage
  - aerobic spores

Unregulated CCL 3 Contaminants
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Bensulide
Benzyl chloride
Butylated hydroxyanisole
Captan
Chlorate (also D-DBP)
Chloromethane (Methyl chloride)
Clethodim
Cobalt
Cumene hydroperoxide
Cyanotoxins (3)
Dicrotophos
Dimethipin
Dimethoate
Disulfoton
Diuron
Equilenin
Equilin
Erythromycin
Estradiol (17-beta)
Estriol
Estrone
Ethinyl Estradiol (17-alpha)
Ethoprop
Ethylene glycol
Ethylene oxide
Ethylene thiourea
Fenamiphos
Formaldehyde (formerly)
Germanium
Halon 1011 (Bromochloromethane)
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n-Propylbenzene
o-Toluidine
Oxirane, methyl
Oxydemeton-methyl
Oxyfluorfen
Perchlorate
Perfluorooctane sulfonic acid (PFOS)
Perfluorooctanoic acid (PFOA)
Permethrin
Profenofos
Quinoline
RDX
sec-Butylbenzene
Strontium
Tebuconazole
Tebufenozide
Tellurium
Terbufos
Terbufos sulfone
Thiodicarb
Thiophanate-methyl
Toluene diisocyanate
Tribufos
Triethylamine
Triphenyltin hydroxide (TPTH)
Urethane
Vanadium
Vinclozolin
Ziram

Adenovirus
Caliciviruses
Campylobacter jejuni
Enterovirus
Escherichia coli (0157)
Helicobacter pylori
Hepatitis A virus
Legionella pneumophila
Mycobacterium avium
Naegleria fowleri
Salmonella enterica
Shigella sonnei

Red = No EPA Method

Blue = Current Method Development

Contaminant Candidate List 4 (CCL 4)

- Spring 2012 - Published FR notice requesting nominations of contaminants to be considered for inclusion on CCL 4
- Summary of Nominations:
  - 59 unique contaminants were nominated by 10 organizations and individuals
    - 5 microbes and 54 chemicals
    - 8 contaminants were nominated more than once
  - EPA is currently evaluating the new data provided for nominated chemicals and microbes, to determine the appropriateness of inclusion on the CCL 4
    - The nomination letters and web site submittals can be found in the CCL 4 docket (EPA-HQ-OW-2012-0217) at www.regulations.gov

Current Status

- 2013 - Expect to publish Draft CCL 4 for public review and comment
- 2014 - Expect to publish Final CCL 4

Method Development Challenges

- Lack of suitable standards, internal standards and surrogate compounds
- Compounds with extremely high water solubility
- Low detection limits are often required
- Timeframe imposed by continual CCL/UCMR cycle
- Lack of previous method development work

EPA United States Environmental Protection Agency

CCL/UCMR Methods Webinar -2013

Development of LC/MS/MS Methods for the Analysis of Chemicals on U.S. EPA's Contaminant Candidate List

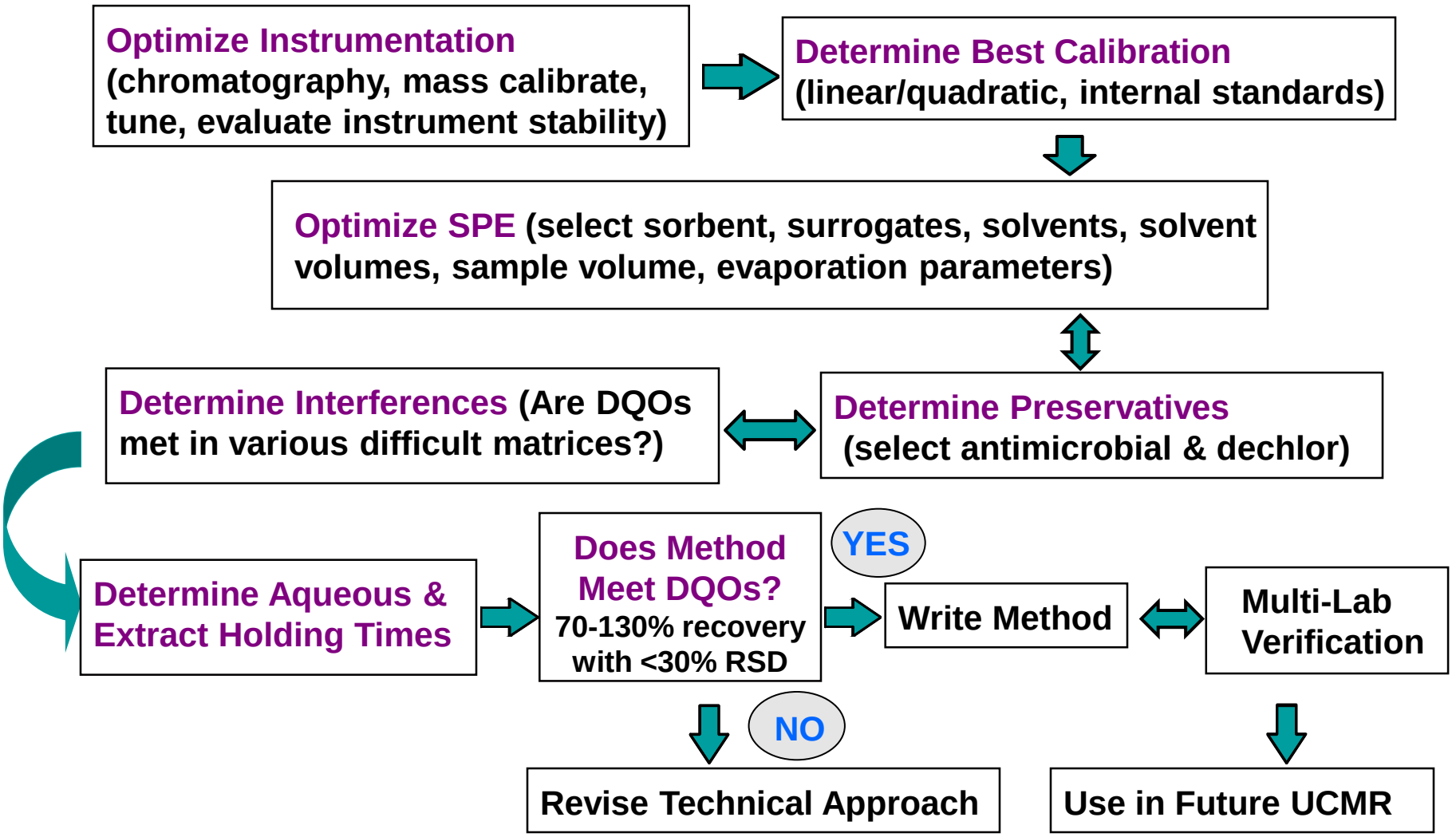
Jody A. Shoemaker and Daniel R. Tettenhorst

Disclaimer: *Mention of trade names or commercial products does not constitute endorsement or recommendation for use.*

Office of Research and Development
National Exposure Research Laboratory

EPA United States Environmental Protection Agency

Method Development Process



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ORD/NERL LC/MS/MS Methods

❖ LC/MS/MS Drinking Water Methods

- ✓ Method 535 – 12 acetanilide degradates (6 CCL)-**UCMR 2**
- ✓ Method 537 – 14 perfluorinated alkyl acids (2 CCL)-**UCMR 3**
- ✓ Method 538 – 11 chemicals (4 CCL)

http://www.epa.gov/nerlcwww/ordmeth.htm

❖ LC/MS/MS Methods Under Development

- ✓ Method 540 – 12 pesticides (5 CCL)
- ✓ Method 543 – 8 pesticides (6 CCL)
- ✓ Method 544 – 8 cyanotoxins (1 CCL)

Method 538: DAI-LC/MS/MS Contains 11 Analytes

Method Analytes

acephate	aldicarb
dicrotophos	aldicarb sulfone
methamidophos	diisopropyl methylphosphonate (DIMP)
oxydemeton-methyl	fenamiphos sulfone
quinoline	fenamiphos sulfoxide
	thiofanox

Analytes in red are on CCL 3

- Most method analytes are pesticides (except for quinoline and DIMP) with the potential to contaminate drinking water sources
- Quinoline is an industrial starting material, a pharmaceutical (anti-malarial) and a flavoring agent
- DIMP is a chemical by-product in the production of sarin gas

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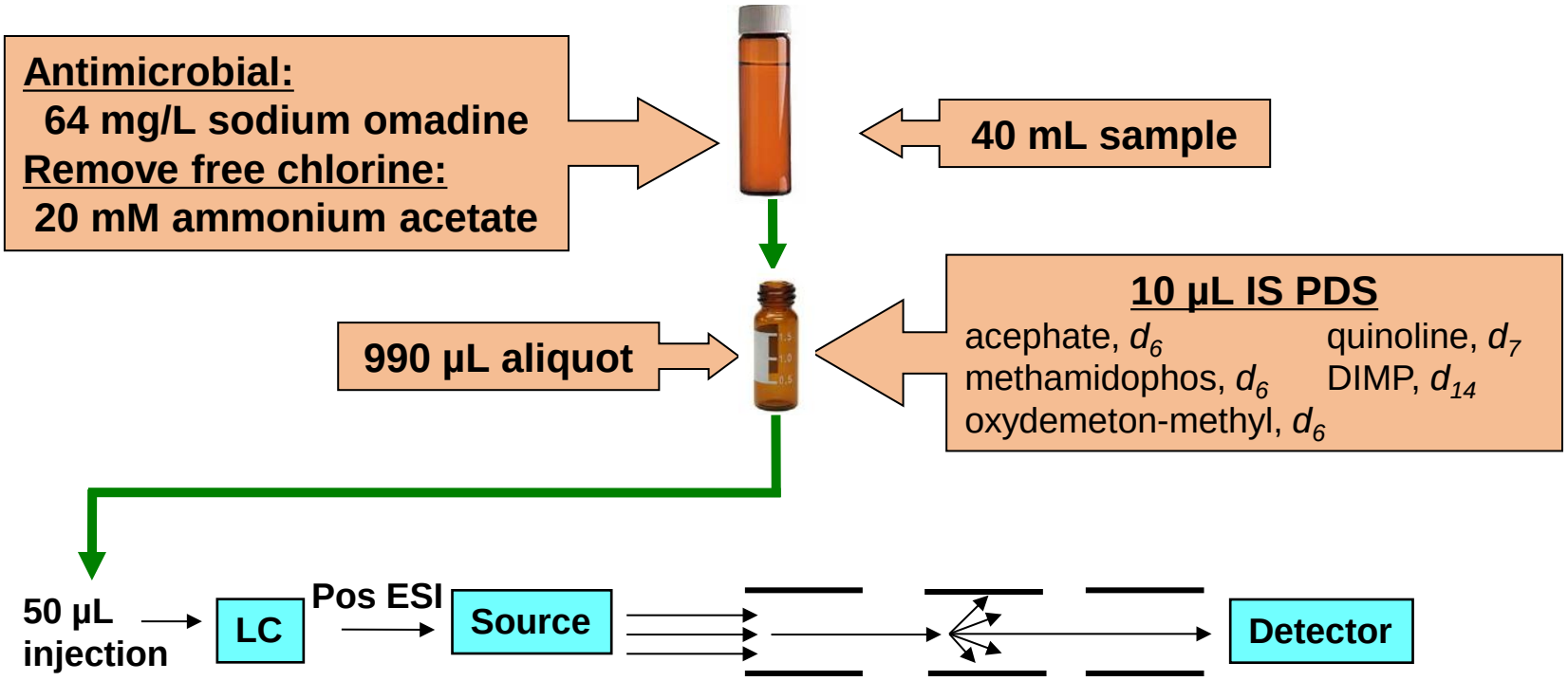
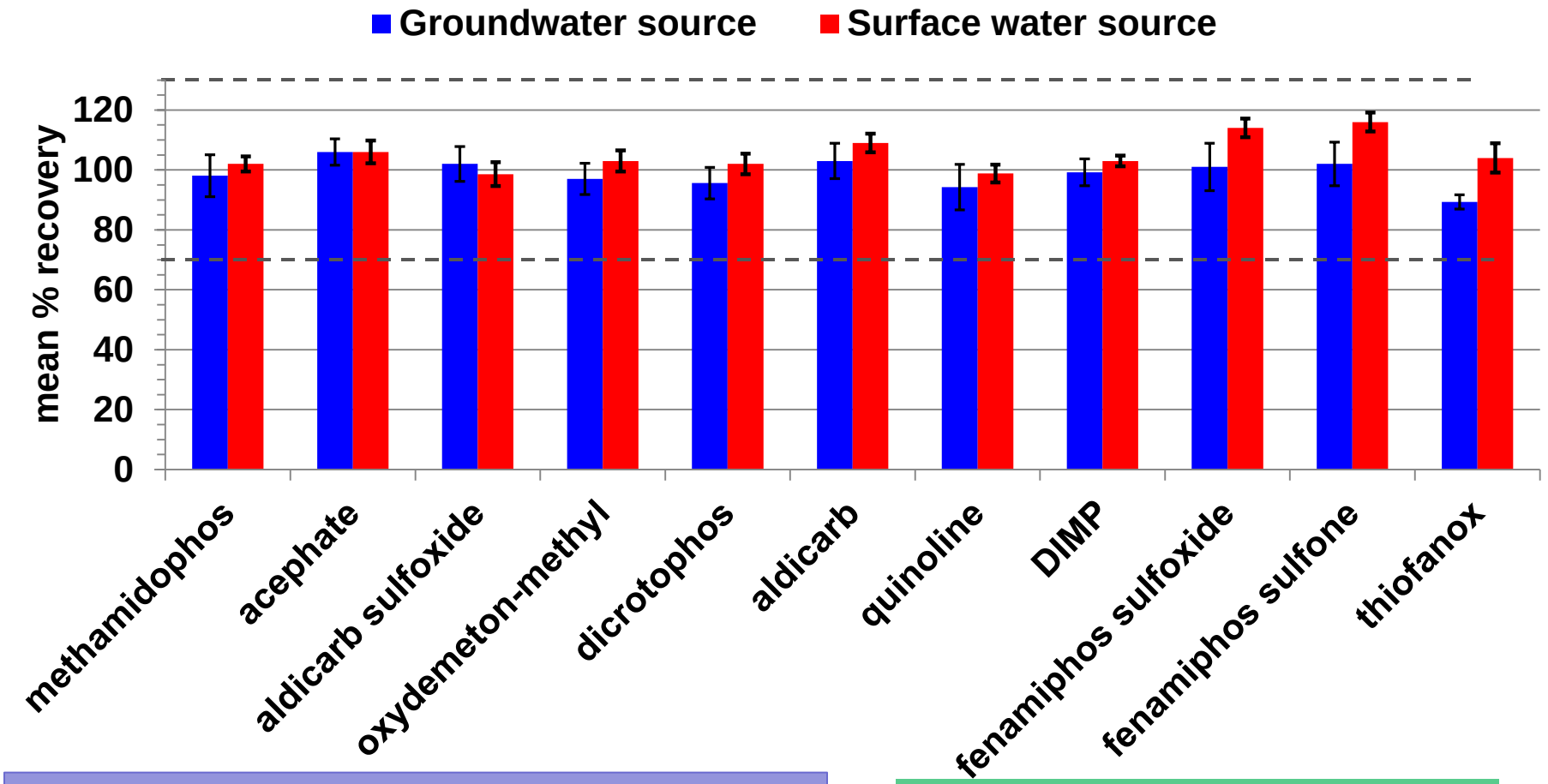
M538
Direct Aqueous Injection Approach



Selected Reaction Monitoring

M538 Performance Data

Fortified at 0.99 – 43 µg/L (n=7)



LCMRL range: 11-180 ng/L (quinoline = 1.5 µg/L)

HRLs: 250 ng/L – 175 µg/L (quinoline 10 ng/L)

Method 540: SPE-LC/MS/MS Contains 12 Analytes

Method Analytes on CCL 3

3-hydroxycarbofuran	fenamiphos sulfone
fenamiphos	fenamiphos sulfoxide
bensulide	methomyl
tebuconazole	chlorpryifos oxon
tebufenozide	phorate sulfone
disulfoton sulfoxide	phorate sulfoxide

Analytes in red are on CCL 3

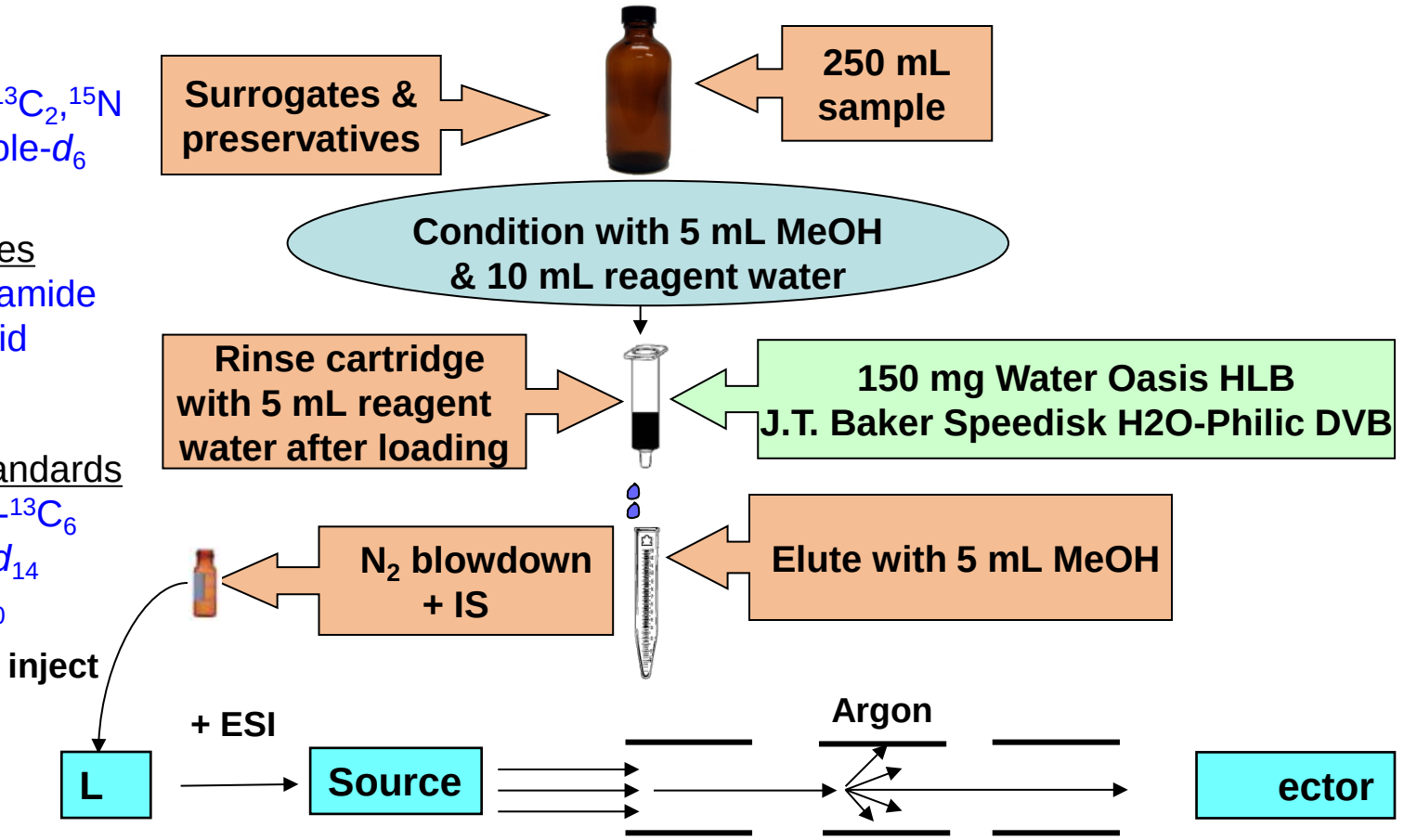
- all method analytes are pesticides or pesticide degradates with the potential to contaminate drinking water sources

Method 540 Analytical Procedure

Surrogates
methomyl-$^{13}C_2$,^{15}N
tebuconazole-d_6

Preservatives
2-chloracetamide
ascorbic acid
Trizma

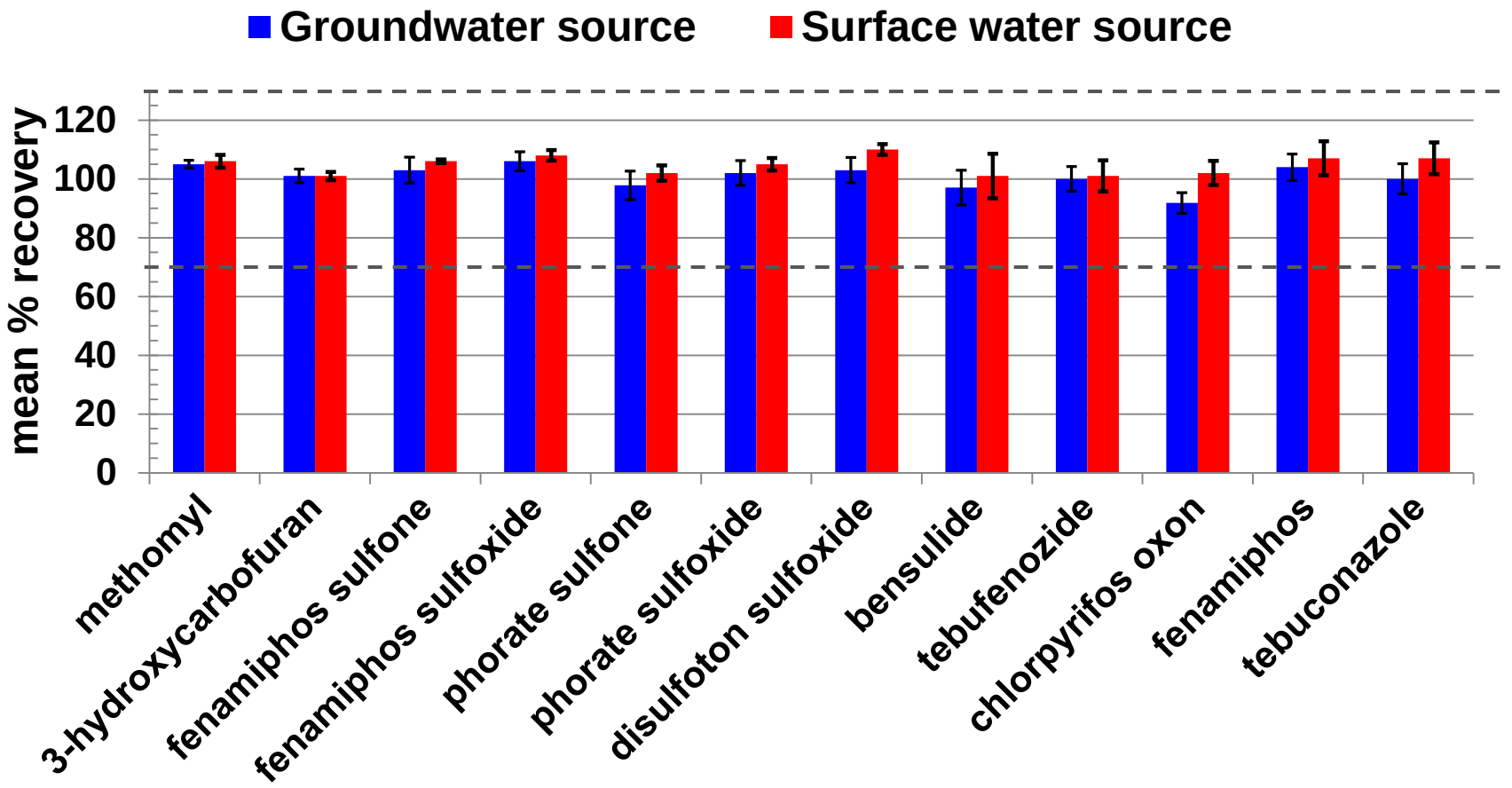
Internal Standards
carbofuran-$^{13}C_6$
bensulide-d_{14}
phorate-d_{10}



LC/MS/MS - Selected Reaction Monitoring

M540 Performance Data

Fortified at 12.8 – 32 ng/L (n=4)



LCMRL range: 0.64 – 2.0 ng/L

HRLs: 420 ng/L – 210 µg/L

Method 543: On-line SPE-LC/MS/MS Contains 8 Analytes

Method Analytes on CCL 3

3-hydroxycarbofuran
fenamiphos
bensulide
quinoline
tebuconazole
tebufenozide
fenamiphos sulfone
fenamiphos sulfoxide

Analytes in red are on CCL 3

- **all method analytes are pesticides or pesticide degradates (except quinoline) with the potential to contaminate drinking water sources**
- **concentration, elution, separation all done by automation**
- **1-5 mL sample volume typical**
- **analysis time/sample is <20 min**
- **high throughput**

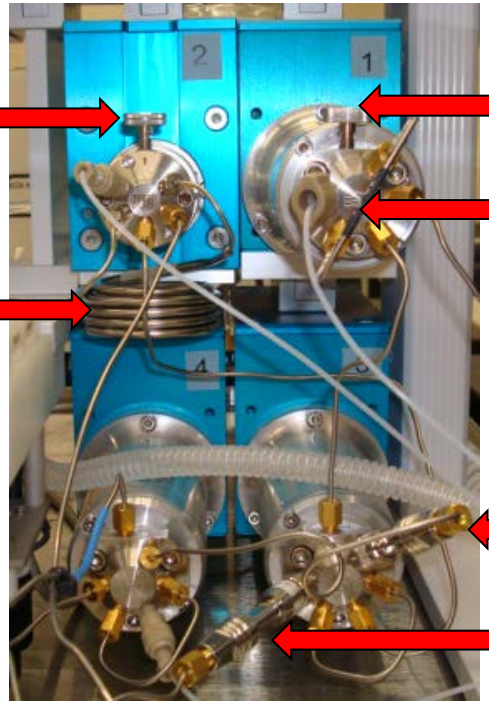
Method 543 Preliminary Procedure

IS(s) & preservatives: potassium citrate ascorbic acid

20 mL sample vial

SPE injection port
2.5 mL injections

SPE 5-mL sample loop



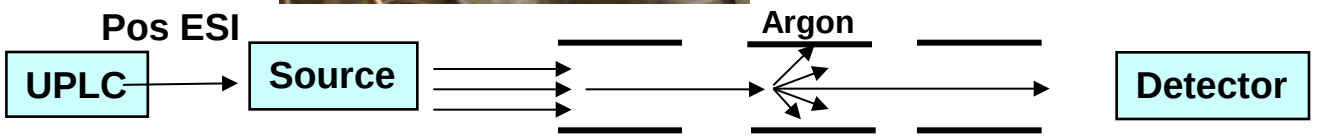
UPLC injection port
1-10 µL range

Analytical sample loop
10 µL

SPE cartridge 1
Oasis HLB

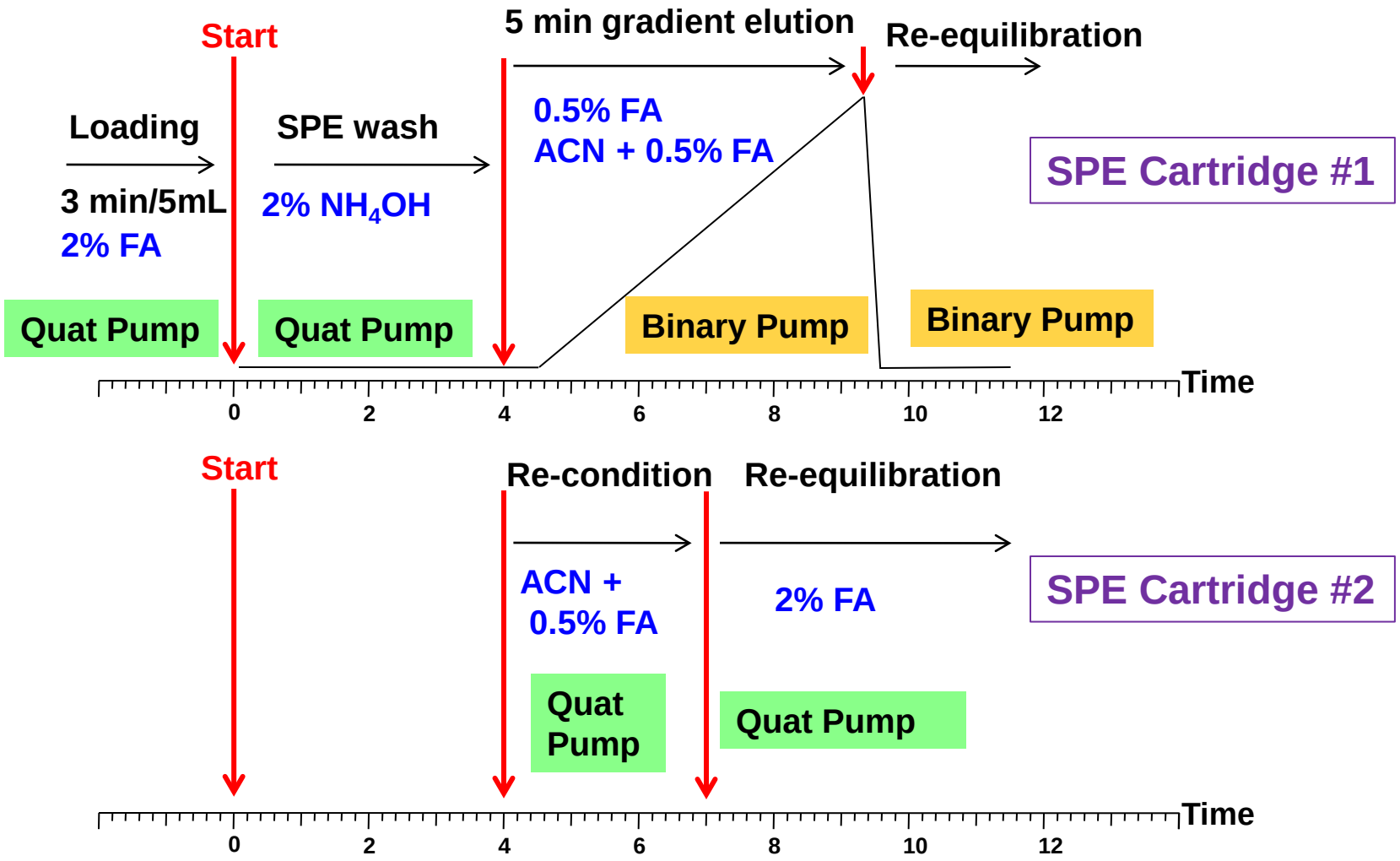
SPE cartridge 2
Oasis HLB

Internal Standards
- quinoline-d_7
- carbofuran-$^{13}C_6$
- tebuconazole-d_6
- bensulide-d_{14}

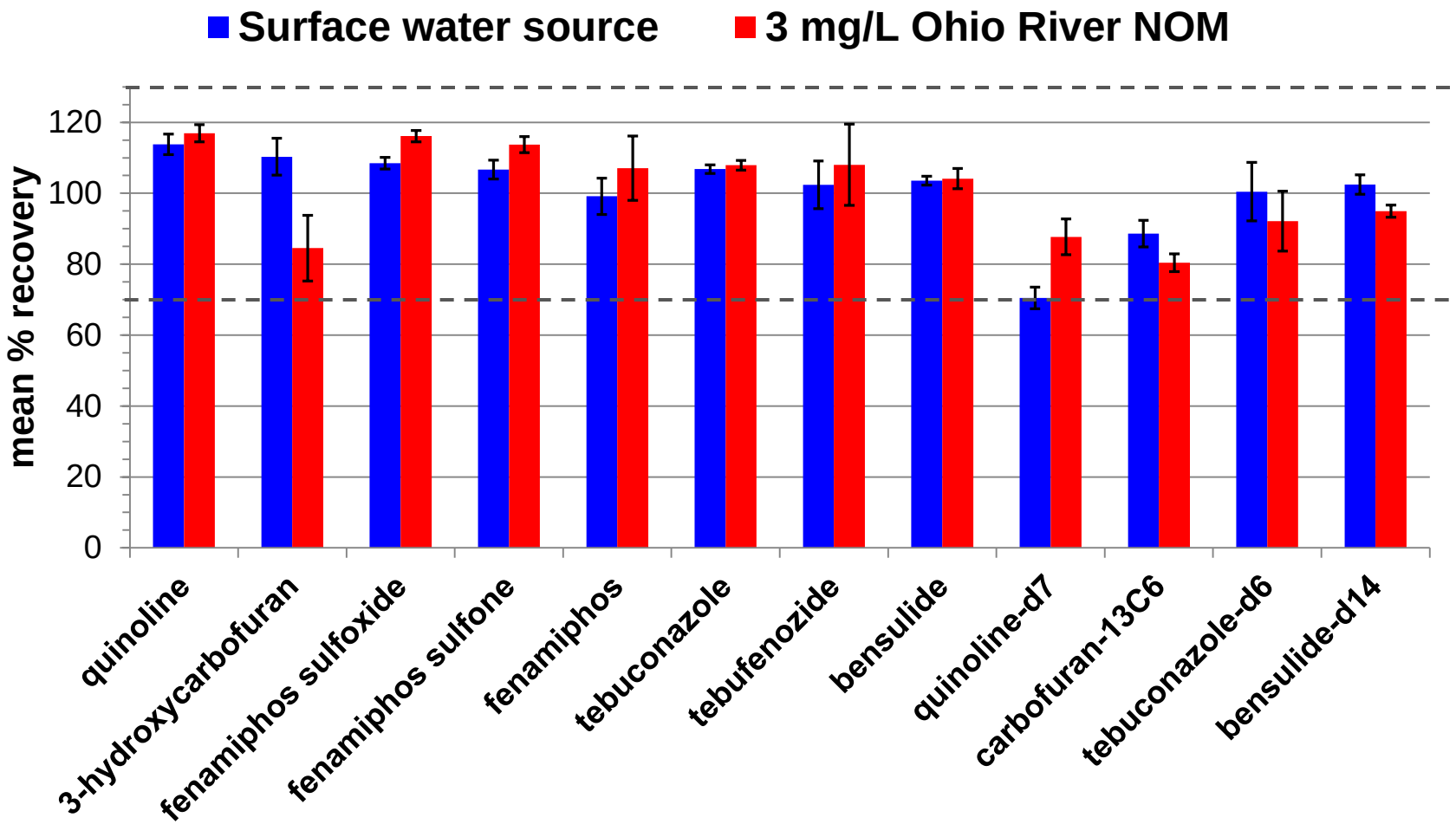


LC/MS/MS - Selected Reaction Monitoring

Method 543: On-line SPE Events



On-line SPE-LC/MS/MS Preliminary Performance Data
Fortified at 4 – 10 ng/L (n=3)



LCMRL range: TBD

HRLs: 10 ng/L – 210 µg/L

Method 544: SPE-LC/MS/MS Potentially 8 Cyanotoxins

Method Analytes

MC-LR	**MC-RR**
MC-YR	**MC-LA**
MC-LW	**MC-LF**
MC-LY	**Nodularin**

- widespread occurrence of blooms
- potential for occurrence in finished drinking water
- toxic – HRL is 21 ng/L for MC-LR
- unique problems in development of robust analytical methods – availability of multiple sources of high purity standards, lack of isotopically labeled IS and SUR, matrix interferences

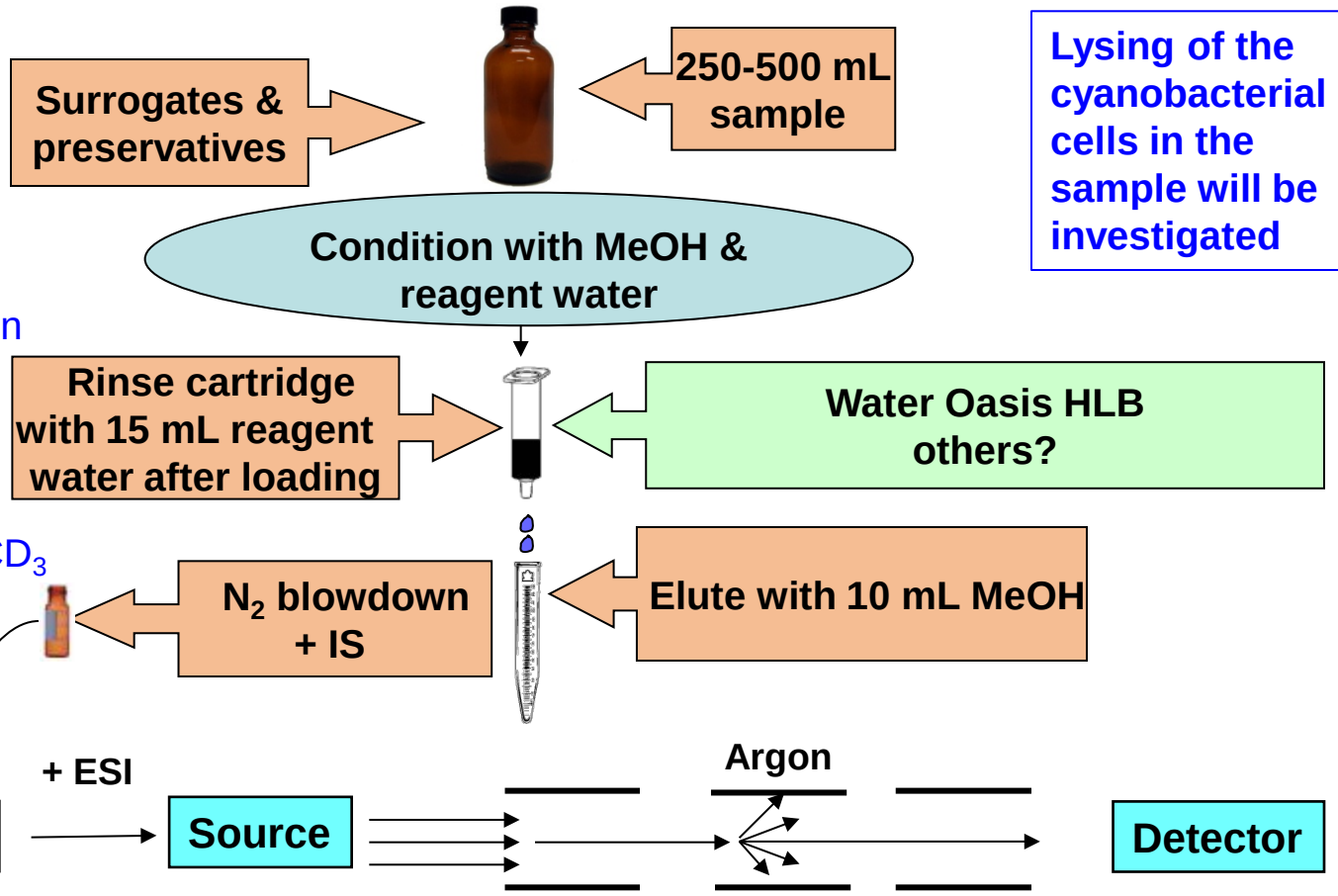
Analytes in red are on CCL 3

Preliminary
Method 544 Analytical Procedure

Preservatives
2-chloracetamide
ascorbic acid
Trizma

Surrogates
TBD
e.g., leucine-enkephalin
cyclo-RADFV

Internal Standards
TBD
Current : Me-MC-LR, CD_3

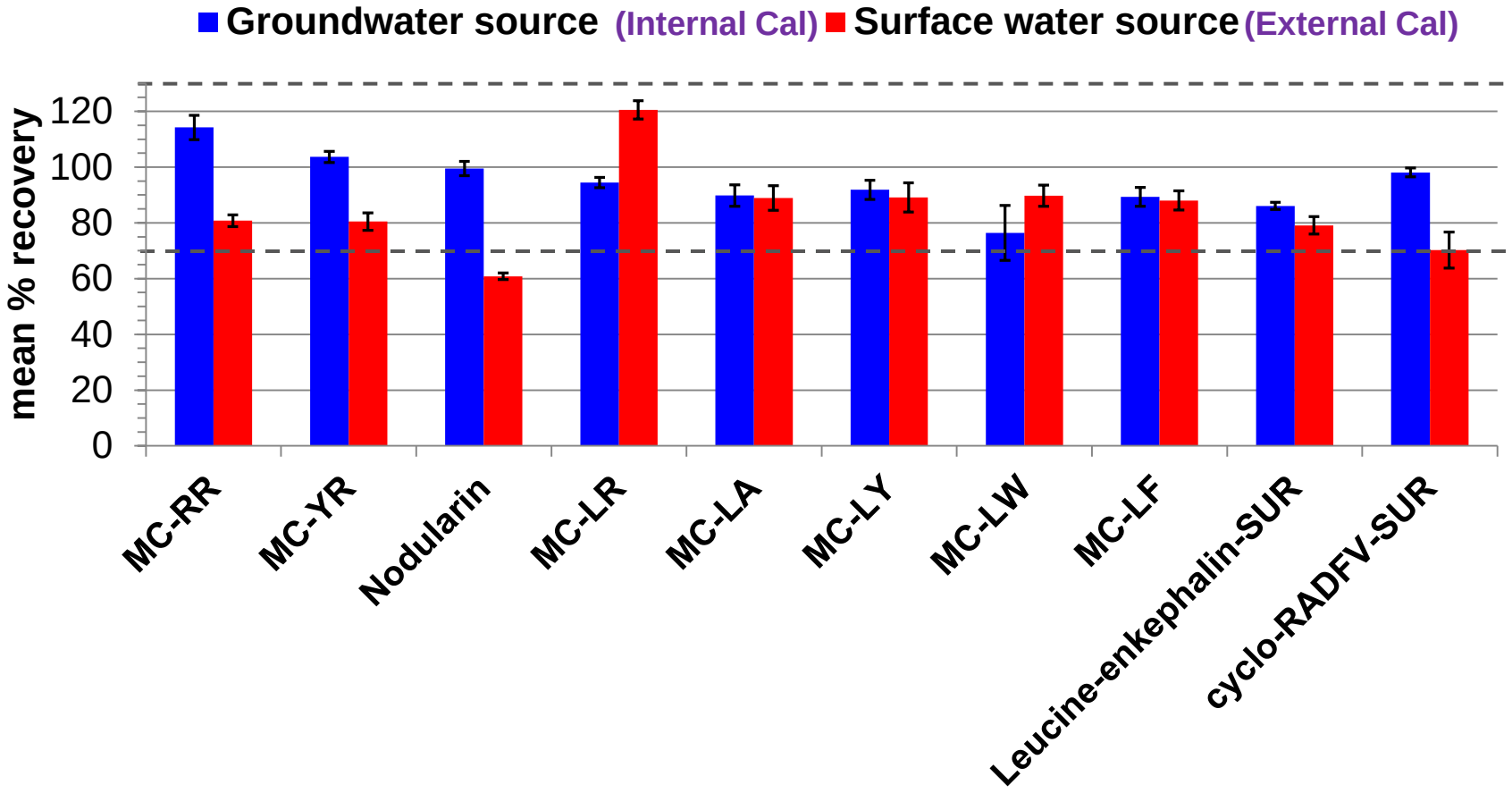


Lysing of the cyanobacterial cells in the sample will be investigated

LC/MS/MS - Selected Reaction Monitoring

Method 544 Preliminary Performance Data

Fortified at 200 – 400 ng/L
except MC-LA at 1000 ng/L (n=3)



LCMRL range: TBD

HRL MC-LR: 21 ng/L

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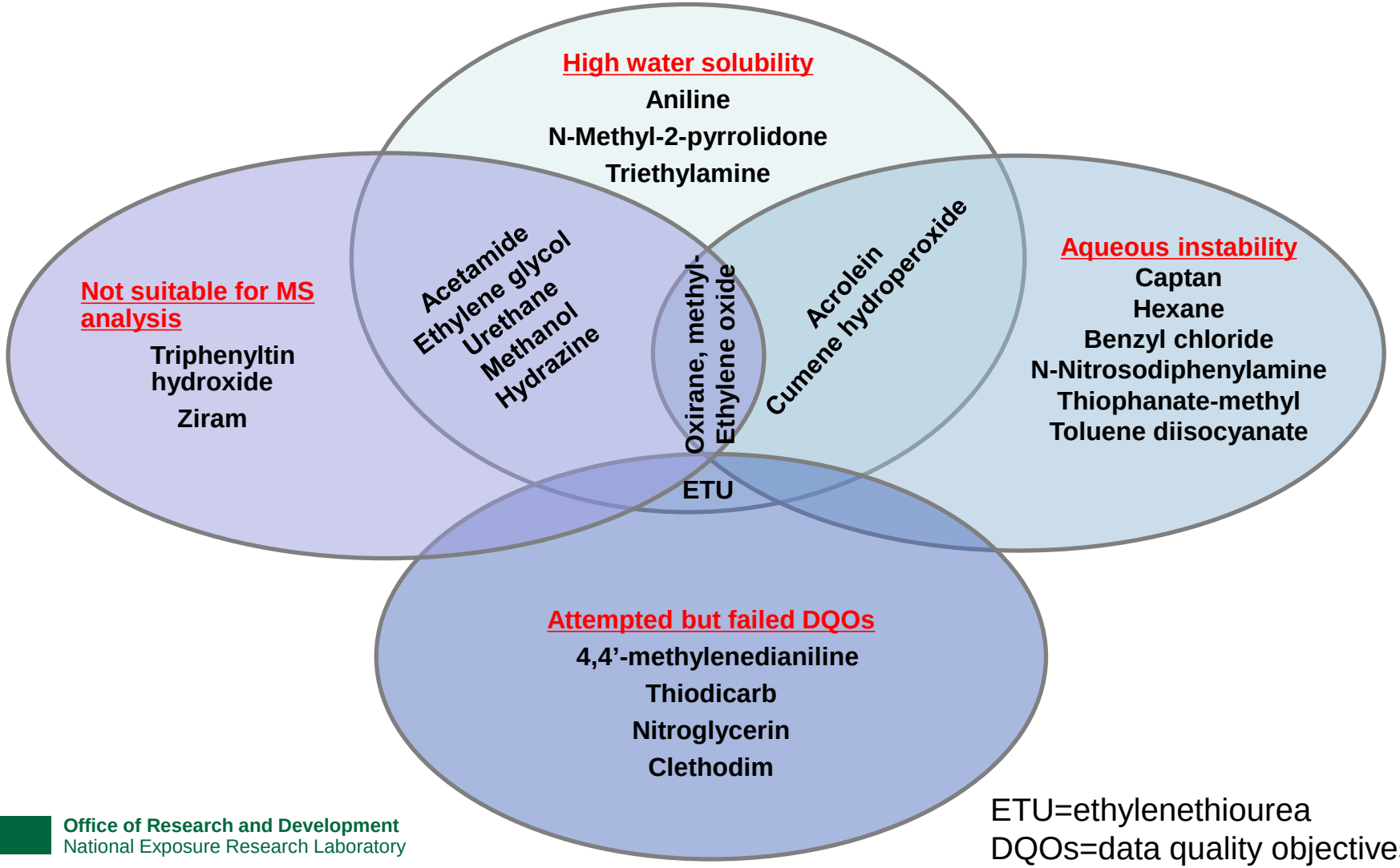
Summary of ORD/NERL LC/MS/MS Methods

- **2 LC/MS/MS methods completed and ready for potential use in UCMR 4 – total of 9 CCL 3 chemicals**
  - **Method 538**
  - **Method 540**

- **2 methods under development for potential use in UCMR 4 – one additional CCL 3 analyte**
  - **Method 543 (optional method for Method 540 analytes)**
  - **Method 544 (MC-LR)**

Remaining CCL 3 Chemicals – Challenges
(as of May 2013)



ETU=ethylenethiourea
DQOs=data quality objectives

EPA
United States
Environmental Protection
Agency

Drinking Water Methods Development by Gas Chromatography-Mass Spectrometry (GC-MS)

Paul E. Grimmett



Office of Research and Development
National Exposure Research Laboratory, 26 W. Martin Luther King Dr., Cincinnati, OH 45268

United States Environmental Protection Agency

Background

- The Safe Drinking Water Act, as amended in 1996, requires EPA to execute a program that identifies, monitors and considers for regulation new and emerging chemicals that may pose a risk to drinking water consumers.
- EPA notifies the public of chemicals it is investigating by publishing the drinking water Contaminant Candidate List (CCL).
- In order for EPA's Office of Water to evaluate CCL chemicals for regulation, it is vital that nationwide occurrence data for these chemicals in drinking water are obtained.
- Nationwide occurrence data are obtained through Unregulated Contaminant Monitoring (UCM).
- Rugged, accurate, and sensitive methods are needed for UCM.

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Method Development Goals

- Determine appropriate surrogates and internal standards
- Establish Data Quality Objectives (DQOs): normally 70-130% recovery with <30% RSD
- Preservation scheme to allow for sample holding: antimicrobial, dechlorinating agent, and pH buffer
- Establish sample and extract holding times – ideally ≥14 days
- Establish detection limits (DLs) and lowest concentration minimum reporting levels (LCMRLs) below health reference levels (HRLs)

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Recently Published GC Methods by ORD-NERL

1. **Method 522: 1,4-dioxane**
   - SPE on coconut carbon sorbent, followed by GC/MS
   - allows for selected ion monitoring (SIM)
   - two sampling/extraction options (100 mL and 500 mL samples)
   - method currently used for occurrence data in UCMR 3 (2013-2015)

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Recently Published GC Methods by ORD-NERL (cont.)

2. Method 525.3: Semi-volatiles

- published in February 2012
- approx. 130 analytes (pesticides, herbicides, PCBs, PAHs, etc)
- includes 16 CCL 3 compounds and 17 regulated contaminants
- SPE on polymeric sorbent (DVB), followed by GC/MS
- improved preservation scheme, updated surrogates and internal standards, addition of SIM option, and new PCB screening procedure

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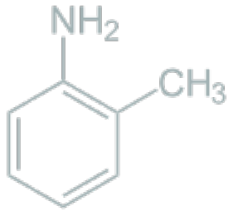
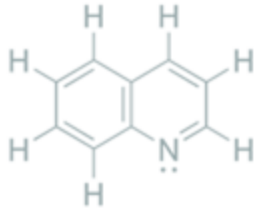
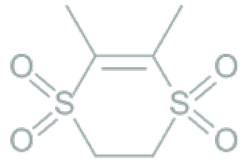


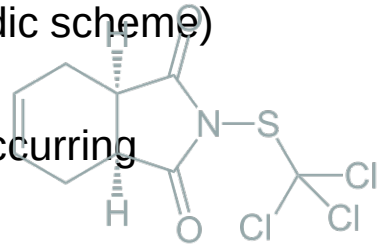


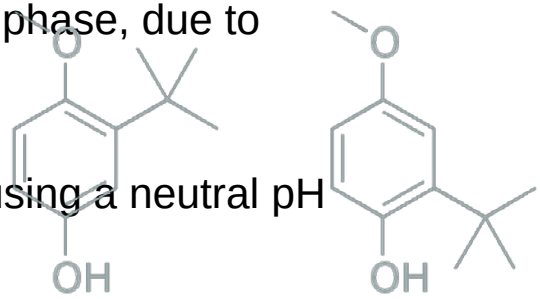
Current Work



Development of drinking water method for *o*-toluidine, quinoline, dimethipin, captan, and butylated hydroxyanisole (BHA)

- *o*-Toluidine and quinoline (both industrial precursors) have basic structures, which led to poor sorbent recovery using Method 525.3 preservatives (acidic scheme)
- BHA (a food and packaging preservative) suffered from randomly occurring extraction inefficiency and was removed from Method 525.3
- Dimethipin was included in EPA Method 525.3, but with limited sorbent options
- Captan was removed from Method 525.3 in the evaluation phase, due to hydrolysis (half life in water is less than 1 day)
- All 5 compounds are being evaluated for an EPA method using a neutral pH preservation scheme





Current SPE Extraction Technique - Cartridges

United States Environmental Protection Agency

Add surrogates:
o-toluidine-d_9
quinoline-d_7

Add preservatives:
ascorbic acid
Trizma buffer (pH 7.0)
EDTA
DZU

Rinse & condition:
methylene chloride
methanol
water

Multiple hydrophilic-modified sorbent options

Add 1 L water sample extract at ~10 mL/min
Rinse with RW

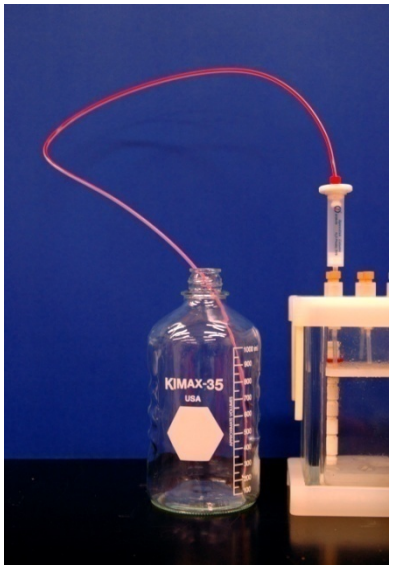
Dry cartridge for 10 min

Elute with methylene chloride

Add IS(s):
acenaphthene-d_{10}
phenanthrene-d_{10}
chrysene-d_{12}

Dry and concentrate extract to 1 mL

GC/MS:
Full scan option or
SIM option



GC/MS Full Scan Analysis Specs

Column: Restek RXI-5sil-MS and J&W DB-1701
30 m x 0.25 mm x 0.25 µm column

Injector: 275 ˚ C (splitless mode), 20 psi pulse

Inj. vol: 1 µL

Flow: 1 mL/min, helium carrier gas

Oven: 60 ˚ C for 1 min, to 300 ˚ C at 10 ˚ C/min, hold 2 min. Total time = 27 min.

MS Transfer Line: 275 ˚ C

MS: scan 50-350 amu, full scan

GC/MS SIM Analysis Specs (Preliminary)

Column: Restek RXI-5sil-MS

Injector: 275 ° C (splitless mode), 20 psi pulse

Inj. vol: 1 µL

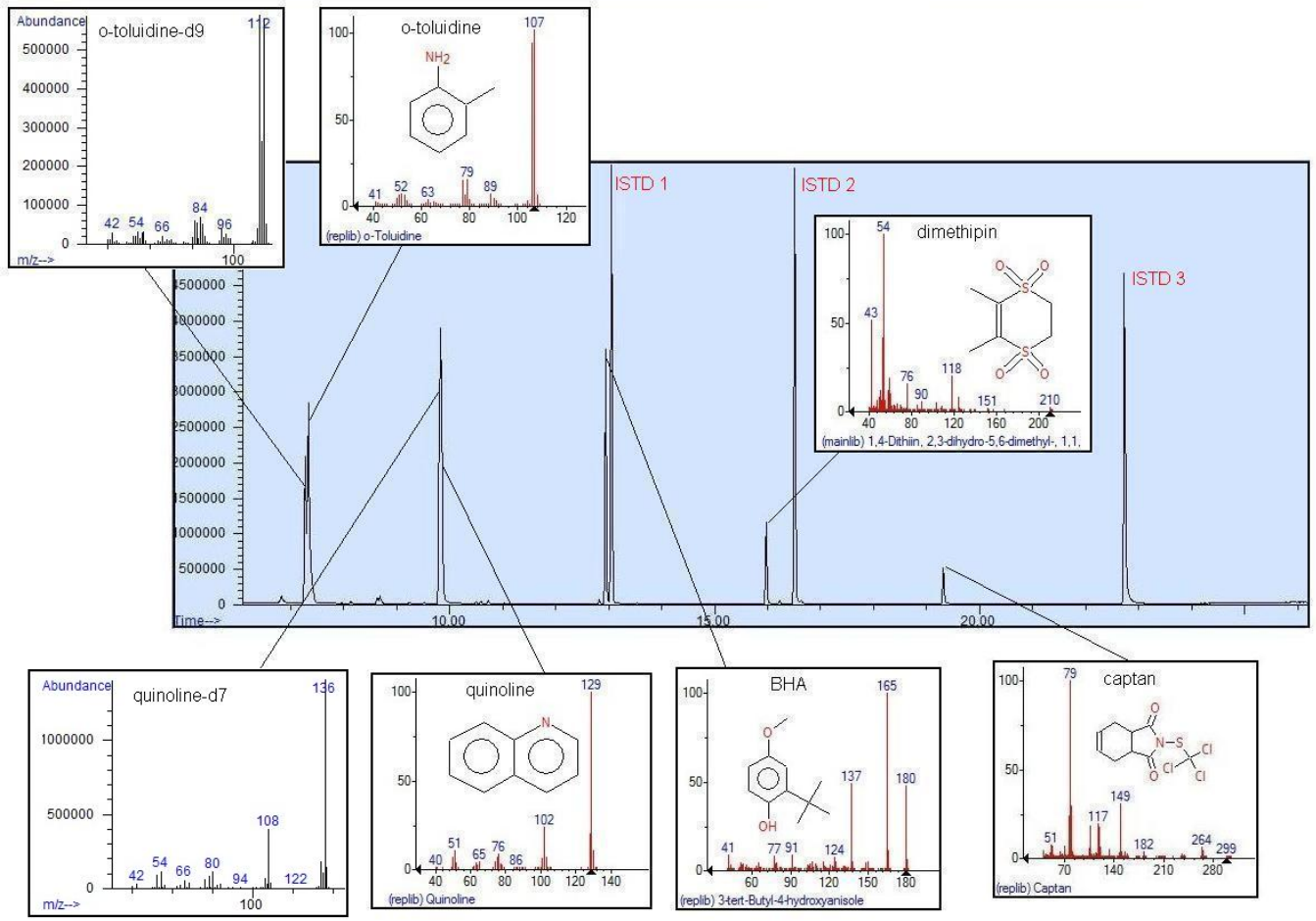
Flow: 1 mL/min, helium carrier gas

Oven: 60 ° C for 1 min, to 300 ° C at 10 ° C/min, hold 2 min. Total time = 27 min.

MS Transfer Line: 275 ° C

MS SIM: 6 windows (amu, dwell time)
WIN1, 5.00 min: (106.10, 25) (107.10, 25) (112.10, 25)(114.10, 25)
WIN2, 7.64 min: (102.00, 25) (108.10, 25) (129.10, 25)(136.10, 25)
WIN3, 10.62 min: (137.10, 25) (162.10, 25) (164.10, 25)(180.10, 25)
WIN4, 13.76 min: (54.10, 75) (118.00, 75) (188.10, 25)
WIN5, 17.16 min: (79.00, 75) (149.00, 75)
WIN6, 20.37 min: (236.20, 25) (240.20, 25)

Sample Total Ion Chromatogram (TIC) with Mass Spectra, 5 µg/mL standard



Internal Standards acenaphthene-d_{10}, phenanthrene-d_{10}, and chrysene-d_{12} labeled as ISTD 1, ISTD 2, and ISTD 3, respectively.

United States Environmental Protection Agency

Quality Control Data at Various Concentrations, Drinking Water Matrices

- Three different water matrices were fortified with CCL 3 compounds of interest, then extracted and analyzed.

  1. Drinking water – surface water source

  2. Drinking water – groundwater source (hardness ~ 350 mg/L)

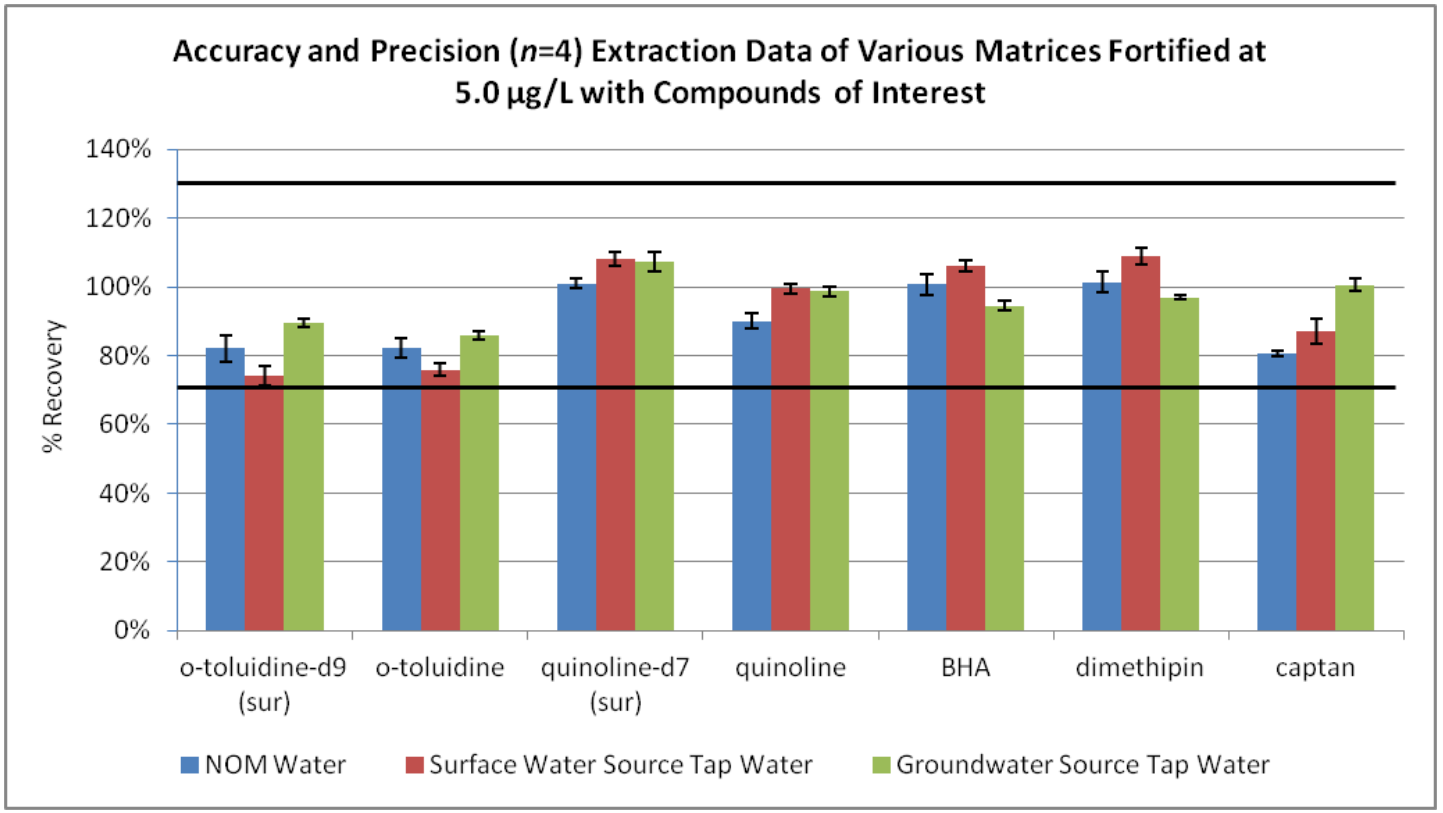
  3. Natural Organic Material NOM water – lab water spiked with concentrated organic matter from the Ohio River (TOC @ 1.8-2.2 mg/L)

- Fortification performed at 5 µg/L, 1 µg/L, and 0.1 µg/L.

- Each matrix was extracted in 4x replicates

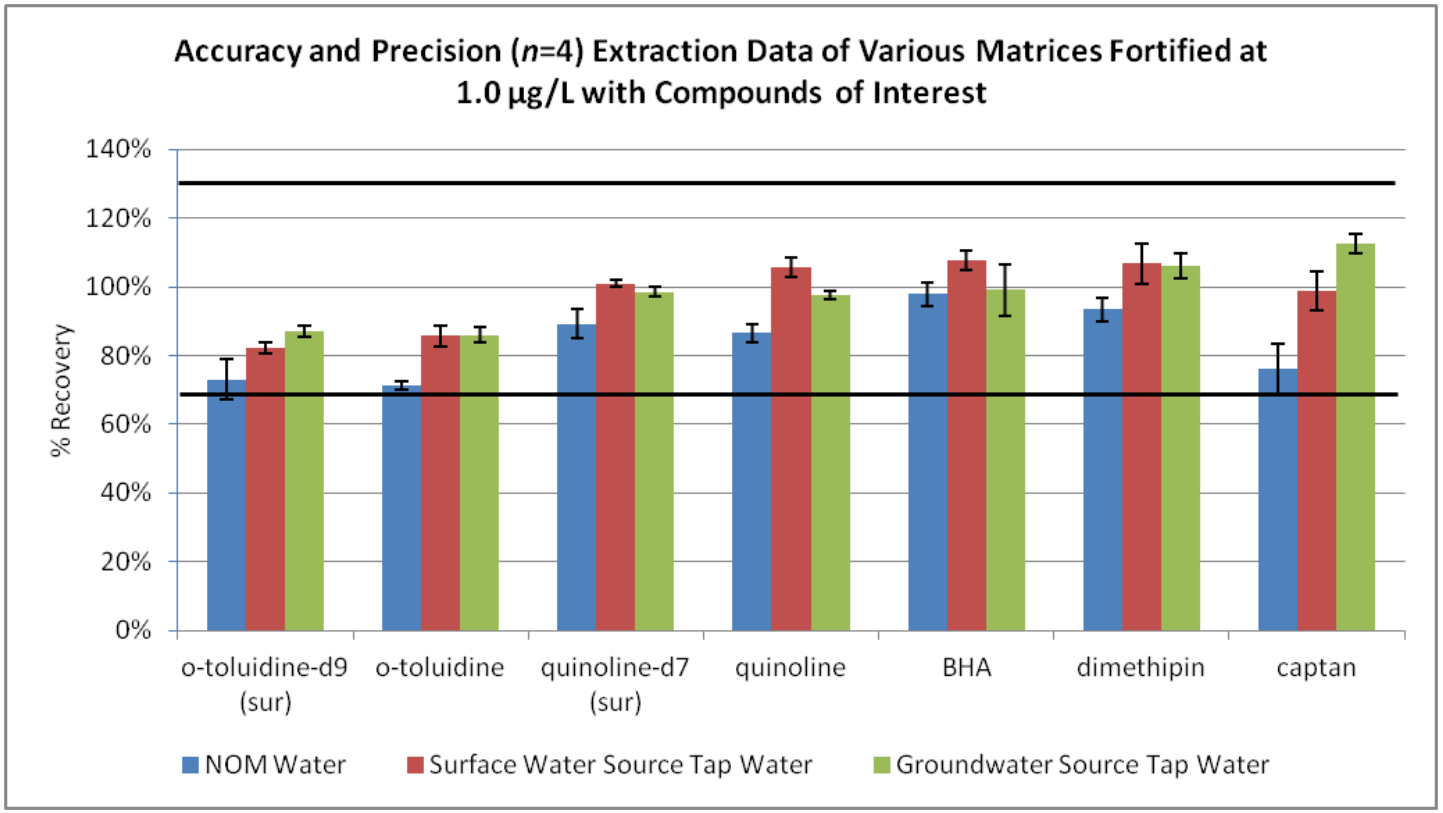
United States Environmental Protection Agency

Quality Control Data from Matrix Extracts at High Level Fortification (Full Scan GC/MS)



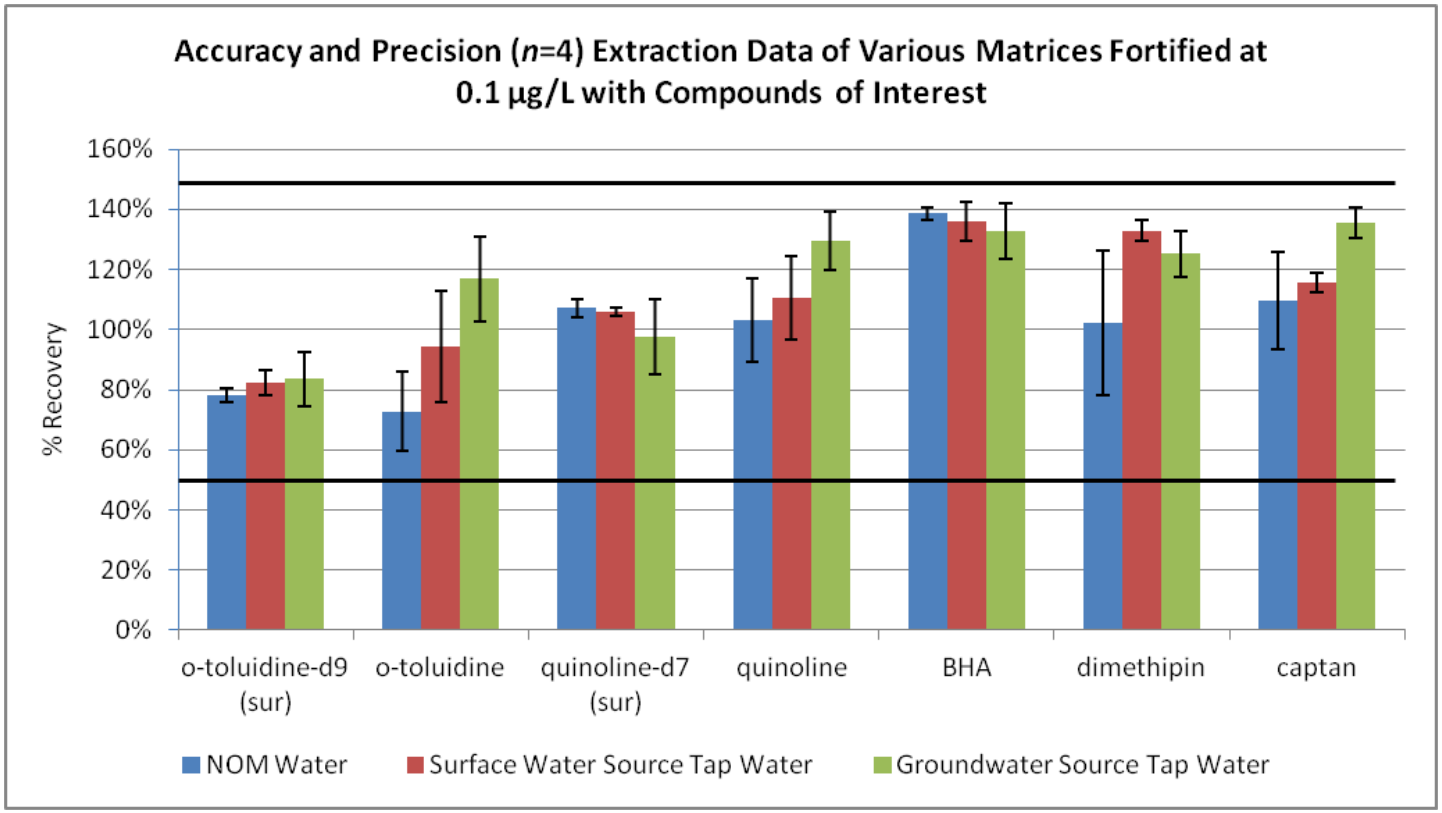
Lower and upper limit bars are set at 70% and 130%, respectively.

Quality Control Data from Matrix Extracts at Mid Level Fortification (Full Scan GC/MS)



Lower and upper limit bars are set at 70% and 130%, respectively.

Quality Control Data from Matrix Extracts at Low Level Fortification (Full Scan GC/MS)



Lower and upper limit bars are set at 50% and 150%, respectively.

United States Environmental Protection Agency

Remaining Laboratory Work with Current Method

- Perform sample and extract holding time study

  - GOALS:14-28 day holding time for samples; 14-28 days for extracts

  - Special attention to captan (hydrolysis issues) and *o*-toluidine (solvent stability issues)

- LCMRL and MDL analysis and calculations

- Repeat sample matrix challenges, holding time studies, and LCMRL/MDL calculations in SIM (work has been initiated)

  - quinoline: HRL of 0.010 µg/L

- Multi-laboratory verification

United States Environmental Protection Agency

Future Research and Potential Challenges

Review of Select Remaining CCL 3 Compounds:

CCL3 Compound	Comments
Acetamide	Not suitable for generic SPE, highly water soluble (2000g/L); Too small molecular weight (59.1) for LC/MS or EI-GC/MS
Acrolein	Volatile, water solubility > 10%. Stability issues in water.
Cumene hydroperoxide	Safety concerns due to high reactivity, low water solubility
Ethylene glycol	Too small molecular weight (62.1) for EI-GC/MS or LC/MS; miscible with water, not suitable for generic SPE
Ethylene oxide	Too small molecular weight (44.0) for EI-GC/MS or LC/MS/MS; gas at room temperature
Hydrazine	Too small molecular weight (32.0) for EI-GC/MS or LC/MS; volatile
Methanol	Too small molecular weight (32.0) for EI-GC/MS or LC/MS; volatile
Nitroglycerin	Not amenable to GC due to thermal instability; cannot ionize by positive ion ESI-LC/MS/MS
N-Methyl-2-pyrrolidone	Miscible with water, not suitable for generic SPE
Oxirane, methyl- (propylene oxide)	Too small molecular weight (58.1) for EI-GC/MS or LC/MS; volatile
Toluene diisocyanate	Reacts immediately with water, stability issues
Urethane	Not suitable for GC; Too small molecular weight (89.1) for LC/MS

METHOD DEVELOPMENT PROCESS FOR DRINKING WATER ANALYSIS

CB&I

Alan Zaffiro

WHAT WE DO TO CREATE A METHOD

PLANNING

AGENCY DIRECTION (e.g.,CCL3)

ANALYTE GROUPING

LITERATURE SEARCH

PRIVATE PARTNERS

RESEARCH PLAN

(NEW OR UPDATE EXISTING METHOD)

EXECUTION

METHOD DEVELOPMENT

VALIDATION

REVIEW

PUBLICATION

WHAT WE DO TO CREATE A METHOD

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CONSIDERATIONS

- MAXIMIZE # OF COMPOUNDS ANALYZED BY A SINGLE TECHNIQUE
- PHYSICAL-CHEMICAL PROPERTIES
- REACTIVITY / FORM
- MONITORING LIMITS
- USE OR ENVIRONMENTAL IMPACT
- RELEVANCE
- EXTEND EXISTING DRINKING WATER METHOD

WHAT WE DO TO CREATE A METHOD

PLANNING

AGENCY DIRECTION (e.g.,CCL3)

⇓

ANALYTE GROUPING

⇓

LITERATURE SEARCH

PRIVATE PARTNERS

⇓

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(NEW OR UPDATE EXISTING METHOD)

EXECUTION

METHOD DEVELOPMENT

⇓

VALIDATION

⇓

REVIEW

⇓

PUBLICATION

SOURCES

- EPA METHODS PUBLISHED BY OTHER DIVISIONS
- JOURNALS
- METHODS PUBLISHED BY OTHER GOVERNMENT AGENCIES, e.g., USGS
- APPLICATION NOTES
- VENDORS
- UNIVERSITIES
- PERSONAL COMMUNICATION
- PAST TSC ATTEMPTS
- PRELIMINARY LAB WORK
- VOLUNTARY CONSENSUS STANDARD BODIES (e.g. ASTM, STANDARD METHODS)

WHAT WE DO TO CREATE A METHOD

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PUBLICATION

ELEMENTS

- LITERATURE SEARCH RESULTS
- PROPOSED TARGET LIST
- PROPOSED QC COMPOUNDS
  - INTERNAL AND SURROGATE STANDARDS
- PROPOSED TECHNIQUE
  - PRESERVATION
  - DIRECT INSTRUMENTAL ANALYSIS?
  - SAMPLE PREPARATION (EXTRACTION, DERIVATIZATION, BUFFER)
- PROPOSED CONDITIONS

PLANNING

AGENCY DIRECTION (e.g.,CCL3)

⇓

ANALYTE GROUPING

⇓

LITERATURE SEARCH

PRIVATE PARTNERS

⇓

RESEARCH PLAN

(NEW OR UPDATE EXISTING METHOD)

EXECUTION

METHOD DEVELOPMENT

⇓

VALIDATION

⇓

REVIEW

⇓

PUBLICATION

BENCH WORK TO OPTIMIZE CONDITIONS

- WORK BACKWARDS ITERATIVELY
  - INSTRUMENTAL DETERMINATION>Sx PPEP>PRESERVATION
  - REAGENT WATER>FIELD SAMPLES
- GOALS
  - ***ROBUST, REPEATABLE, TRANSLATABLE METHOD***
  - 70 TO 130% RECOVERY; ±30% PRECISION
  - AT LEAST 14 DAYS STORAGE STABILITY
  - DETECTION LIMITS MEET MONITORING / REGULATORY OBJECTIVES
- MINIMIZE COST AND HAZARDOUS MATERIALS
- UTILIZE GENERIC LABORATORY MATERIALS

PLANNING

AGENCY DIRECTION (e.g.,CCL3)

ANALYTE GROUPING

LITERATURE SEARCH

PRIVATE PARTNERS

RESEARCH PLAN

(NEW OR UPDATE EXISTING METHOD)

EXECUTION

METHOD DEVELOPMENT

VALIDATION

REVIEW

PUBLICATION

VERIFY PERFORMANCE AT EPA AND MULTIPLE OUTSIDE LABORATORIES

- SINGLE LABORATORY--MOST EXTENSIVE SCOPE
  - LCMRL, P&A (CHALLENGING MATRIXES), MRL CONFIRMATION
  - STORAGE STABILITY WITH MICROBIAL INOCULANT PRESENT
  - DATA PUBLISHED IN SECTION 17 DRINKING WATER METHODS
- MULTI-LABORATORY VALIDATION
  - LCMRL, P&A (ONE MATRIX), MRL CONFIRMATION
  - DATA PUBLISHED IN RESEARCH SUMMARY
  - LABORATORIES RECOGNIZED IN METHOD

WHAT WE DO TO CREATE A METHOD

PLANNING

AGENCY DIRECTION (e.g.,CCL3)

ANALYTE GROUPING

LITERATURE SEARCH

PRIVATE PARTNERS

RESEARCH PLAN

(NEW OR UPDATE EXISTING METHOD)

EXECUTION

METHOD DEVELOPMENT

VALIDATION

REVIEW

PUBLICATION

METHOD DOCUMENT AND PERFORMANCE DATA

- PEER REVIEW OF METHOD
  - CONCURRENT WITH MULTI-LABORATORY VALIDATION
- AGENCY
  - INTERNAL (CB&I)
  - EPA PO
  - EPA MANAGEMENT
  - EPA HEADQUARTERS
  - VERIFY PDF CONVERSION (SECTION 508 COMPLIANT)

WHAT WE DO TO CREATE A METHOD

PLANNING

AGENCY DIRECTION (e.g.,CCL3)

⇓

ANALYTE GROUPING

⇓

LITERATURE SEARCH

PRIVATE PARTNERS

⇓

RESEARCH PLAN

(NEW OR UPDATE EXISTING METHOD)

EXECUTION

METHOD DEVELOPMENT

⇓

VALIDATION

⇓

REVIEW

⇓

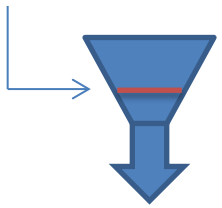
PUBLICATION

12 CANDIDATES

INITIAL ANALYTE GROUPING

- Ethylene oxide, propylene oxide, methanol, 1-butanol, allyl alcohol, 2-methoxy ethanol, ethylene glycol, acrolein, hexane, o-toluidine, triethylamine, benzyl chloride
- SMALL, POLAR MOLECULES POSSIBLY AMENABLE TO GC
- MOST POSSIBLY ISOLATED FROM H_2O USING CARBON- OR CMS-BASED EXTRACTION MEDIA

12 CANDIDATES



LITERATURE SEARCH

- PHYSICAL PROPERTIES
  - HENRY'S LAW CONSTANT
  - MW
  - BOILING POINT
- HRL
- METHODS PUBLISHED BY OTHER GOVERNMENT AGENCIES
- TECHNIQUES REPORTED IN:
  - APPLICATION NOTES
  - JOURNALS
  - PROCEEDINGS OF CONFERENCES
  - PERSONAL COMMUNICATION

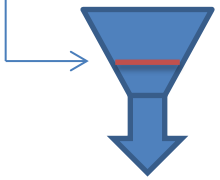
LITERATURE SEARCH SUMMARY TABLE

Table 1. Physical properties, published methods, and literature summary.

Analyte(s)	Henry's Law Constant, M/atm [a]	MW	bp, °C	HRL, µg/L	SW846	Literature techniques (author reference in parentheses)
Ethylene oxide Propylene oxide	7.1 5.2	44 58	10.7 34	0.1 0.2	5031[b], 8015D (direct aqueous injection—DAI)	Air and pharmaceutical residual methods
Methanol	2.3×10^{2}	32	64.7	3,500	8015D DAI	P&T w/FID Ether extraction (Woo) Headspace in blood (numerous citations) Derivatization to alkyl nitrite/extraction/GC/ECD (Nguyen) SPME (Supelco application notes)
1-Butanol	1.2×10^{2}	74	118	700	5031, 5030C[c] w/heat,	Ether extraction (Woo)
Allyl alcohol	2×10^{2}	58	97	35	5031, 5030C w/heat, 8015D DAI	
2-Methoxyethanol	12.1	76	125	21	Not found	SPE-carbon molecular sieve (Supelco application notes)
Ethylene glycol	1.7×10^{4}	62	197	14,000	8015B DAI	SPE-carbon molecular sieve (Supelco), DAI-GC-FID (Turner, Restek, Teske)
Acrolein	7 – 8	56	53	3.5	5030C, 8015D DAI, 8261A vacuum distillation	EPA Method 603 (heated purge and trap)
Hexane	5.5×10^{-4}	86	69	420	Not found	P&T (524.3 Research Summary)
Ortho-toluidine	Not available	107	200	0.19	8015D DAI	SPME (Supelco), IC (Zhu)
Triethylamine	Not available	101	90	2.3	8015D DAI	SPME (Alltech application note)
Benzyl chloride	1.6	127	179	0.2	5030C	P&T (524.2)

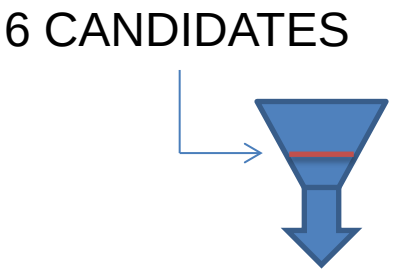
a. Reference: ethanol (1.9×10^{2}), benzene (1.8×10^{-1}), 1-propanol (1.4×10^{2}), MtBE (1.7), 1,4-dioxane (2.1×10^{2}), TBA (8.4).
b. Azeotropic distillation
c. Purge and trap technique

12 CANDIDATES



INITIAL LAB WORK AND PROPOSED METHOD

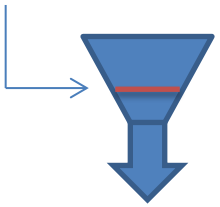
- PRIORITY CONTAMINANT: 2-methoxyethanol
- LIMITED INFORMATION OR EXPENSIVE INSTRUMENTS NEEDED
  - methanol, hexane, acrolein, triethyl amine, o-toluidine (not practical)
- INITIAL LAB INVESTIGATION REQUIRED
  - EtO AND ptO: HEADSPACE, EXTRACTION, AND P&T FAILED
  - THESE TWO ELIMINATED
- REMAINING 5 COMPOUNDS AMENABLE TO EXTRACTION
  - SIMILAR TECHNIQUE TO EPA METHOD 522
  - ORD-NERL ORIGINALLY PROPOSED MULTIPLE TARGETS FOR THIS METHOD



PROPOSED METHOD (CONT.)

- PROPOSE EXTENDING M-522 beyond 1,4-dioxane
- NEW TARGETS: n-butanol, allyl alcohol, **2-methoxyethanol**, ethylene glycol, benzyl chloride
  - CONTAINS PRIORITY CONTAMINANT
- QC COMPOUNDS
  - LABELED ISOTOPES OF 1,4-dioxane, n-butanol, allyl alcohol
- PROPOSED CONDITIONS
  - CARBON-EXTRACTION, GC-MS DETERMINATION

6 CANDIDATES

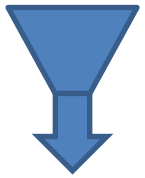


BENCH WORK TO OPTIMIZE CONDITIONS

- ETHYLENE GLYCOL NOT RETAINED BY ANY EXTRACTION MEDIA EVALUATED
- BENZYL CHLORIDE FAILED PRELIMINARY STORAGE STABILITY
- ONLY ONE OF THE EXTRACTION MEDIA IN M-522 SUITABLE FOR REMAINING COMPOUNDS
- MODIFICATION OF M-522 EXTRACTION SOLVENT REQUIRED TO RECOVER ADDITIONAL TARGETS
- 1,4-dioxane, n-butanol, allyl alcohol**, 2-methoxyethanol**

VERIFY PERFORMANCE AT EPA AND MULTIPLE OUTSIDE LABORATORIES

- SUCCESSFULLY DEMONSTRATED AT EPA
- METHOD CURRENTLY BEING WRITTEN
- CURRENTLY RECRUITING OUTSIDE LABORATORIES



4
ANALYTES

ABILITY TO MEET MONITORING GOALS

MRL's and HRLs

Analytes	MRL *	CCL3 HRL
1,4-dioxane	0.2 µg/L	3.0 µg/L (cancer)
allyl alcohol	1.0 µg/L	35 µg/L
n-butanol	1.0 µg/L	700 µg/L
2-methoxyethanol	1.0 µg/L	21 µg/L

* This is the aqueous concentration equivalent to the low calibration standard.

METHOD PARAMETERS

- **Preservation:** identical to EPA method 522
- **SPE:** 0.1 L Sx; neutralized with 5 mL x 0.8 M $NaHCO_3$; Supelco Envi-Carb Plus (400 mg); **60-min air dry in reverse direction**; elution 20% MeOH:DCM; 2-mL extract volume
- **Extract analysis**: 30 m x 0.25 mm i.d. x 0.5 µm d_f column (Agilent P/N CP9222 VF-WAXms); 1 µL injection @ 200 °C inlet; temperature- programmed separation; MS detection in SIM mode

ANALYTE LIST AND CALIBRATION RANGES

Analytes	Range, µg/L	Range, ng/mL (equivalent extract concentration)
I.S.: 1,4-dioxane-d_8	added to extract	250
I.S.: chlorobenzene-d_5	added to extract	250
Surr: allyl alcohol-d_6	5.0	250 (also serves as isotope dilution internal standard)
Surr: n-butanol-d_{10}	5.0	250 (also serves as isotope dilution internal standard)
1,4-dioxane	0.10 — 8.0	5.0 — 400
allyl alcohol	0.50 —20	25 — 1000
allyl alcohol*	0.50 —20	25 — 1000
n-butanol	0.50 —20	25 — 1000
n-butanol*	0.50 —20	25 — 1000
2-methoxyethanol	0.50 —20	25 — 1000

* Reported with isotope dilution quantitation

CHALLENGES

- WATER MANAGEMENT
  - EXTRACTION MEDIA IS DIFFICULT TO DRY
  - WET EXTRACTS CAUSE RETENTION TIME SHIFTS (COMPROMISING QUALITATIVE ID) AND CAN AFFECT ANALYTE RESPONSE (QUANTITATIVE AND QC)
  - EXCESSIVE DRYING CAUSES LOW RECOVERY
- ONLY 1 EXTRACTION MEDIUM AVAILABLE FOR THE ANALYSIS OF THESE ANALYTES
- LOT TO LOT VARIATION
  - LOADING TIME
  - DRYING TIME

ANALYSIS OF ERYTHROMYCIN AND OTHER PHARMACEUTICALS BY LC-MS/MS

CB&I

William A. Adams, Ph.D.

PHARMACEUTICAL TARGET ANALYTES

- Carbamazepine, Diazepam, Diclofenac (sodium salt), Enalapril (maleate salt), Fluoxetine (HCl), Gemfibrozil, Naproxen, Phenytoin, Sulfamethoxazole, Triclosan, Trimethoprim, and Erythromycin (*measured as Erythromycin–H_2O*)

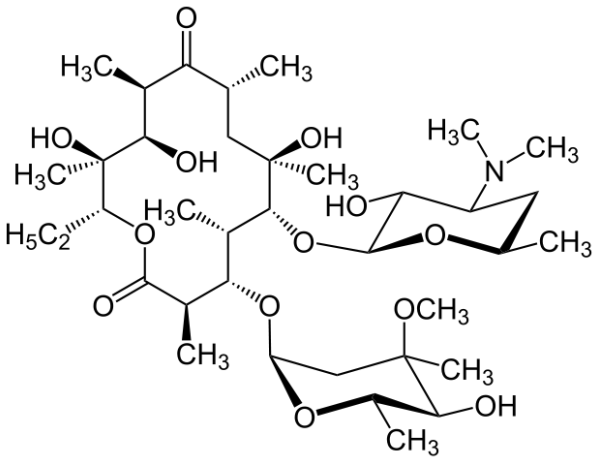
- Variety of chemically unrelated analytes
  - Both ESI positive and ESI negative modes

- Analysis by LC-MS/MS using an 5 mM ammonium acetate and methanol gradient

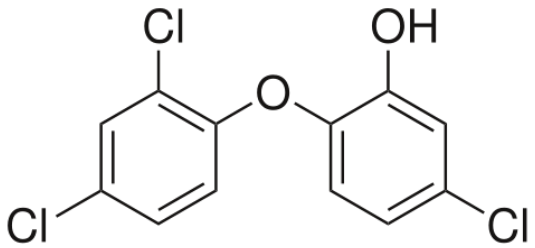
- SPE (6 mL, 200 mg HLB cartridge)

- ^{13}C-Naproxen-d_3, Triclosan-d_3, Carbamazepine-d_{10}, chosen as internal standards; ^{13}C-Trimethoprim-d_3 and Diclofenac-d_4 chosen as surrogate standards

- Erythromycin and triclosan
- Only erythromycin listed on CCL3

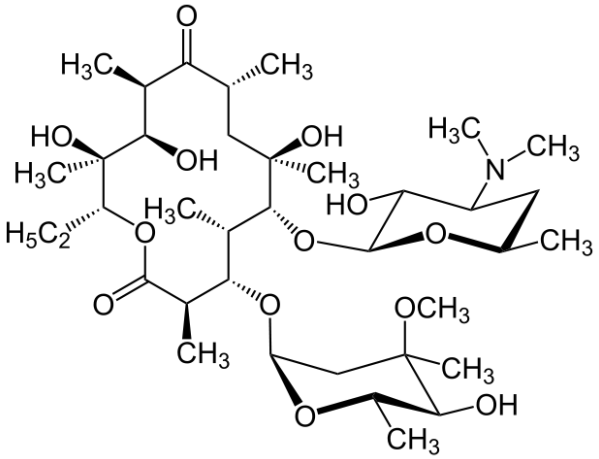


Erythromycin



Triclosan

- Erythromycin
  - At pH <7, water is removed and compound no longer exhibits antibiotic properties (Hirsch et al., 1999)
  - For analysis, erythromycin is measured as erythromycin–H_2O (717.0 > 158.3 *m/z*)



Erythromycin

- Store samples in refrigerator (6°C)

- 100 mg/L ascorbic acid
  - Reduces free chlorine present in tap water samples
  - Easy to handle
  - Solid can be added to bottles before sampling

- 350 mg/L EDTA
  - Chelates metals in tap water samples
  - Prevents metal hydrolysis
  - Solid can be added to bottles before sampling

- 9.4 g/L potassium citrate
  - Acts as a microbial inhibitor
  - pH ~3.8
  - Solid can be added to bottles before sampling

- Solid phase extraction (SPE)
  - 6 cc, 200 mg HLB cartridges
  - Vacuum manifold
  - 1 liter samples extracted and eluted with 5 mL 1:1 methanol/acetone
- Dilution
  - 5 mL reagent water added to extract
  - 100:1 sample to extract concentration factor

CHROMATOGRAPHIC CONDITIONS

ESI positive

HPLC		
Column: Waters Xterra® MS C18, 2.1 x 150 mm, 3.5 µm		
Column temperature: 30 °C		
Column flow rate: 0.200 mL/min		
Autosampler temperature: 10 °C		
Injection volume: 10 µL		
Gradient:		
Time (min)	**%5 mM ammonium acetate in 10% MeOH/90% reagent water**	**%MeOH**
0.00	90	10
0.50	90	10
0.51	50	50
8.00	25	75
8.01	0	100
10.00	0	100
14.00	90	10
24.00	End Run	

ESI negative

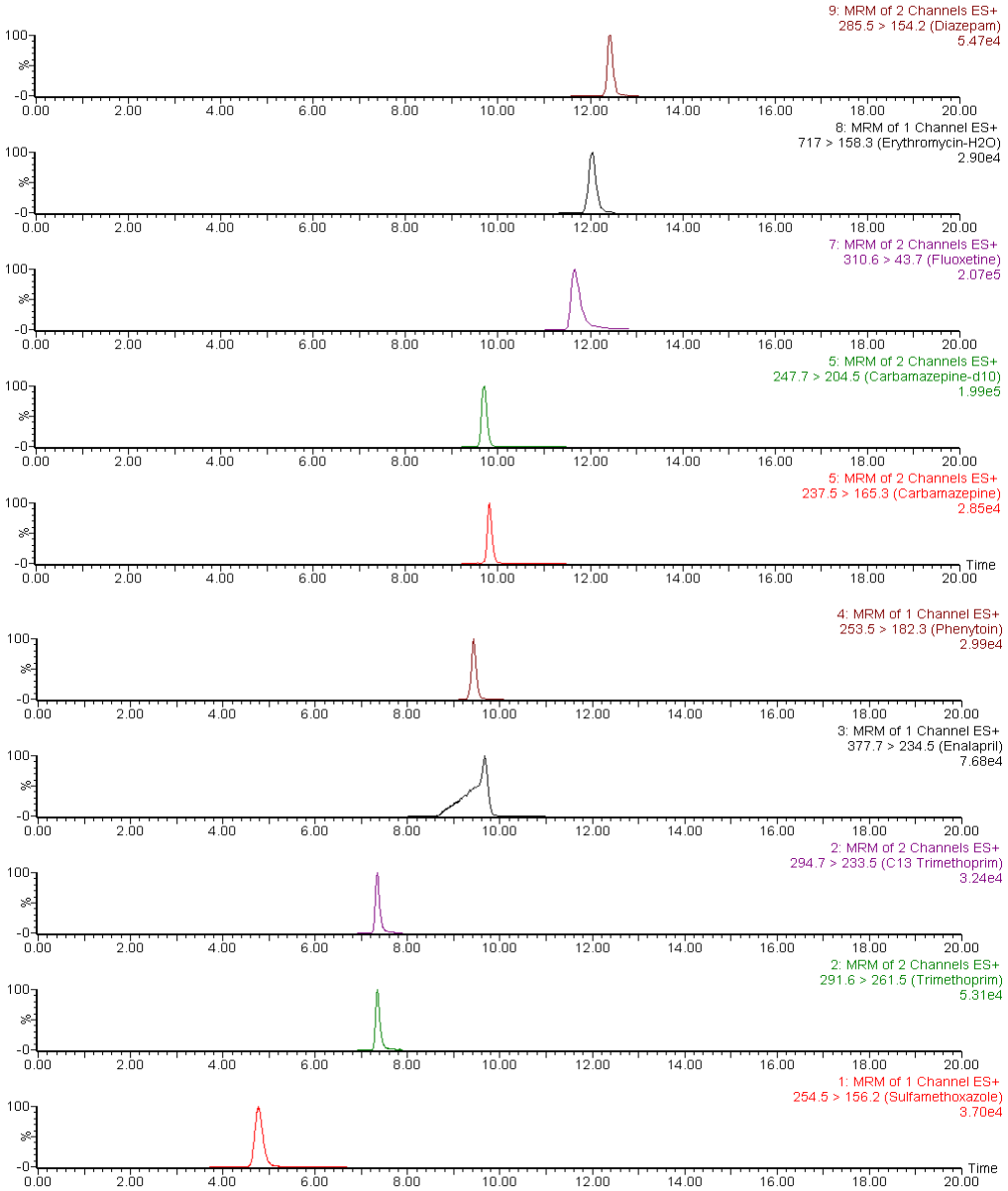
HPLC		
Column: Waters Xterra® MS C18, 2.1 x 150 mm, 3.5 µm		
Column temperature: 30 °C		
Column flow rate: 0.200 mL/min		
Autosampler temperature: 10 °C		
Injection volume: 50 µL		
Gradient:		
Time (min)	**%5 mM ammonium acetate in 10% MeOH/90% reagent water**	**%MeOH**
0.00	90	10
0.50	90	10
0.51	40	60
8.00	0	100
11.00	0	100
15.00	90	10
25.00	End Run	

MS CONDITIONS

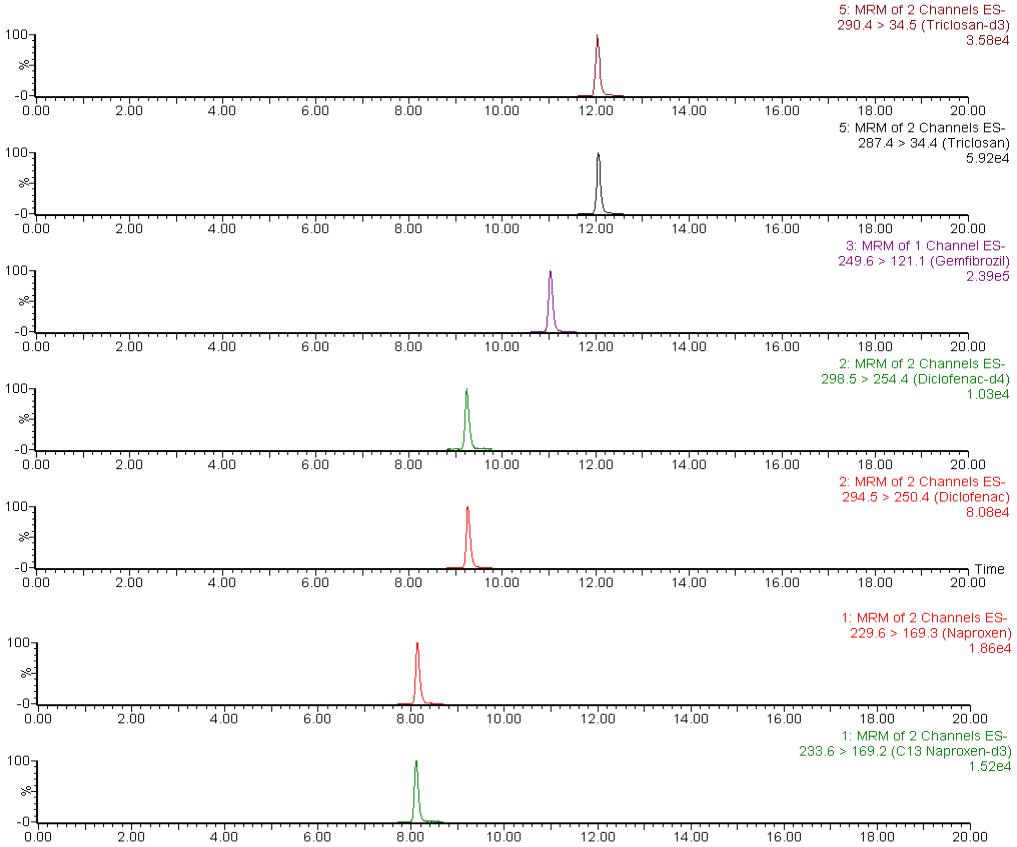
MS Parameter	HPLC-MS/MS
Polarity	Positive ion electrospray
Capillary Voltage, kV	2.50
Source Temperature, ^{o}C	120
N_2 Desolvation Temperature, ^{o}C	400
N_2 Desolvation Gas Flow, L/hr	900
Cone Gas Flow, L/hr	50
Extractor Lens, V	2.00
RF Lens, V	0.2

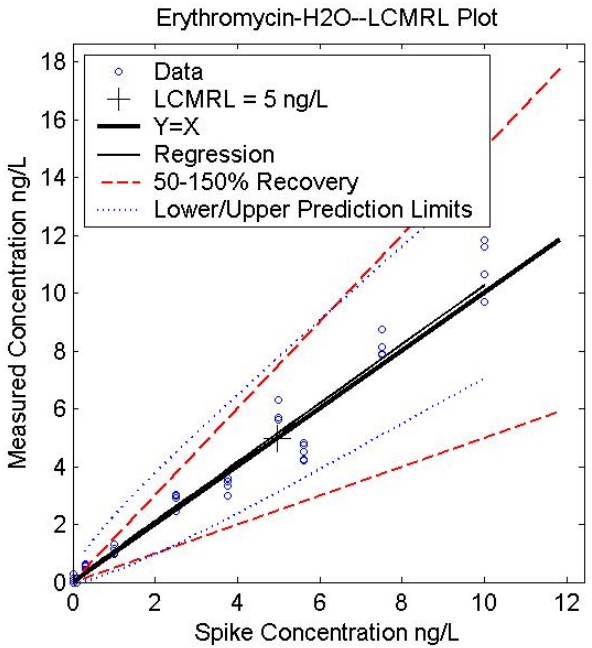
MS Parameter	HPLC-MS/MS
Polarity	Negative ion electrospray
Capillary Voltage, kV	2.50
Source Temperature, ^{o}C	120
N_2 Desolvation Temperature, ^{o}C	400
N_2 Desolvation Gas Flow, L/hr	900
Cone Gas Flow, L/hr	50
Extractor Lens, V	1.00
RF Lens, V	0.1

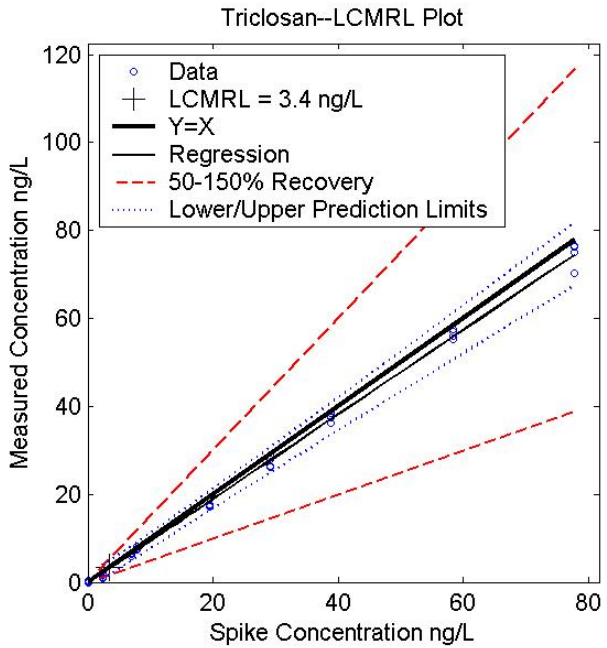
ESI POSTIVE CHROMATOGRAM



ESI NEGATIVE CHROMATOGRAMS







Analyte	**LCMRL (ng/L)[a]**
Carbamazepine	2.4
Diazepam	0.27
Diclofenac	1.1
Enalapril	0.60
Fluoxetine	0.98
Gemfibrozil	1.4
Naproxen	4.5
Phenytoin	1.4
Sulfamethoxazole	0.28
Triclosan	**3.4**
Trimethoprim	4.1
Erythromycin	**5.0**

- Precision and accuracy measured in different matrixes (e.g. reagent water, high TOC tap water, hard tap water)
- Measured at two concentrations
  - Low and Mid/High of calibration range
- Accuracy
  - Low: 50–150% recovery
  - Mid/High: 70–130% recovery
- Precision
  - Low: ≤30% RSD
  - Mid/High: ≤20% RSD

- Reagent water
  - 9.4 g/L potassium citrate, 350 mg/L EDTA, and 100 mg/L ascorbic acid

Analyte	Low Concentration			High Concentration		
	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD
Carbamazepine	4.94	87.9	4.0	31.3	91.5	0.88
Diazepam	0.340	72.9	7.8	20.0	92.1	3.4
Diclofenac	2.04	97.2	4.2	102	95.1	1.2
Enalapril	1.22	105	6.0	40.4	89.7	1.6
Fluoxetine	1.24	70.3	8.2	104	84.6	2.3
Gemfibrozil	3.00	92.7	1.4	100	95.6	2.1
Naproxen	9.20	101	4.0	100	98.5	1.2
Phenytoin	2.42	76.0	4.5	30.7	90.2	4.1
Sulfamethoxazole	0.410	92.2	8.0	29.4	90.9	0.51
Triclosan	**7.00**	**91.5**	**5.0**	**311**	**99.7**	**1.5**
Trimethoprim	8.80	90.5	4.2	30.4	91.1	2.4
Erythromycin	**5.60**	**80.4**	**6.2**	**40.0**	**83.9**	**1.7**
Diclofenac-d_4	100	94.0	2.4	100	93.9	1.8
$^{13}C_3$-Trimethoprim	20.0	86.6	2.3	20.0	87.3	3.3

- Ground source tap water
  - 9.4 g/L potassium citrate, 350 mg/L EDTA, and 100 mg/L ascorbic acid
  - Free chlorine: 0.76 mg/L
  - Total chlorine: 0.97 mg/L
  - Hardness: 331 mg/L
  - TOC: 0.75 ppm

Analyte	Low Concentration			High Concentration		
	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD
Carbamazepine	4.94	93.0	2.5	31.3	95.3	1.2
Diazepam	0.340	73.5	13	20.0	97.7	1.8
Diclofenac	2.04	104	7.6	102	97.4	1.4
Enalapril	1.22	94.3	2.9	40.4	94.4	1.1
Fluoxetine	1.24	86.1	6.4	104	94.9	2.4
Gemfibrozil	3.00	92.7	4.1	100	96.4	2.0
Naproxen	9.20	89.0	3.8	100	104	2.1
Phenytoin	2.42	83.0	5.9	30.7	80.2	2.1
Sulfamethoxazole	0.410	87.8	12	29.4	93.8	1.3
Triclosan	**7.00**	**88.5**	**10**	**311**	**96.7**	**1.0**
Trimethoprim	8.80	92.4	5.2	30.4	94.3	1.2
Erythromycin	**5.60**	**69.8**	**7.3**	**40.0**	**78.7**	**5.5**
Diclofenac-d_4	100	98.9	1.5	100	88.9	2.2
$^{13}C_3$-Trimethoprim	20.0	98.4	1.8	20.0	97.7	1.8

- Surface source tap water
  - 9.4 g/L potassium citrate, 350 mg/L EDTA, and 100 mg/L ascorbic acid
  - Free chlorine: 1.16 mg/L
  - Total chlorine: 1.65 mg/L
  - Hardness: 124 mg/L
  - TOC: 4.23 ppm

Analyte	Low Concentration			High Concentration		
	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD
Carbamazepine	4.94	83.7	2.7	31.3	91.1	2.8
Diazepam	0.340	54.7	27	20.0	95.7	0.92
Diclofenac	2.04	71.8	5.6	102	73.6	1.4
Enalapril	1.22	99.5	8.8	40.4	94.1	0.42
Fluoxetine	1.24	96.6	4.3	104	105	1.8
Gemfibrozil	3.00	104	3.3	100	111	0.91
Naproxen	9.20	92.7	4.8	100	98.6	1.9
Phenytoin	2.42	95.4	6.6	30.7	95.4	1.6
Sulfamethoxazole	0.410	92.2	28	29.4	93.3	0.96
Triclosan	**7.00**	**93.5**	**12**	**311**	**99.2**	**0.94**
Trimethoprim	8.80	69.6	5.2	30.4	72.5	2.1
Erythromycin	**5.60**	**85.0**	**6.3**	**40.0**	**87.2**	**2.0**
Diclofenac-d_4	100	72.3	0.92	100	75.3	1.7
$^{13}C_3$-Trimethoprim	20.0	80.2	1.6	20.0	81.4	1.3

- Surface source tap water

Analyte	Fortified Conc. (ng/L)	Day 0		Day 7		Day 14		Day 21		Day 28	
		Avg. %Rec	%RSD	% Change from Day 0	%RSD	% Change from Day 0	%RSD	% Change from Day 0	%RSD	% Change from Day 0	%RSD
Carbamazepine	7.82	106	6.1	-1.3	6.6	-2.2	3.0	-6.7	3.4	-8.4	0.46
Diazepam	5.00	114	1.1	-8.9	1.5	-6.7	1.8	-6.7	1.8	-11	1.7
Diclofenac	25.6	105	0.84	-[c]	-[c]	4.3	1.8	3.3	0.92	-13	1.2
Enalapril	10.1	90.8	2.3	0.29	2.9	-2.6	2.8	-1.3	0.78	1.1	2.2
Fluoxetine	26.0	113	2.4	0.56	7.8	15	41	-6.6	3.6	-4.1	1.9
Gemfibrozil	25.0	104	1.2	7.3	2.3	3.3	2.1	4.1	1.1	-5.3	2.5
Naproxen	25.0	106	1.2	3.4	2.0	2.5	2.7	0.39	2.7	0.50	2.2
Phenytoin	7.68	94.8	11	-[c]	-[c]	-30	3.4	-15	9.2	-18	2.8
Sulfamethoxazole	7.34	93.0	3.3	-1.2	5.5	-2.7	0.53	-7.8	0.56	-6.1	2.6
Triclosan	**77.8**	**103**	**2.8**	**3.1**	**3.5**	**5.1**	**2.2**	**1.5**	**1.3**	**-1.1**	**0.76**
Trimethoprim	7.60	74.8	2.0	1.7	4.1	-10	3.0	-10	4.5	-16	3.4
Erythromycin	**10.0**	**102**	**1.9**	**-1.3**	**22**	**-6.2**	**4.5**	**-0.62**	**1.5**	**-3.7**	**2.8**
Surrogate	**Fortified Conc. (ng/L)**	**Avg. %Rec**	**%RSD**	**Avg. %Rec**	**%RSD**	**Avg. %Rec**	**%RSD**	**Avg. %Rec**	**%RSD**	**Avg. %Rec**	**%RSD**
Diclofenac-d_4	100	85.5	1.2	103	5.1	97.8	1.7	99.7	0.55	82.2	0.68
$^{13}C_3$-Trimethoprim	20.0	79.4	0.84	78.9	0.84	74.7	0.43	76.7	2.4	79.1	1.9

- Surface source tap water

Analyte	Fortified Conc. (ng/L)	Day 0		Day 7		Day 14		Day 21		Day 28	
		Avg. %Rec	%RSD	% Change from Day 0	%RSD	% Change from Day 0	%RSD	% Change from Day 0	%RSD	% Change from Day 0	%RSD
Carbamazepine	7.82	106	6.1	-10	3.8	-9.4	3.5	-12	2.3	-14	3.5
Diazepam	5.00	114	1.1	-14	2.5	-12	4.1	-11	2.4	-13	3.0
Diclofenac	25.6	105	0.84	-6.4	1.1	-6.4	3.2	-0.74	1.0	-17	1.1
Enalapril	10.1	90.8	2.3	4.4	2.2	0.69	0.50	2.4	0.79	4.3	1.5
Fluoxetine	26.0	113	2.4	13	2.2	2.6	4.6	8.8	4.9	18	6.0
Gemfibrozil	25.0	104	1.2	-2.5	1.8	-3.3	1.7	3.0	0.41	-11	0.63
Naproxen	25.0	106	1.2	-0.48	1.3	3.6	3.1	4.0	1.8	3.2	2.8
Phenytoin	7.68	94.8	11	-	-	-35	7.4	-20	4.3	-16	13
Sulfamethoxazole	7.34	93.0	3.3	-12	4.0	-9.6	1.2	-7.6	2.2	-8.2	1.6
Triclosan	**77.8**	**103**	**2.8**	**-1.0**	**1.8**	**-0.10**	**1.1**	**1.5**	**1.6**	**-4.1**	**2.7**
Trimethoprim	7.60	74.8	2.0	-3.1	3.0	-12	3.1	-9.7	0.41	-11	3.4
Erythromycin	**10.0**	**102**	**1.9**	**-24**	**6.6**	**-14**	**4.5**	**-12**	**1.8**	**-16**	**4.8**
Diclofenac-d_4	100	85.5	1.2	-0.94	2.4	-1.8	5.2	10	5.1	-11	4.2
$^{13}C_3$-Trimethoprim	20.0	79.4	0.84	11	3.7	-1.9	4.5	1.6	2.8	1.2	2.0

CONCLUSIONS

- Various pharmaceuticals/personal care products (PPCPs) analyzed in both ESI positive and ESI negative modes
- Erythromycin measured as erythromycin–H_2O
  - Likely transforms prior to analysis
- Extraction necessary to reach desired concentration levels
  - HLB cartridges found to provide best overall extraction recovery for both positive and negative analytes
- All analytes meet precision and accuracy QC requirements in three different matrixes
- Sample and extract hold times change less than 20% after 28 days

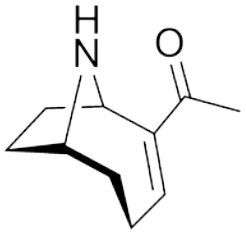
ANALYSIS OF CYLINDROSPERMOPSIN AND ANATOXIN-A BY LC-MS/MS

CB&I

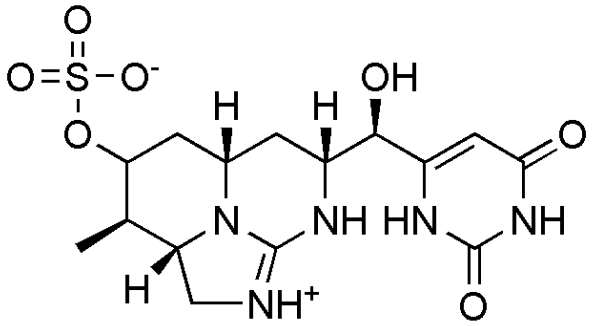
William A. Adams, Ph.D.

CCL3 ALGAL TOXIN TARGET ANALYTES

- Cylindrospermopsin and anatoxin-a
  - Toxins produced by blue-green algae
- Water soluble, polar molecules
- Analysis by LC-MS/MS using a high aqueous composition eluent
  - Cylindrospermopsin: 416.2 > 194.0 *m/z*
  - Anatoxin-a: 165.8 > 148.8 *m/z*
- Direct injection



Anatoxin-a



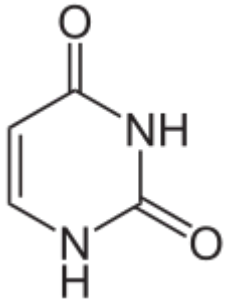
Cylindrospermopsin

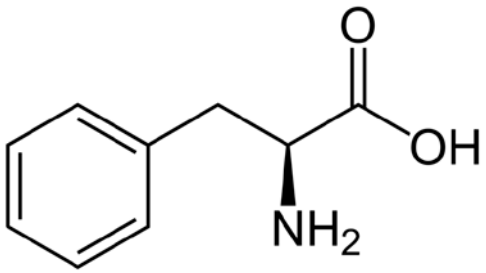
- Isotopically labeled standards not available for cylindrospermopsin or anatoxin-a

- Cylindrospermopsin is a uracil derivative
  - Deuterated uracil readily available and elutes near retention time of cylindrospermopsin (Uracil-d_5)

- Anatoxin is isobaric and structurally similar to L-phenylalanine
  - Deuterated phenylalanine is readily available and appears to track well with anatoxin-a (L-phenylalanine-d_4)

Uracil





L-phenylalanine

CHROMATOGRAPHIC CONDITIONS

HPLC		
Column: Waters XSelect® HSS T3, 2.1 x 150 mm, 3.5 µm		
Column temperature: 30 °C		
Column flow rate: 0.200 mL/min		
Autosampler temperature: 10 °C		
Injection volume: 50 µL		
Gradient:		
Time (min)	**%100 mM acetic acid in reagent water**	**%MeOH**
0.00	100	0
0.50	90	10
8.50	100	0
13.50	End Run	

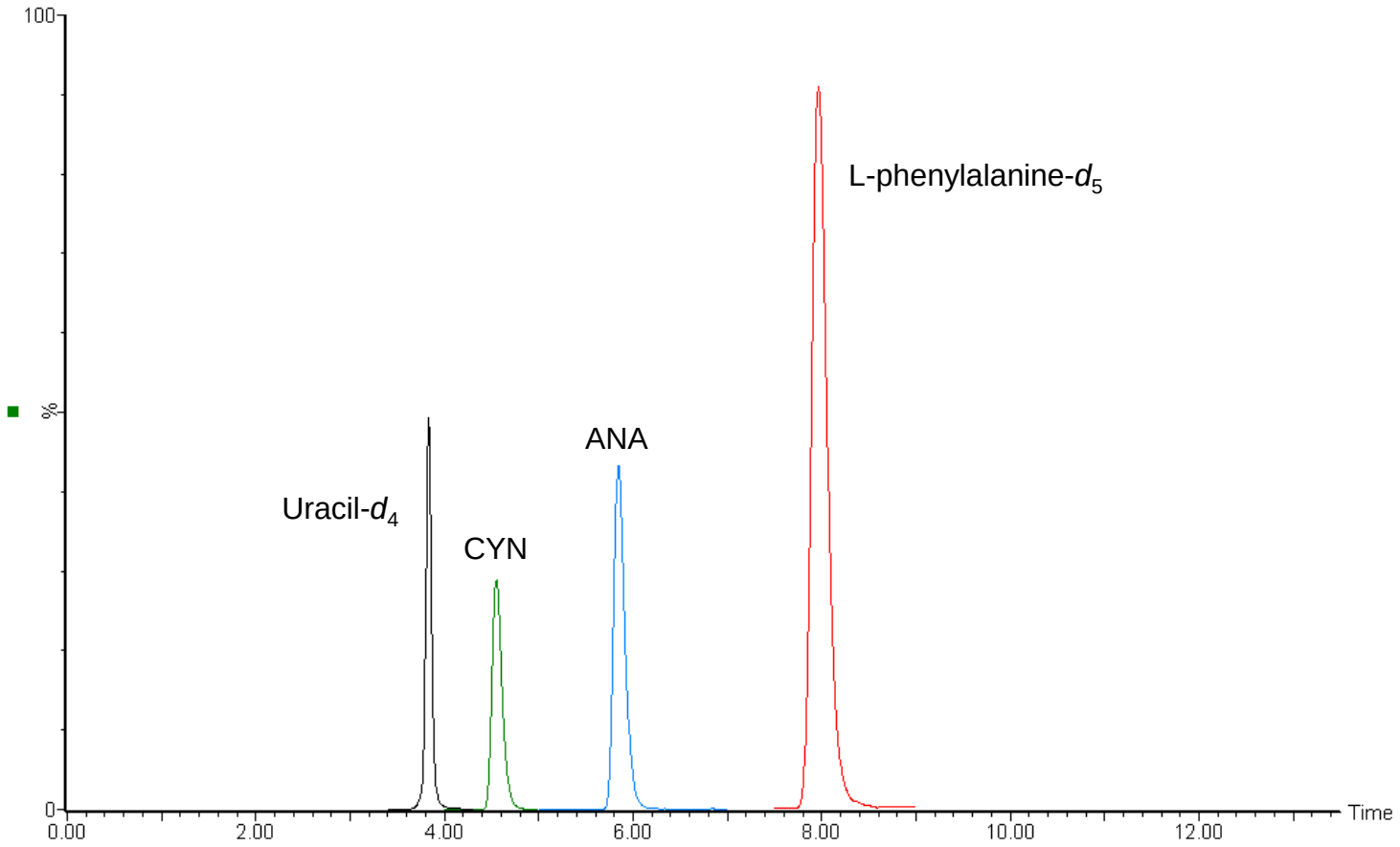
MS CONDITIONS

MS Parameter	**HPLC-MS/MS**
Polarity	Positive ion electrospray
Capillary Voltage, kV	2.50
Source Temperature, °C	120
N_2 Desolvation Temperature, °C	400
N_2 Desolvation Gas Flow, L/hr	900
Cone Gas Flow, L/hr	50
Extractor Lens, V	2.00
RF Lens, V	0.2

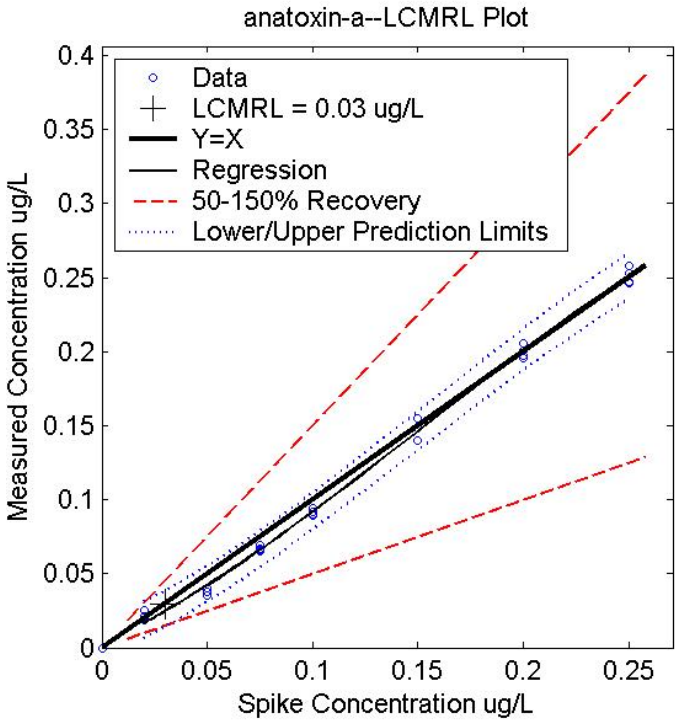
Preservation

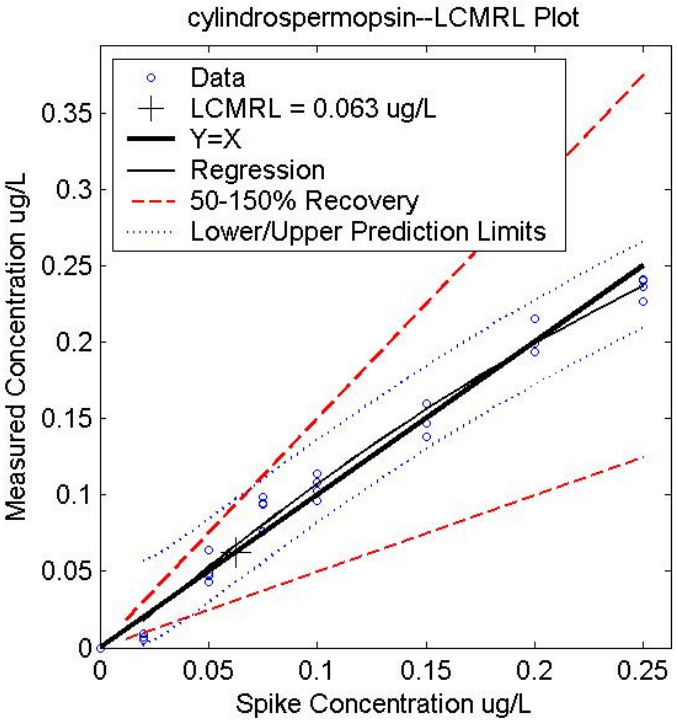
- Store samples in refrigerator
- 100 mg/L ascorbic acid
  - Reduces free chlorine present in tap water samples
  - Easy to handle
  - Solid can be added to bottles before sampling
- 1000 mg/L sodium bisulfate
  - Acts as a microbial inhibitor
  - pH less than 3
  - Solid can be added to bottles before sampling

CHROMATOGRAM – 5 ng/mL ANALYTES



- HRL values FROM CCL3
  - Cylindrospermopsin – 0.21 µg/L
  - Anatoxin-a – 3.5 µg/L
- Calibration range
  - Cylindrospermopsin – 0.050 to 10.0 µg/L
  - Anatoxin-a – 0.050 to 10.0 µg/L
- MRL confirmed at 0.100 µg/L for both analytes


anatoxin-a--LCMRL Plot
Data
LCMRL = 0.03 ug/L
Y=X
Regression
50-150% Recovery
Lower/Upper Prediction Limits
Measured Concentration ug/L
Spike Concentration ug/L
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4


cylindrospermopsin--LCMRL Plot
Data
LCMRL = 0.063 ug/L
Y=X
Regression
50-150% Recovery
Lower/Upper Prediction Limits
Measured Concentration ug/L
Spike Concentration ug/L
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35

- Precision and accuracy measured in different matrixes (e.g. reagent water, high TOC tap water, hard tap water)

- Measured at two concentrations
  - Low and Mid/High of calibration range

- Accuracy
  - Low: 50–150% recovery
  - Mid/High: 70–130% recovery

- Precision
  - Low: ≤30% RSD
  - Mid/High: ≤20% RSD

- Reagent water
  - 1000 mg/L sodium bisulfate and 100 mg/L ascorbic acid

Reagent Water Low													
Compound	Samples (ug/L)							Mean (ug/L)	SD	Unfortified blank (ug/L)	Theoretical spike (ug/L)	%RSD	%Rec
ESI (+)	1	2	3	4	5	6	7						
cylindrospermopsin	0.117	0.128	0.117	0.103	0.100	0.094	0.102	0.109	0.012	0.000	0.100	**11.136**	**108.7**
anatoxin-a	0.108	0.116	0.108	0.106	0.102	0.127	0.111	0.111	0.008	0.000	0.100	**7.391**	**111.1**

Reagent Water High													
Compound	Samples (ug/L)							Mean (ug/L)	SD	Unfortified blank (ug/L)	Theoretical spike (ug/L)	%RSD	%Rec
ESI (+)	1	2	3	4	5	6	7						
cylindrospermopsin	2.837	2.609	2.834	2.767	2.614	2.681	2.664	2.715	0.097	0.000	2.500	**3.588**	**108.6**
anatoxin-a	2.757	2.689	2.810	2.580	2.688	2.798	2.709	2.719	0.079	0.000	2.500	**2.898**	**108.7**

- Ground source tap water
  - 1000 mg/L sodium bisulfate and 100 mg/L ascorbic acid
  - Free chlorine: 0.69 mg/L
  - Total chlorine: 0.97 mg/L
  - Hardness: 325 mg/L
  - Conductivity: 798 µS
  - pH: 7.76

Ground Water Low													
Compound	Samples (ug/L)							Mean (ug/L)	SD	Unfortified blank (ug/L)	Theoretical spike (ug/L)	%RSD	%Rec
ESI (+)	1	2	3	4	5	6	7						
cylindrospermopsin	0.137	0.132	0.120	0.128	0.119	0.122	0.125	0.126	0.007	0.000	0.100	**5.247**	**126.1**
anatoxin-a	0.102	0.080	0.087	0.099	0.092	0.096	0.093	0.093	0.007	0.000	0.100	**8.016**	**92.7**

Ground Water High													
Compound	Samples (ug/L)							Mean (ug/L)	SD	Unfortified blank (ug/L)	Theoretical spike (ug/L)	%RSD	%Rec
ESI (+)	1	2	3	4	5	6	7						
cylindrospermopsin	2.406	2.516	2.413	2.435	2.380	2.487	2.508	2.449	0.054	0.000	2.500	**2.206**	**98.0**
anatoxin-a	2.316	2.394	2.370	2.324	2.382	2.419	2.424	2.376	0.043	0.000	2.500	**1.790**	**95.0**

- Surface source tap water
  - 1000 mg/L sodium bisulfate and 100 mg/L ascorbic acid
  - Free chlorine: 0.64 mg/L
  - Total chlorine: 1.14 mg/L
  - Hardness: 142 mg/L
  - Conductivity: 344 µS
  - pH: 7.20
  - TOC 3.04 mg/L

Surface Water Low													
Compound	Samples (ug/L)							Mean (ug/L)	SD	Unfortified blank (ug/L)	Theoretical spike (ug/L)	%RSD	%Rec
ESI (+)	1	2	3	4	5	6	7						
cylindrospermopsin	0.139	0.124	0.111	0.112	0.119	0.109	0.104	0.117	0.012	0.000	0.100	**10.070**	**116.9**
anatoxin-a	0.106	0.091	0.096	0.101	0.101	0.093	0.090	0.097	0.006	0.000	0.100	**6.178**	**96.9**

Surface Water High													
Compound	Samples (ug/L)							Mean (ug/L)	SD	Unfortified blank (ug/L)	Theoretical spike (ug/L)	%RSD	%Rec
ESI (+)	1	2	3	4	5	6	7						
cylindrospermopsin	2.762	2.768	2.699	2.714	2.715	2.649	2.679	2.712	0.043	0.000	2.500	**1.570**	**108.5**
anatoxin-a	2.450	2.506	2.383	2.585	2.484	2.565	2.548	2.503	0.071	0.000	2.500	**2.833**	**100.1**

CONCLUSIONS

- Cylindrospermopsin and anatoxin-a show good response with method
- Labeled analogs of target analytes not available
  - Internal standards chosen based on tracking ability and response
  - Uracil-d_4 has shown some inconsistency in dirtier matrixes and may be removed
- Precision and accuracy are within the acceptable range in three different matrixes
- MRL is confirmed below HRL of both analytes and LCMRL gives reasonable results
- Method development will continue with storage/stability study and second laboratory demonstration

Update to Method 539

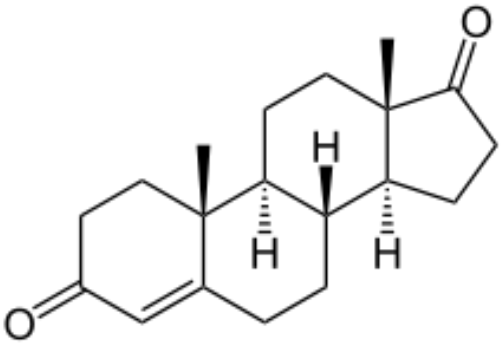
CB&I

Steven D. Winslow

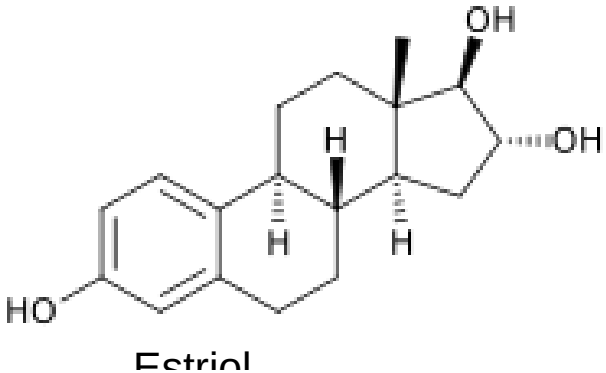
- EPA Method 539: LC-MSMS analysis , multiple reaction monitoring (MRM) , SPE extraction
- Original Target Analyte List

Method 539	CASRN
4-Androstene-3,17-dione	63-05-8
Equilin	474-86-2
17β-Estradiol	50-28-2
Estriol	50-27-1
Estrone	53-16-7
17α-Ethynylestradiol	57-63-6
Testosterone	58-22-0

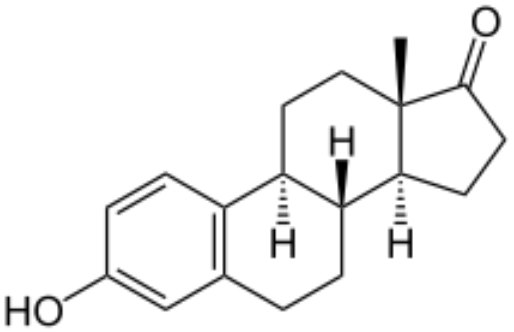
- **EPA METHOD 539 TARGET ANALYTES**



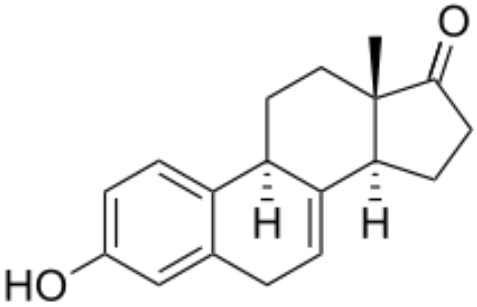
Androstenedione



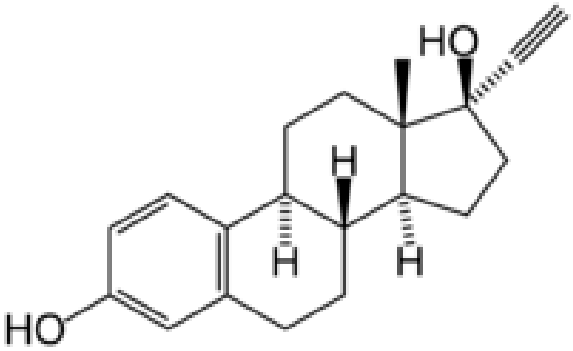
Estriol



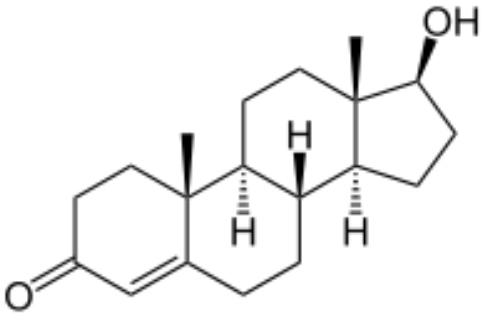
Estrone



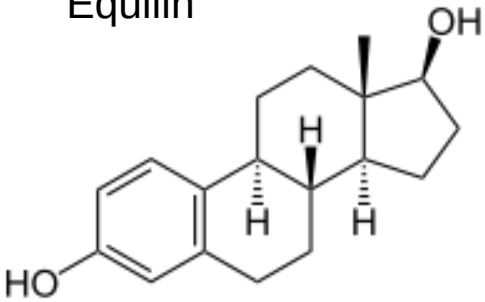
Equilin



Ethynylestradiol



Testosterone



β-estradiol

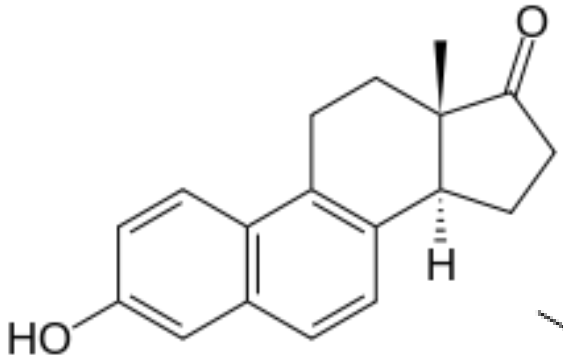
Proposed additional hormones to Method 539

- Five compounds, introduced late in Method 539 development, were initially rejected from method

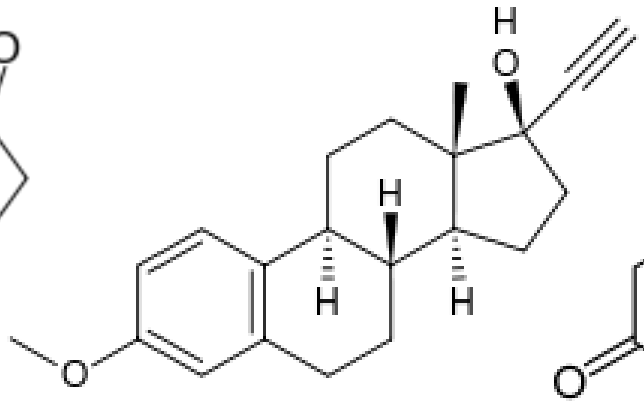
COMPOUNDS	CAS#	ISSUES	HRL (ug/L)
alpha- Estradiol	57-91-0	M539: enhancement issue	0.35
Equilenin	517-09-9	M539: recovery in finished ground water failed QC	0.35
Mestranol	72-33-3	M539: did not work with ESI	0.28
Norethindrone	68-22-4	M539: ESI+ internal standard did not track	0.04
Progesterone	57-83-0	M539: ESI+ internal standard did not track Nominated for CCL4	---

- Internal standards now available
  - Norethindrone-2,2,4,6,6,10-d_6
  - Progesterone-2,3,4-$^{13}C_3$

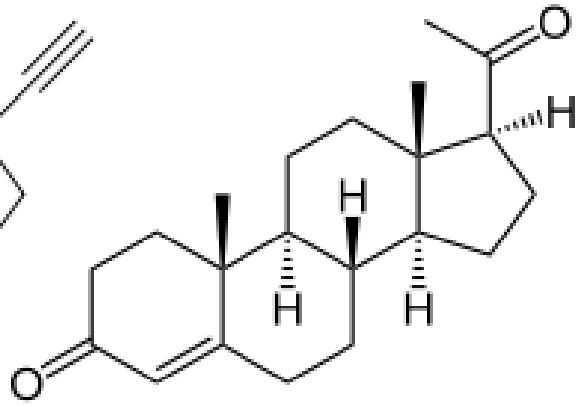
- Structures of new hormones



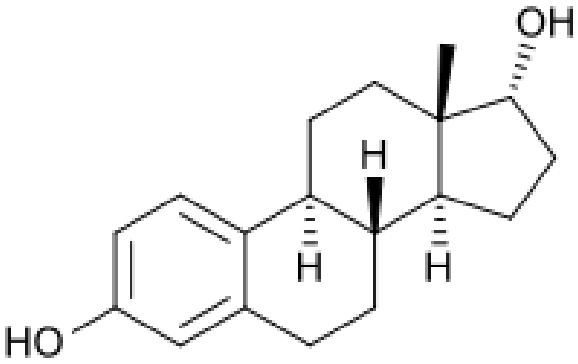
Equilenin



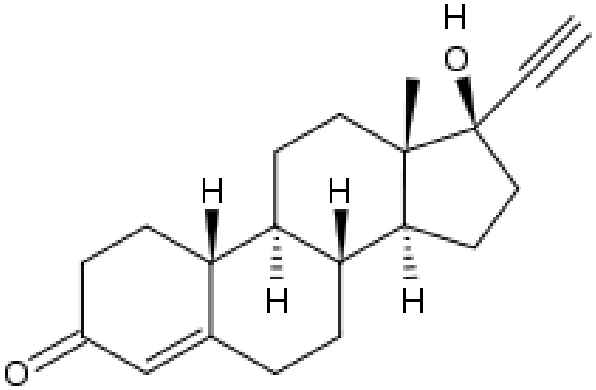
Mestranol



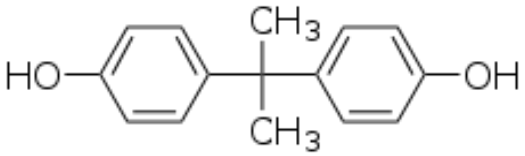
Progesterone



α-Estradiol

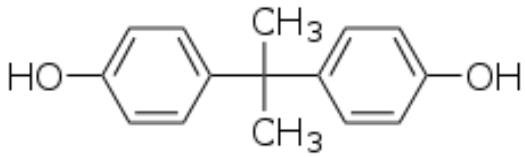


Norethindrone



Bisphenol A

- Also add bisphenol A to method
  - Nominated for CCL4
  - Deuterated bisphenol A used as surrogate M539
    - Extraction not an issue
  - But, bisphenol A contamination from instrument and solvent at least 8 ppb
  - Quantitation level can be no lower than 3 times LRB level
    - MRL would be above 24 ppb



Bisphenol A

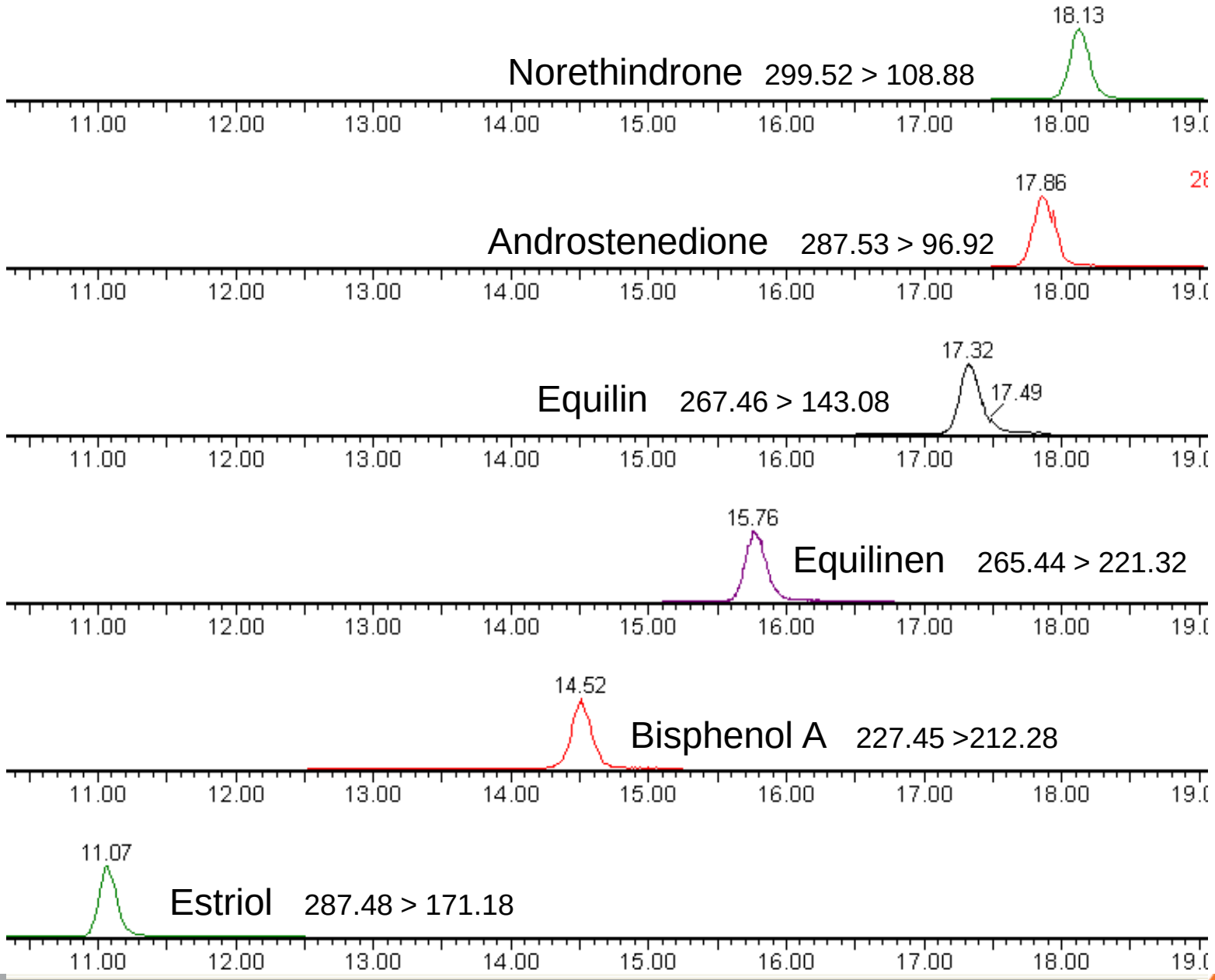
- Instrument manufacturer suggests use of an "isolator" column for bisphenol A
  - Contamination temporarily retained in short column placed between solvent mixer & pumps
  - Contamination separated on chromatogram by later elution
- Would bisphenol A require too many precautions for a "hormone" method? Separate method?

- Proposed target analyte list for Method 539 revision with additional five hormones & bisphenol A (in bold)

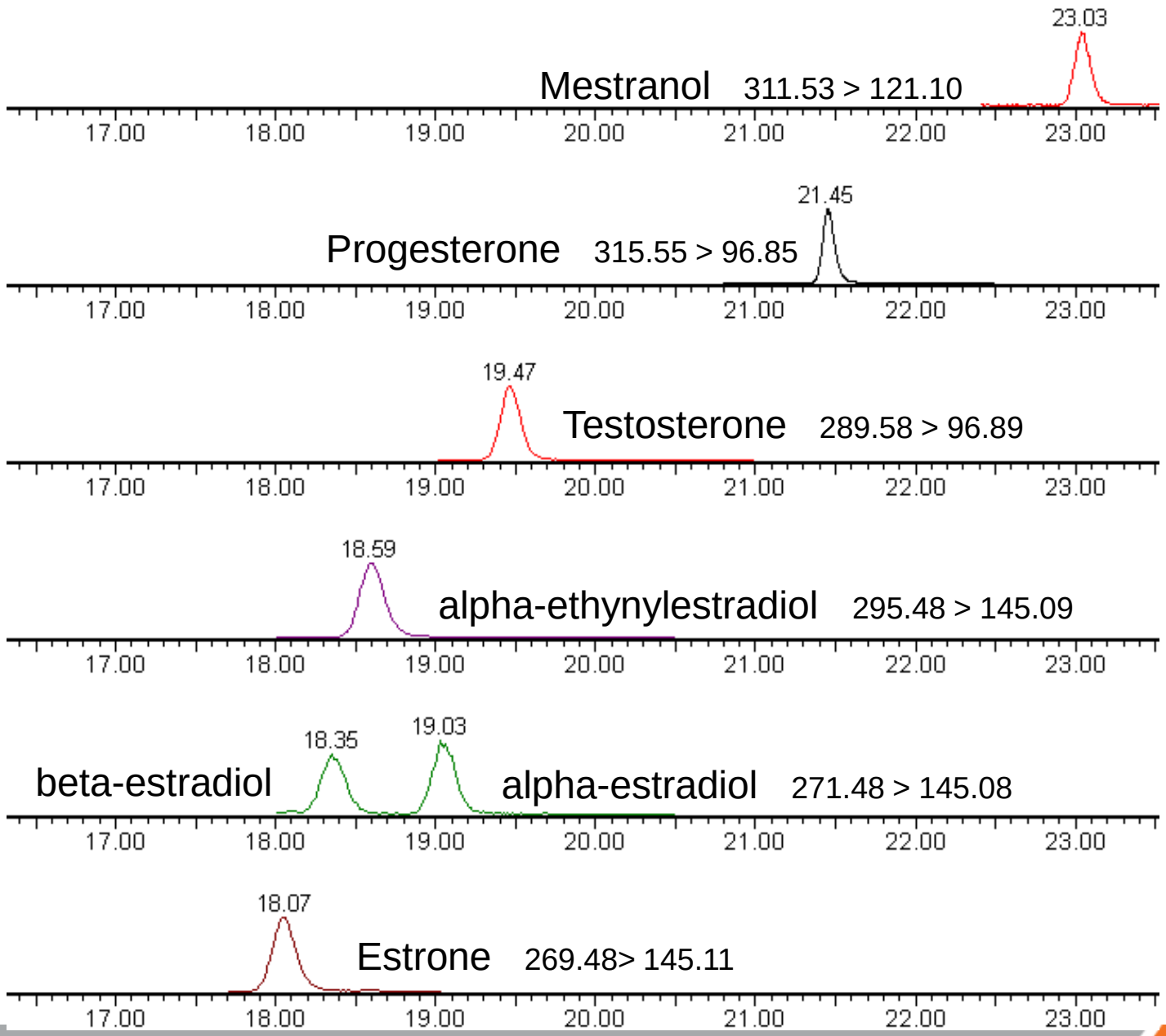
	CAS#
Androstenedione	63-05-8
Bisphenol A	80-05-7
Equilenin	517-09-9
Equilin	474-86-2
α-Estradiol	57-91-0
β-Estradiol	50-28-2
Estriol	50-27-1
Estrone	53-16-7
α-Ethynylestradiol	57-63-6
Mestranol	72-33-3
Norethindrone	68-22-4
Progesterone	57-83-0
Testosterone	58-22-0

- Initial chromatographic challenges
  - alpha- & beta-estradiol must be well separated, because precursor and product ions are same
  - Must use high water content (90%) in initial segment of gradient mobile phase program to focus bisphenol A contamination
- Develop calibration range
  - Test for reproducibility
  - Estimate lowest calibration level
- Extract spiked samples with C18 disks
  - If needed, use Oasis HLB disks
- Check for contamination or interference in Laboratory Reagent Blanks (LRBs) that contain preservation reagents, sodium omadine & sodium thiosulfate

- First 6


18.13
Norethindrone 299.52 > 108.88
11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.0
17.86
Androstenedione 287.53 > 96.92
11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.0
17.32
17.49
Equilin 267.46 > 143.08
11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.0
15.76
Equilinen 265.44 > 221.32
11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.0
14.52
Bisphenol A 227.45 >212.28
11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.0
11.07
Estriol 287.48 > 171.18
11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.0

- Last 7


23.03
Mestranol 311.53 > 121.10
17.00 18.00 19.00 20.00 21.00 22.00 23.00
21.45
Progesterone 315.55 > 96.85
17.00 18.00 19.00 20.00 21.00 22.00 23.00
19.47
Testosterone 289.58 > 96.89
17.00 18.00 19.00 20.00 21.00 22.00 23.00
18.59
alpha-ethynylestradiol 295.48 > 145.09
17.00 18.00 19.00 20.00 21.00 22.00 23.00
18.35
19.03
beta-estradiol
alpha-estradiol 271.48 > 145.08
17.00 18.00 19.00 20.00 21.00 22.00 23.00
18.07
Estrone 269.48> 145.11
17.00 18.00 19.00 20.00 21.00 22.00 23.00

- Collect data for demonstration of capability
  - Determine Lowest Concentration Minimum Reporting Level (LCMRL)
  - Run Precision & Accuracy studies at a low and a mid level in reagent water, surface water and ground water
  - Conduct holding time study for samples with preservatives
    - Holding time study for extracts
  - Conduct Multi-Laboratory Validation
    - At least two other labs
    - Use data to calculate MRL for method

Microbiology Methods Overview

Sandhya Parshionikar, Ph.D.

Office of Ground Water and Drinking Water
Standards and Risk Management Division
Technical Support Center

May 15, 2013

Candidate Contaminant List 3

Microbial Contaminant Name	Information
Adenovirus	Virus -respiratory and gastrointestinal illness
Caliciviruses Virus (includes Norovirus)	Virus - gastrointestinal illness
Campylobacter jejuni	Bacterium - gastrointestinal illness
Enterovirus	Group of viruses - mild respiratory illness
Escherichia coli (0157)	Toxin-producing bacterium - gastrointestinal illness and kidney failure
Helicobacter pylori	Bacterium - ulcers and cancer
Hepatitis A virus	Virus - liver disease and jaundice
Legionella pneumophila	Bacterium -lung diseases when inhaled
Mycobacterium avium	Bacterium - lung infection in those with underlying lung disease, and disseminated infection in the severely immuno compromised
Naegleria fowleri	Protozoan parasite -warm surface and ground water causing primary amebic meningoencephalitis
Salmonella enterica	Bacterium -gastrointestinal illness
Shigella sonnei	Bacterium -gastrointestinal illness and bloody diarrhea

Challenges to Microbial monitoring under UCMR

- Pathogen presence in biofilms
  - Biofilm sampling presents a challenge for UCMR
- Could be present in low concentrations
  - Need to sample large volumes of water
    - Presents logistical challenges for UCMR
- Pathogen presence in unconventional locations
  - Hospital and nursing home hot water tanks and storage tanks
    - Public health concern but challenging to monitor
- Some pathogens hard to grow in culture
  - Risk assessment with PCR data is a challenge

Method Development Rationale

- Depends on which pathogen to target for UCMR monitoring
  - What is the likelihood of a pathogen being found in finished drinking water?
  - Which pathogen has a validated method ready?
  - Which public health issue to address during UCMR monitoring?
    - Outbreaks
    - Other public health concerns
  - Under what conditions do we monitor?
    - Frequency
    - Volume of water
    - Seasonality
    - Type of public water system

Current Method Development Efforts

- *H.pylori*
- *E.coli* O157:H7
- Adenovirus
- *Mycobacterium*

Development of Methods to detect *H. pylori* and *E. coli* O157:H7 in Water

CCL Methods Meeting/Webinar

Keya Sen,

Technical Support Center, SRMD, OGWDW, OW US EPA

May 15, 2013

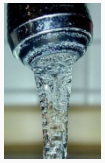
Facts about *H. pylori*

- *H. pylori* a gram negative bacteria
  - Causes chronic active and persistent gastritis
  - Stomach and duodenal ulcers and in few of these cases, gastric cancer
- Approximately 25 Million Americans suffer from the disease
  - Each year 500,000 to 850,000 new cases
- Culturable from stomach but unculturable from water
  - necessitates the use of PCR based methods

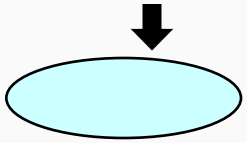
H. Pylori Method Development

- Analyzes 1 liter of sample collected
- Detects a segment of the gene *ure*A, conserved in all *H. pylori*, by qPCR
- Uses an internal control which is a process control and is added to every sample- a Bioball
  - Confirms whether a sample is a true negative

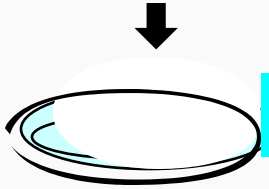
Detection of *H. pylori* from Water



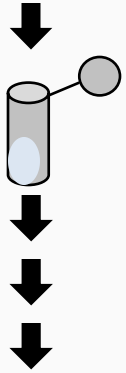
Collect 1 liter of drinking water. Spike with 10-1000 cells of *H. pylori*. Add 1 Bioball



Filter through 47mM, 0.4 micron



Extract filter with lysis solution



Remove solution to centrifuge tube.

Extract DNA into 33 µl water.

Use 11 µl (x3) for qPCR

Inter-laboratory validation of qPCR method for *H. pylori* detection

- Five volunteer laboratories selected
- Three drinking water samples (1L) tested
- One sterile reagent grade water (1L)
- 2 levels of spike used
  - 15 or 100 cells of *H. pylori*
- BioBall-KS 10 containing 30 cells of *E. coli* used as matrix spike (process control)

Initial and Ongoing Precision and Recovery (IPR and OPR) Acceptance Criteria[1]

Performance test*	*E. coli* KS10 BioBall™ acceptance criteria
Initial precision and recovery (IPR) • Mean percent recovery • Precision (as maximum relative standard deviation)	 25% - 379% (8-114 gene copies)[2] 107%
Ongoing precision and recovery (OPR) as percent recovery	Detect - 482% (145 gene copies)

[1]IPR requires the analysis of 4 reagent-grade water samples; OPR requires the analysis of a single reagent water sample

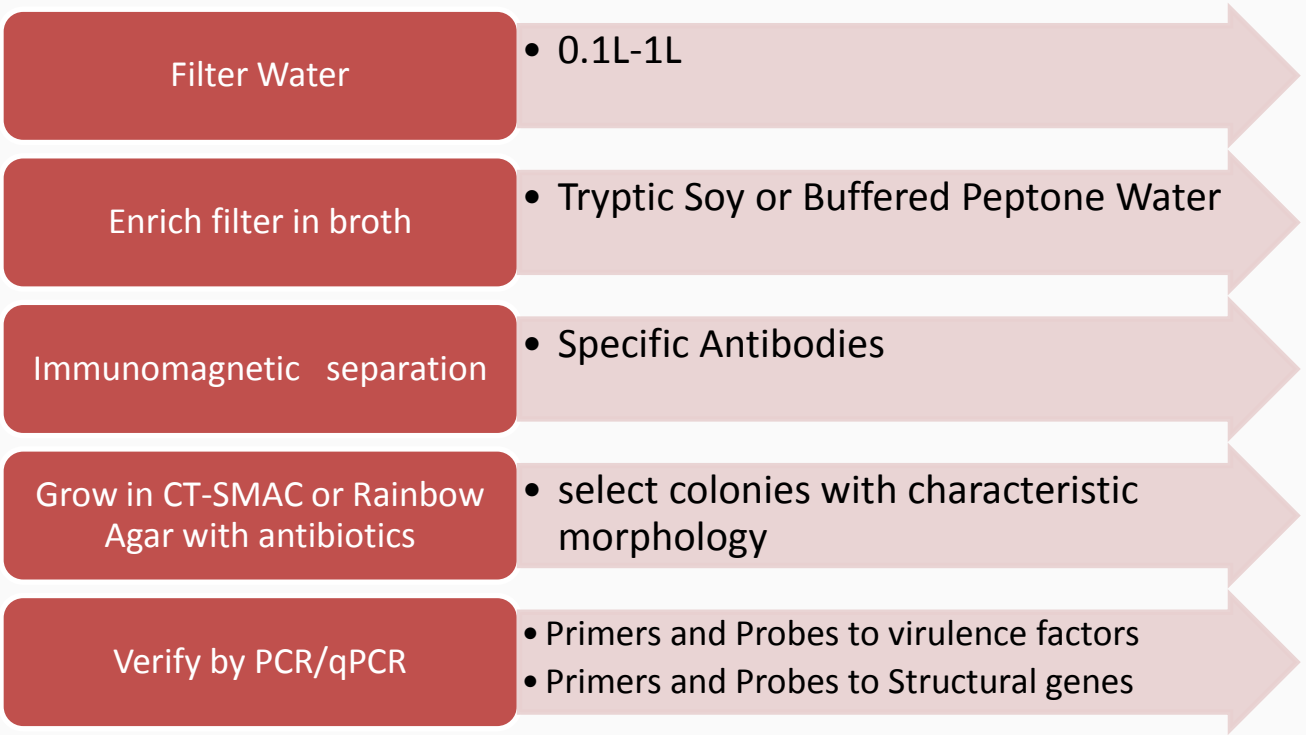
[2]One gene copy can be considered to be equivalent to one cell since each *E. coli* KS10 cell has the target cloned into a single copy plasmid.

E. Coli O157:H7 Method Development

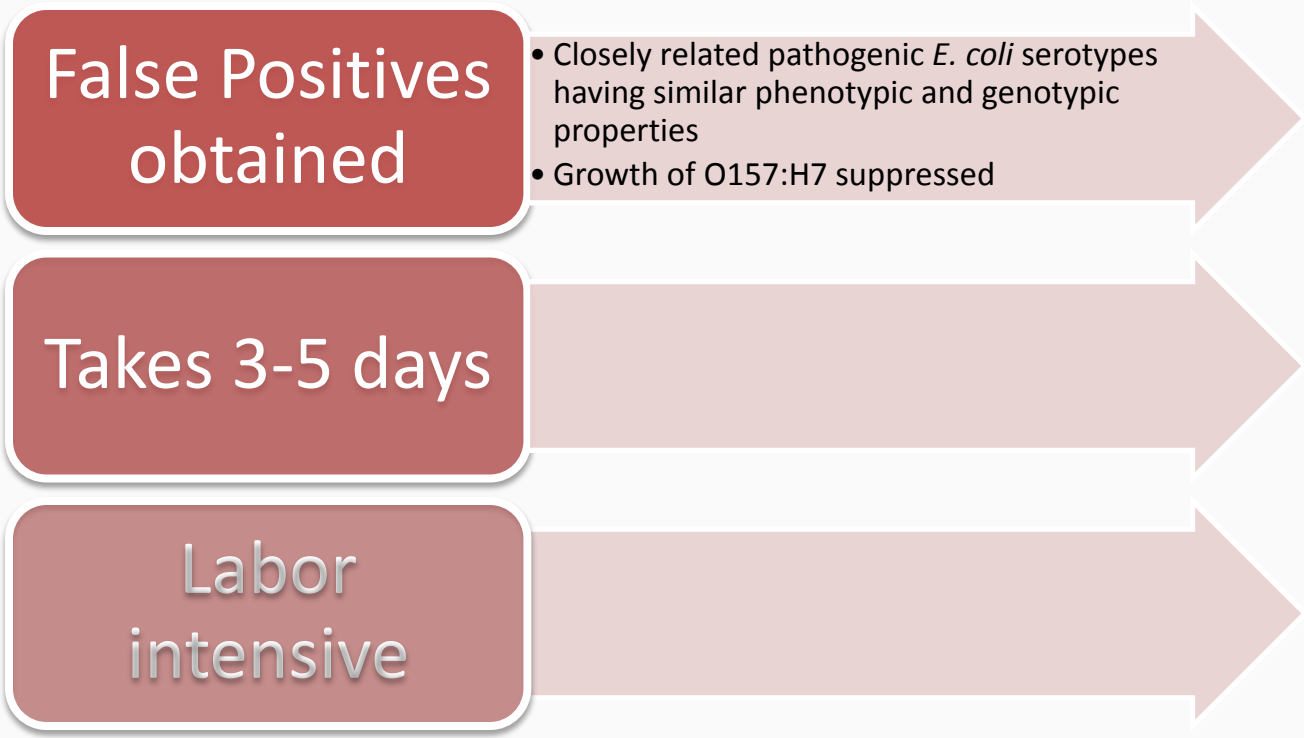
Facts about *E. coli* O157:H7

- Gram negative bacteria that lives in the guts of ruminants
- Causes severe illness
  - bloody diarrhea and vomiting
  - About 10 % develop hemolytic uremic syndrome (HUS) that can result in non-functioning kidneys
- About 95,400 cases each year from *E. coli* O157:H7 in the US
- Bacteria can be cultured from water but can be difficult to identify
  - Needs specialized approach

Traditional Methods for Detection of *E. coli* O157:H7



Issues With Detection of *E. coli* O157:H7 by Traditional Methods



Goals for EPA Method for *E. coli* O157:H7

Rapid method	• Detect in source and drinking water within a day
Highly Sensitive	• Able to detect 1-5 cells
Highly specific	• No false positives • Appropriate QC reagents
Able to detect viable cells	• Allow better risk assessment

Strategy

- Add an enrichment medium directly to water sample
- Provide conditions for stressed cells (starvation, chlorination induced), if present , to recover and grow
- Perform qPCR before and after enrichment without isolation of *E. coli* cells
  - Change in Ct value from pre to post enrichment will indicate viable cells
  - A decrease of 3.3 Ct value corresponds to 1 log increase in gene copies, if the amplification efficiency is 100%

Development of *E. coli* O157: H7 qPCR assays

- Searched literature for primers and probes
- Tested primers and probes with an ATCC strain
- Tested 4 commercially available kits with an ATCC strain
- Obtained 61 strains from USDA, FDA and in-house collection
  - Tested kits and probes

Selection of Targets/kits

- ABI MicroSeq kit produced no false positives
  - Targets two non-coding regions on the E. coli genome
  - Has an internal control for qPCR
- Additional triplex qPCR reaction optimized
  - Targets genes stx1, stx2, eae
  - Developed MGB probes
- Additional monoplex assay that targets *rfb*E gene optimized for confirmation
  - *rfb*E specific to O157 serotype
  - Developed MGB probes

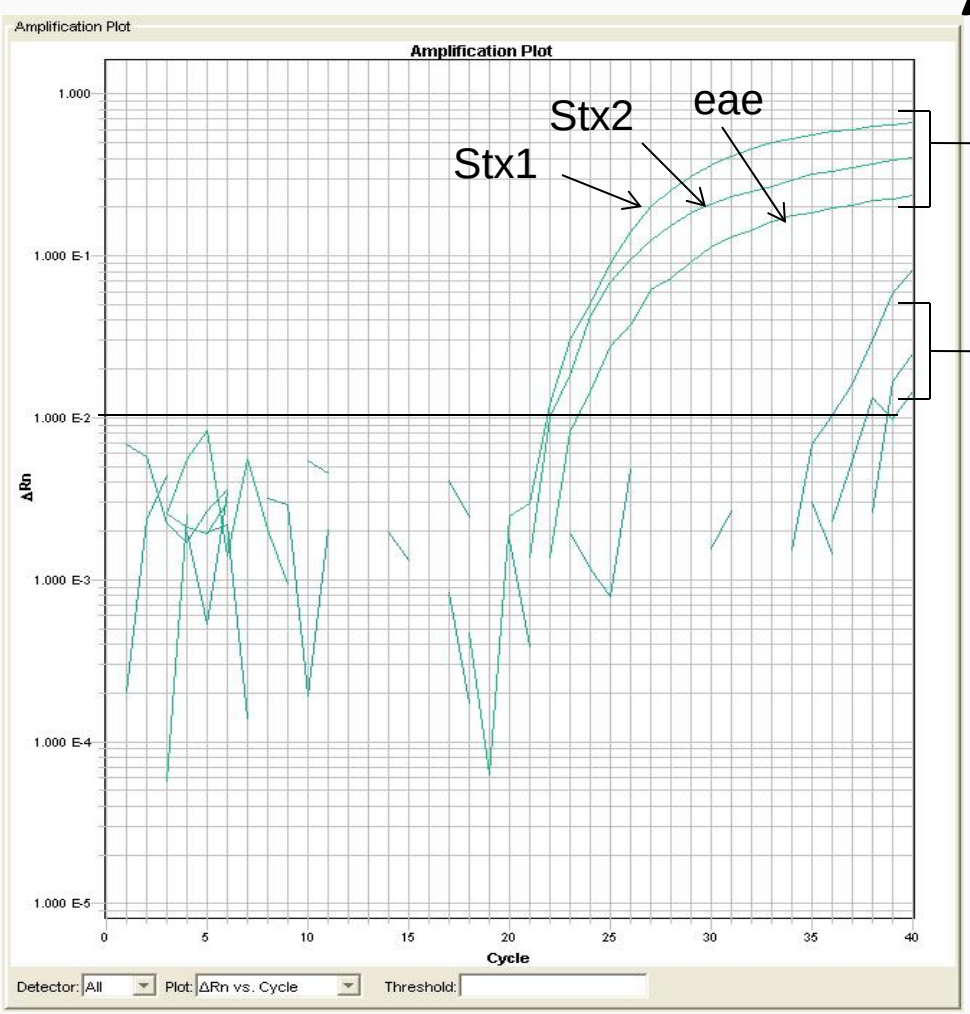
(*Environmental Science and Technology (2011): 45, 2250-2256*
Sen, K[1]. Sinclair[1], J, Boczek, L[2], Rice, E.G[3].)

Demonstration of Viability

- Bacterial Cells starved for 12- 14 days
- Dilutions made, 4-400 cells
- Spiked water samples with different dilutions
- Added 6X Presence-Absence broth to sample

  -1 mL aliquot removed and stored at – 20^0 C (pre-enrichment).
- Allowed to grow:35 ^{0}C for 18-23 hrs
  - 1 mL aliquot removed
- DNA extracted from both aliquots
  - Centrifuge to pellet cells
  - Add 100 μL of PrepMan buffer; Heat at 99 ^{0} C
- Perform 3 qPCR assays with 3 μL extract.
- Examine whether there is a shift in Ct values from pre to post enrichment samples

Demonstration of Viability



- Detects less than 10 cells
- Examines only 30 μL of sample

Addition of Process Control

- Bioball-KS10 used for *H. pylori* considered good for the purpose since they are *E. coli* cells but not expected to occur naturally
- Optimized the number of cells to add
- BTF-Biomerieux packaged 5000 cells into Bioball.
- Bioball added at the time of extraction
- Detected in a separate Monoplex assay,
  - Used same primers and probe used in *H. pylori* assay

Collection to Detection

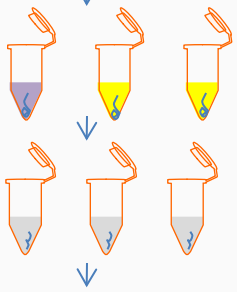
Collect 1L water

Add 200 mLPresence /Absence broth

Remove 1 mL. Store at -20 ^{0}C

Grow 1199 mL x 18-23 hrs at 35 ^{0}C till yellow.

Remove 2 x 1 mL



Add 1 Bioball-5000 to all three tubes
Centrifuge all tubes
Remove supernatant
Suspend Pellet in PrepMan Buffer
Heat to 95^{0} C x 10 mins
Centrifuge tubes x 2 mins

Use 3 μL of supernatant from each tube in 3 qPCR assays

ID → ABI MicroSeq (Triplex 1)
ID → Stx1, Stx2, eae (Triplex 2) → rfbE
ID → SPC (Monoplex)

Testing Method With Field Samples

- Six Surface water tested
  - Four had pathogenic *E. coli*
  - Only one sample had *E. coli* O157:H7

Inter-laboratory validation of qPCR method for detection

- Five volunteer laboratories selected
- Three drinking (ground) water samples (1L) tested by all 5 labs
- One sterile reagent grade water (1L) by all 5 labs
- One spike level used <10 cells

Initial and Ongoing Demonstration of Capability (IDC and ODC) Acceptance Criteria

Performance Test	Acceptance Criteria	
Initial Demonstration of Capability (IDC) (as Ct difference between pre and post enrichment samples). IPR requires the analysis of 4 reagent-grade water samples		
Target	**Ct difference**	**Precision (as standard deviation of Ct difference)**
MicroSEQ FAM	15.86- 25.21	1.79
MicroSEQ VIC	17.51-23.90	1.49
eae	15.11- 22.85	2.46
stx1	16.42-25.02	2.38
stx2	10.40-29.01	3.85
Ongoing Demonstration of Capability (IDC) (as Ct difference between pre and post enrichment samples). OPR requires the analysis of a single reagent water sample		
Target	Ct difference	
MicroSEQ FAM	15.74-25.33	
MicroSEQ VIC	17.44-23.97	
eae	13.40-24.56	
stx1	14.95-26.49	
stx2	10.55-28.86	

Conclusions and Next Steps

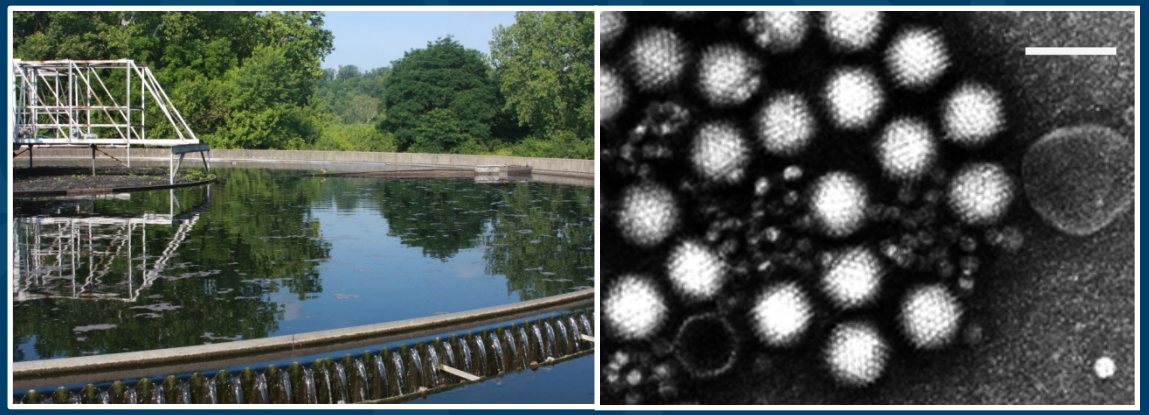
- Both methods have been developed and validated
- Currently being processed for EPA publication

United States Environmental Protection Agency

Adenovirus Methods: Method 1615 Modification and Small Volume Approach

G. Shay Fout, Jennifer L. Cashdollar, and Nichole E. Brinkman

U.S. Environmental Protection Agency, Cincinnati, OH



Office of Research and Development
National Exposure Research Laboratory, Cincinnati, Ohio

May 2013

Presentation Outline

- Method 1615
  - Rationale for the development and enhancement of Method 1615
  - Method overview
  - Water Cluster modifications
  - Method performance
- Small Volume Approaches
  - Method overview and performance
- Future development

EPA Method 1615 Rationale

Groundwater-Borne Disease Outbreaks in the USA

- From 1971 to 2008 there were nearly 846 outbreaks associated with an infectious agent in drinking water; about 50% of the outbreaks were attributable to groundwater
- During the same period, 8% of the outbreaks were caused by viruses and another 43% had a likely viral cause
- Numerous studies have found human enteric viruses in about 25% of wells studied
- Two studies focused on small community systems that use untreated groundwater
  - A study of communities in Southeast Michigan found virus presence in 24% of the wells (Francy et al., 2004)
  - A study of communities in Wisconsin found virus in 95% of the wells (Borchardt et al. 2012)

Virus Types, Frequencies, and Concentrations in Tap Water

Virus Type	Number qPCR Positive Samples	Virus Concentration Genomic copies/L		Number Culture Positive Samples
		Mean	Maximum	
Adenovirus	157 (13%)	0.07	9.5	40/157 (25%)
Enterovirus	109 (9%)	0.8	851.1	31/109 (28%)
GI Norovirus	51 (4%)	0.6	115.7	
GII Norovirus	0 (0%)	0	0	
Hepatitis A	10 (1%)	0.006	4.1	
Rotavirus	1 (0.1%)	2×10^{-5}	0.03	
All Viruses	287 (24%)	1.5	853.6	

N = 1,204 samples from 14 Communities; Infectivity was measured on qPCR positive samples only; Reference: Borchardt *et al.* 2012.

EPA Method 1615 Rationale

- The Wisconsin study demonstrated that **22% of the AGI** in the study communities was from virus-contaminated tap water (Borchardt et al. 2012. Environmental Health Perspectives 120:1272-1279)
- For children < 5 yrs in the spring of 2006, the fraction of AGI from norovirus in drinking water was **63%**!

EPA Method 1615 Rationale

Virus in U.S. Source Waters
Percent Cell Culture Positive

Region	Samples	Plant Intakes
East	28	85
South	22	86
Midwest	21	90
West	20	83
Totals	24	87

Average titer all samples: 1.5 MPN/100 L (range: 0-1974)

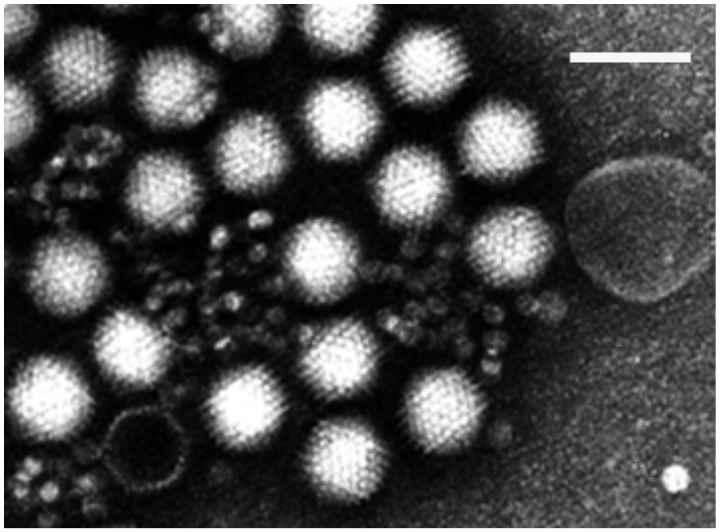
EPA Method 1615 Enhancement

Adenovirus in Wastewater

Adenovirus	Genomic Copies/Liter
Raw/Primary Effluent	2,000 – 800,000,000
Tertiary Effluent	930 – 8,900,000

References:

Bofill-Mas et al., 2006
Katayama et al., 2008
Dong et al., 2009
Kuo et al., 2010
Hewitt et al., 2011
Sidhu et al., 2013
Brinkman et al., submitted



EPA Method 1615 Overview

Filtration/Sample Concentration

Samples:

NanoCeram filters (1800 L of groundwater)

Controls:

PT and PE: Filters containing unknown amounts of virus (10 L spiked)

QC: Filters containing 0 or 500 MPN of virus (10 L spiked)

Matrix: Filters containing 1,000 MPN of virus (1790 + 10 L spiked)

Elution

2 × 500 mL 1.5% beef extract, 0.05 M glycine, pH 9.0

Organic flocculation

pH 3.5 for 30 min

Resuspend floc in 30 mL 0.15 M sodium phosphate, pH 9.0

Subsample 1: approx. 9 mL

Cell Culture

Quantal assay

MPN/L

Subsample 3 Approx. 12 mL

Archive

Subsample 2: approx. 9 mL

Tertiary Concentration

Vivaspin 20, 30 kDa MWCO

RNA Extraction

QIAamp DNA Blood Mini Kit

RT-qPCR

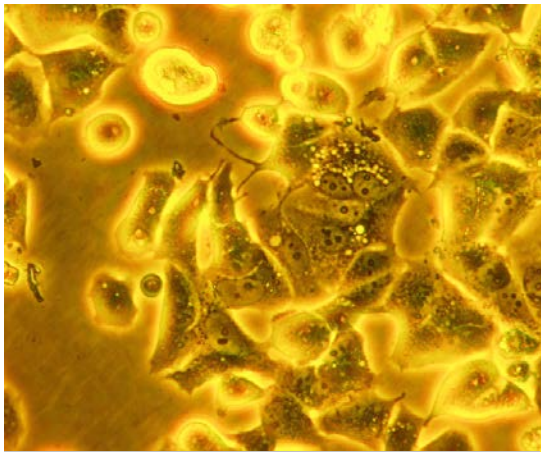
Genomic copies/L

References:

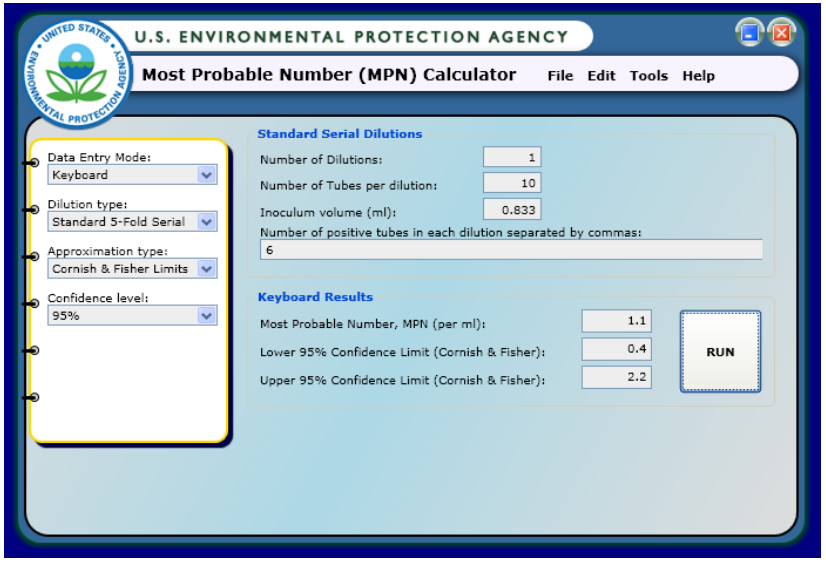
Fout *et al*. 2012. EPA/600/R-10/181, January 2012 revision

Cashdollar *et al*. 2012. Applied Environmental Microbiology 79:215-223

Total Culturable Virus Assay



BGM cells showing early cytopathic effect from poliovirus



QUANTITATION OF TOTAL CULTURABLE VIRUS DATA SHEET					
Sample Number: Sample 1					
Sample	Number Replicates Inoculated	Number with CPE (1)	MPN/mL (2)	95% Confidence Limits/mL	
				Lower	Upper
Undiluted	**10**	**6**	**1.1**	**0.4**	**2.2**
1:5 Dilution	**0**				
1:25 Dilution	**0**				
1:125 Dilution	**0**				

$$M_L = \frac{M_{mL}\ S}{D} = (1.1\ \text{MPN/mL} * 8.33\ \text{mL})/1800\ \text{L} = 0.02\ \text{MPN/L}$$

Additional Concentration for the Molecular Assay

Tertiary Concentration with Vivaspin Concentrators

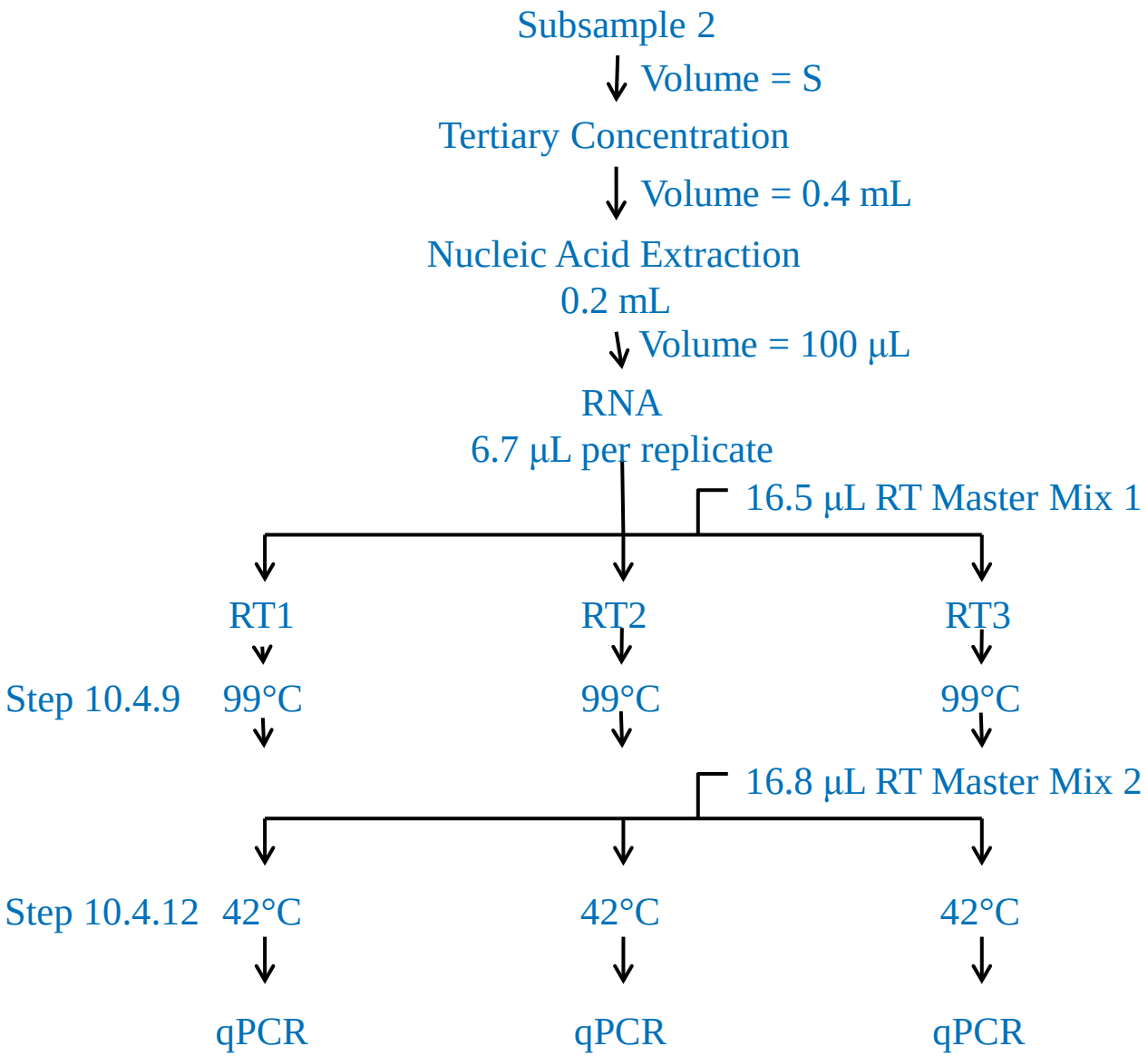
- Fill Vivaspin 20 unit with PBS, 0.2% BSA
- Soak overnight at 4 ° C. Discard PBS
- Rinse with sterile water. Discard water
- Add an amount of Subsample 2 = S
- Centrifuge at 3,000 – 6,000 x g to a volume less than 0.4 mL
- Add 1 mL of 0.15 M sodium phosphate, pH 7-7.5
- Centrifuge at 3,000 – 6,000 x g to a volume less than 0.4 mL
- Add 1 mL of 0.15 M sodium phosphate, pH 7-7.5
- Centrifuge at 3,000 – 6,000 x g to a volume less than 0.4 mL
- Transfer sample to a 1.5 mL microcentrifuge tube
- Bring to a volume of 400 ± 2 µL

Tertiary Concentration

Recovery of Poliovirus and Murine Norovirus Using Vivaspin 20

	Poliovirus	Murine Norovirus
30,000 MWCO	80.9%	45.6%
100,000 MWCO	55.1%	26.1%

Reverse Transcription – Quantitative PCR



Reverse Transcription

Reverse Transcription

- Prepare master mixes
- Add 16.5 µL RT Mix 1
- Add 6.7 µL of sample
- Add 6.7 µL PCR-grade water as no template controls
- Heat at 99 ° C for 4 min
- Quench on ice
- Add 16.8 µL RT Mix 2
- Run at 25 ° C for 15 min, 42 ° C for 60 min, 99 ° C for 5 min, hold of 4 ° C

Ingredient	Volume per reaction (1)	Final concentration	Volume per Master Mix (2)
		RT Master Mix 1	
Random primer (Item 7.5.11)	0.8	10 ng/µL	84.0
Hepatitis G Armored RNA (3) (Item 7.5.12)	1.0		105.0
PCR grade water (Item 7.5.2)	14.7		1543.5
Total	16.5		1732.5
		RT Master Mix 2	
10X PCR Buffer II (Item 7.5.13)	4.0	10 mM tris, pH 8.3, 50 mM KCL	420.0
25-mM $MgCl_2$ (Item 7.5.13)	4.8	3 mM	504.0
10-mM dNTPs (Item 7.5.14)	3.2	0.8 mM	336.0
100-mM DTT (Item 7.5.15)	4.0	10 mM	420.0
RNase Inhibitor (Item 7.5.10)	0.5	0.5 units/µL	52.5
SuperScript II RT (Item 7.5.16)	0.3	1.6 units/µL	31.5
Total	16.8		1764.0

(1) The volumes given are for 40-µL RT assays.

(2) Reagent amounts sufficient for a 96-well PCR plate are given. The volumes shown were calculated by multiplying the volume per reaction amount by the number of assays to be performed, plus an additional 9 assays to account for losses during transfer of the master mix to plates (Item 6.6.15) using items 6.6.9 and 6.6.12. The amount of additional assays to add can be reduced if experience shows that lower amounts are adequate.

(3) Hepatitis G Armored RNA is supplied as an untitered stock. The amount to use must be determined for each lot, as described in Step 13.6.1.

Quantitative PCR

Ingredient	Volume per reaction (µL) [1]	Final concentration	Volume per Master Mix (µL) [2]
2X LightCycler 480 Probes Master Mix (Item 7.4.17) [3]	10.0	Proprietary	1050.0
ROX reference dye (Item 7.4.18) [4]	0.4	0.5 mM	42.0
PCR grade water (Item 7.4.2)	1.0		105.0
10 µM EntF	0.6	300 nM	63.0
10 µM EntR	1.8	900 nM	189.0
10 µM EntP	0.2	100 nM	21.0
Total	14.0		1470.0

(1) The volumes given are for using 6 µL of cDNA from Step 12.5.3 in a qPCR assay using a total qPCR volume of 20 µL.

(2) Reagent amounts sufficient for a 96-well PCR plate are given. The volumes shown were calculated by multiplying the volume per reaction amount by the number of assays to be performed, plus an additional 9 assays to account for losses during transfer of the master mix to tubes or plates. The amount of additional assays to add can be reduced if experience shows that lower amounts are adequate.

(3) 10X PCR Buffer II , 25-mM $MgCl_2$, and AmpliTaq Gold can be substituted for the LightCycler 480 Probe Master Mix.

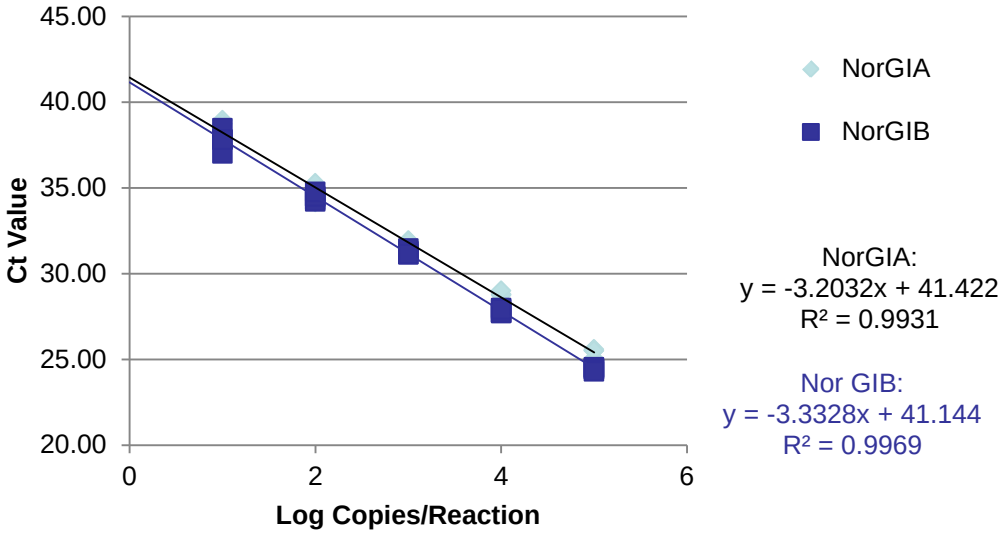
(4) This reagent is necessary for use with Applied Biosystems and similar instruments. It should be substituted with PCR grade water for use with the LightCycler and similar instruments.

Quantitative PCR

Standard Curves Using Armored RNA® EPA-1615

- All results must be based upon standard curves run in duplicate on every RT-qPCR plate
- Standard curves are prepared at concentrations of 2.5×10^8 to 2.5×10^3, giving 502,500 to 5 genomic copies per standard
- Acceptable standard curves must have a R^2 value of > 0.97 and an efficiency of 80-110%
  - R^2 and slope are calculated by the thermocycler
  - Efficiency is calculated by the thermocycler or as shown in the example below
  - For example, a curve having a slope of -3.29 has a % efficiency of $100 \times (10^{-1/-3.29} - 1) = 101.3\%$

Armored RNA® EPA-1615 Norovirus GI Standard Curve



Target	Efficiency	Theoretical Detection Limit (# Particles)
NorGIA	105.4%	3
NorGIB	99.7%	2

Mean Recovery (%) of Spiked Poliovirus and Murine Norovirus from Ground, Surface, and Reagent-Grade Water Samples

Assay	Groundwater n=7		Reagent-Grade Water (High Titer[b]) n=6		Reagent-Grade Water (Low Titer[c]) n=6	
	Recovery	CV[a]	Recovery	CV[a]	Recovery	CV[a]
Total Culturable Virus Assay	58	79	42	34	122	96
Poliovirus RT-qPCR	20	64	48	36	39	29
Murine Norovirus RT-qPCR	30	75	0.6	100	8	83

[a]CV is % coefficient of variation
[b]High titer spike at 1000 MPN of poliovirus and 1000 PFU of murine norovirus per 10 L sample
[c]Low titer spike at 300 MPN of poliovirus and 300 PFU of murine norovirus per 10 L sample

Reference: Cashdollar et al., 2012

Issues with EPA 1615

- Filter clogging in turbid waters
- An assay for adenoviruses was not included in the method
- Poor virus recovery from turbid waters
- Turbid waters have RT-qPCR inhibitors

Water Cluster Modifications

Project P1.5.3A.D.1: Improvements, Field Evaluation, and Commercialization of EPA Method 1615

Subobjective 1.A.i. Prefilter and electropositive filter options: Enhance ability to collect required sample volume when testing turbid waters

Subobjective 1.A.ii. Ultrafiltration versus NanoCeram – Determine the relative effectiveness of using ultrafiltration versus the NanoCeram electropositive filter for concentration of secondary and tertiary effluent

Subobjective 1.A.iii. Celite versus NanoCeram – Determine the relative effectiveness of the NanoCeram versus the small volume method

Subobjective 1.A.iv. Addition of Sea Salts – Determine whether the addition of sea salts can enhance recovery of norovirus recovery

Subobjective 1.A.v. Sonification, Dispersants, and Surfactants – Determine if sonication or treatment with dispersants and surfactants will improve recovery as measured by plaque assay and RT-qPCR

Water Cluster Modifications

Subobjective 1.B. Dilutions for Samples with High Virus Levels – quantify the loss of precision resulting from the use of 10-fold rather than 5-fold dilutions when assaying samples with high virus titer

Subobjective 1.C. Tertiary Concentration - Large Volume Extraction Option – Examine if virus recoveries can be improved by replacing the tertiary concentration followed by a small volume RNA extraction approach with a large volume RNA extraction

Subobjective 1.D. Inhibitor Removal - Proprietary versus EPA Options: improve ability to remove RT-qPCR inhibitors from turbid waters

Subobjective 2.A. and 2.B. Add an adenovirus culture and molecular assay to 1615

Water Cluster Modifications

Objective 3. Develop a RT-qPCR Commercial Kit and compare it to our current approach

Objective 4. Develop an Infectious Virus RT-qPCR assay using PMA

Objective 5. Measurement of Virus Occurrence

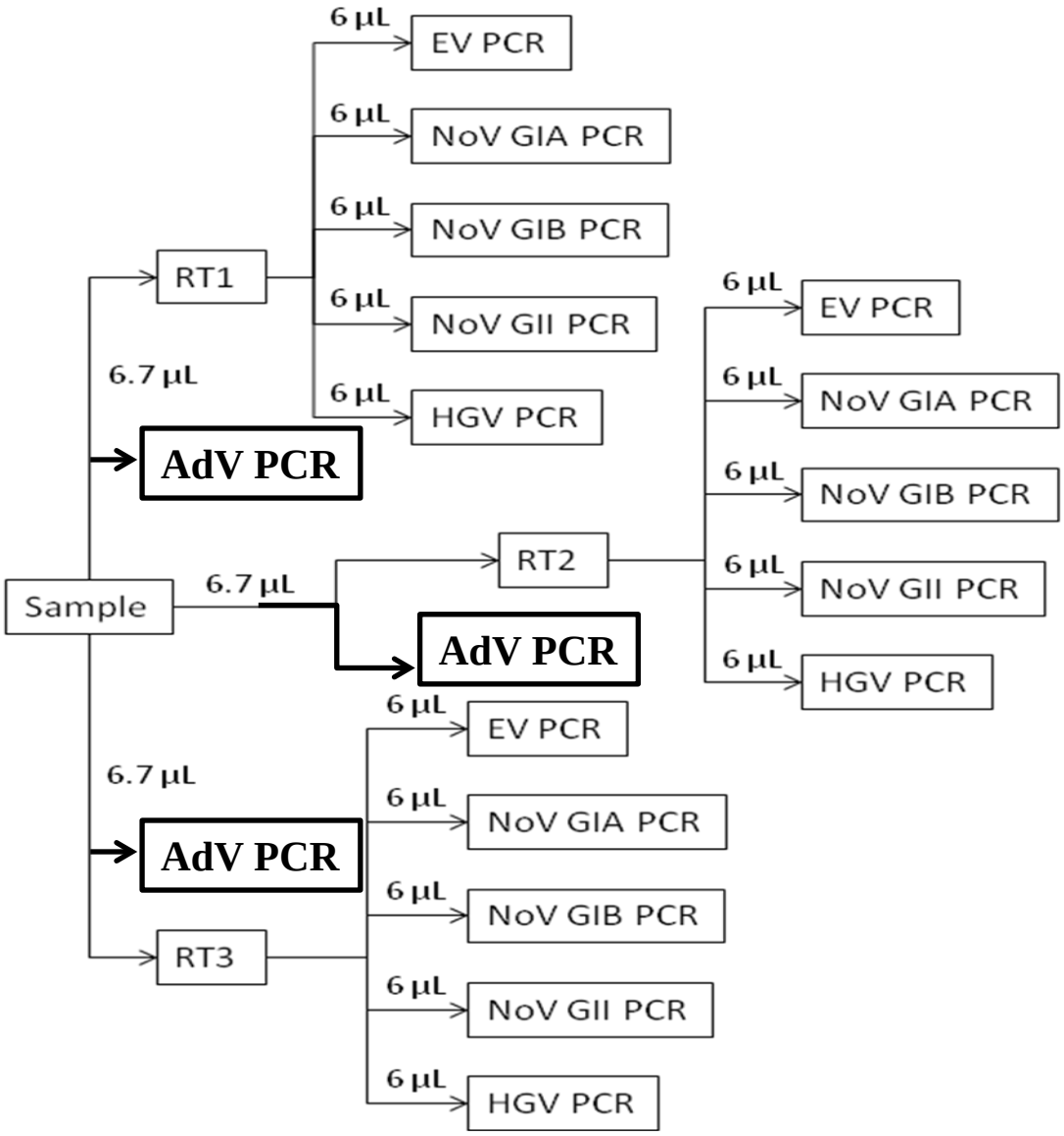
- Measure virus in primary, secondary, and tertiary effluents at AAA Wastewater Services, Hamilton Water Reclamation Facility (biological with chlorine treatment), Sycamore Wastewater Treatment Plant (nutrient removal plant), Twin Creek Preserve, and Upper Mill Creek Regional Water Reclamation Facility (biological with UV)
- Compare virus occurrence with indicator occurrence

Addition of Adenovirus to Method 1615

Adenovirus Culture Assay using A549 cells

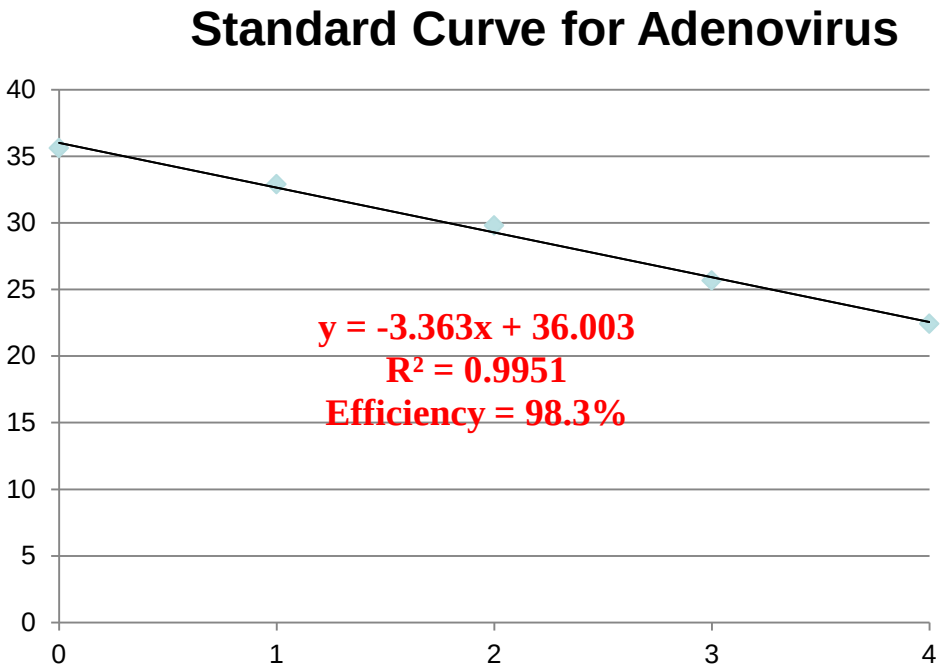
Process Control	% Recovery
Negative	-
Positive 1	18
Positive 2	32
Positive 3	28
Positive 4	12
Positive 5	7
Positive 6	3
Positive 7	12
Positive 8	96
Positive 9	29
Mean Recovery	26
CV	108

Addition of Adenovirus to Method 1615

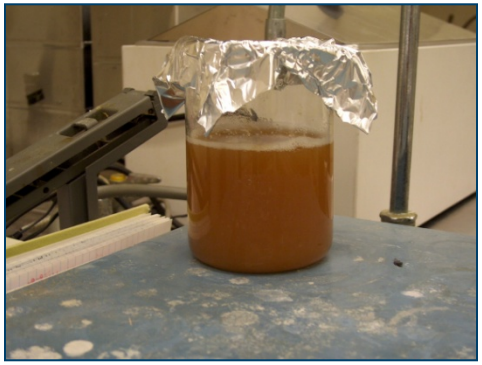


Adenovirus PCR

Prepare Standard Curves for Adenovirus using quantified Adenovirus 5 from OD260, Inc.



Small Volume Method



Celite Method

1. Add 1.5g of celite to the eluent
2. Drop the pH to 4 and mix for 10-30 minutes
3. Collect celite on a sterile pre-filter
4. Elute virus from celite with 40-80 ml of 0.15M sodium phosphate
5. Adjust pH to 7-7.5. Filter sterilize. Freeze for analysis

Recoveries range from 60-90% for enteroviruses and 65-69% for adenovirus

Future Development

- As part of the Water Cluster Project
  - Improve virus detection for turbid waters
  - Test robustness of EPA Method 1615 for measuring virus occurrence at wastewater “End-of-Pipe” settings and in recreational settings
- Test whether the small volume method is scalable to 10 L of secondary or tertiary effluent

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Questions?

Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Detection Methods for Mycobacteria

Stacy Pfaller

Ohio River and Downtown Cincinnati, OH

Disclaimer: This presentation does not necessarily reflect official U.S. EPA policy

Office of Research and Development
National Exposure Research Laboratory, Microbial and Chemical Exposure and Assessment Research Division

May 15, 2013

United States Environmental Protection Agency

Background

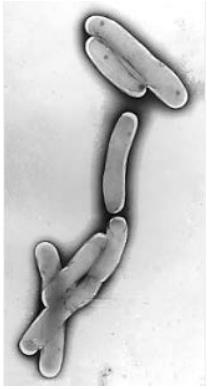
- Waterborne illness caused by nontuberculous mycobacteria (NTM) costs the US nearly $500 M in hospitalizations per year ([a]Collier et al, 2012).
- Pulmonary NTM infections account for almost half of all NTM hospitalizations in the US, and are typically caused by *Mycobacterium avium* (MA) and *M. intracellulare (MI)*
- In addition to pulmonary infections, cause skin, soft tissue, lymph node, systemic infections, among others
- Primary source of human exposure: **WATER**[a]
- **CCL's 1 and 2:** *Mycobacterium avium* Complex (MAC)
- **CCL 3:** *M. avium*

  4 subspecies: *M. avium* subsp. *hominissuis*

  M. avium subsp*. avium*

  M. avium subsp*. silvaticum*

  M. avium subsp*. paratuberculosis*



[a]Collier et al (2012) Epidemiology and Infection, 140: 2003-2013

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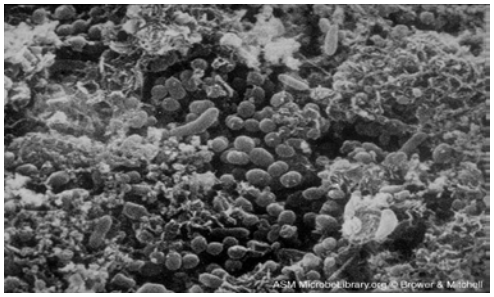
Culture and Molecular Methods



- **Culture Method**

  Standard Methods for the Examination of Water and Wastewater[a] section 9260M

  Modifications described in Covert et al (1999) Appl Environ Microbiol 65:2492-2496

- **Quantitative PCR (qPCR)**

  Beumer et al, (2010) Appl Environ Microbiol 76:7367-7370 and Figure S1, Supplemental Material http://aem.asm.org/content/76/21/7367/suppl/DC1

  Primers and probe for *M. avium* and *M. intracellulare* have been submitted for publication

[a]Eaton, A. D., L. S. Clesceri, E. W. Rice, and A. E. Greenberg (ed.). 2005. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Washington, DC.

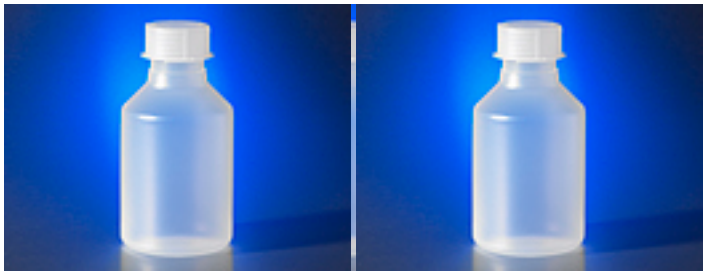
Sample Collection

- Sample collection is identical for both culture and qPCR
- Bulk Water: collected in 1L sterile polypropylene bottles according to sections 9060A and B of Standard Methods for the Examination of Water and Wastewater[a], except NO preservative ($Na_2S_2O_3$).
- Biofilm: collected by swabbing surface of tap, pipe, tubing, etc.
- Samples transported back to lab on ice, stored at 4 ° C until processing, within 48 hours.

[a]Eaton, A. D., L. S. Clesceri, E. W. Rice, and A. E. Greenberg (ed.). 2005. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Washington, DC.

Culture method

- 1L water split into 2 x 500 ml aliquots or some volume of biofilm slurry (1 – 2 ml typically)
- cetyl pyridinium chloride (CPC) is added to a final concentration of 0.04% for reduction of background organisms
- Samples shaken and incubated at room temperature for 30 min
- Samples filtered through 0.45 um pore-size, 47 mm black-grid, cellulose ester filter by vacuum filtration, washing the filter with sterile deionized water.
- filter aseptically transferred to Middlebrook 7H10 agar containing 500 mg L^{-1} cycloheximide
- Plates are incubated a **minimum 8 weeks** at 37 ° C and inspected weekly for growth



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QC samples for Culture method

❖**Sterile medium negative control**

- Performed when medium is made, in advance of samples arriving
- Incubation of un-inoculated medium to ensure sterility

❖**Method blank negative control**

- Sterile deionized water filtered processed at the same time in the same way as unknowns

❖**No CPC control**, if possible

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Benefits of Culture Method

- Many NTM can grow on medium
- Live only detection
- Obtain a culture collection for future characterization
  - Genotype
  - Virulence genes

Drawbacks of Culture Method

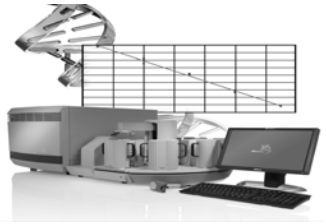
- Method has not been characterized for specificity or sensitivity
- ***medium is not selective for mycobacteria***
- CPC disinfection may reduce recovery of target by 70%

  (Personal communication: Terry Covert)
- Every colony is an unknown in need of identification
- Only a subset of colonies can be chosen for identification
- ***method is not quantitative***
- Months to years before results are obtained
- Performs poorly on biofilm

qPCR Method

- Water/biofilm slurry is vacuum filtered through 47.0 mm, 0.45 um polycarbonate membrane (no CPC treatment necessary)
- Membrane rolled and placed in 2.0ml tube containing 0.3g glass beads and buffer
- Microorganisms trapped on membrane lysed physically by bead beating
- DNA from crude lysate extracted using WaterMaster kit reagents from (EpiCenter Biotechnologies, Madison, WI)
- DNA resuspended in sterile, molecular biology-grade water
- Three replicate qPCR reactions analyzed/ DNA extract
  - Two replicates must be positive for a sample to be considered positive

MA/MI/MAP-specific assay



Data Analysis
Absolute Quantification

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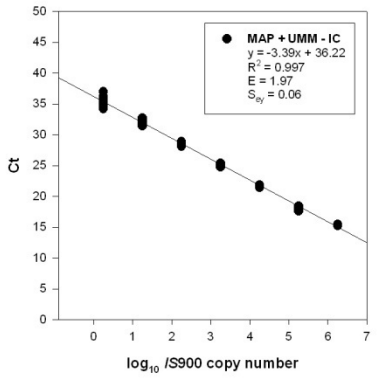
Absolute Quantification from Master Standard Curves

- Generated from six independents series of 10-fc serial dilutions of purified genomic DNA from ATCC Type strains of MA, MI, and MAP
- Each dilution series contains eight standards, ranging in concentration from 10^6 target copies t 1 copy, run in triplicate = 18 measurements/ standard
- C_T measurements plotted against log target number and analyzed by linear regression to generate line equation
- Target number in unknown sample estimated frc line equation

A



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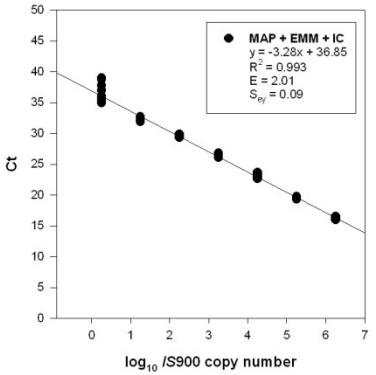

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QC Samples for qPCR

❖**Method Blank** (negative control)

- Sterile molecular biology grade water or sterile swab filtered and processed at the same time in the same way as unknowns

❖**Standards** (positive control)

- Purified genomic DNA from target, serially diluted

❖**No Template Control** (negative control)

- Sterile molecular biology grade water added to qPCR reaction instead of DNA extract

❖**Internal positive Control** (IC)

- Commercially available kit (TaqMan Exogenous Internal Positive Control Kit, Life Technologies, Carlsbad, CA)

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Characteristics of qPCR assays for drinking water and biofilm

qPCR assay	Target (copies/ genome)	E¶ Amplification Efficiency	LOD‡ Targets/qPCR reaction	LOQ† Targets/qPCR reaction	Specificity£	Sensitivity § Drinking Water	Sensitivity § Biofilm
M. avium	16S rDNA (1)	1.92	10	10	100%	100 cells/L	1000 cells/swab
M. avium subsp. paratuberculosis	IS*900* (14-20)	2.01	1.8	1.8	100%	100 cells/L	Not determined
Beumer et al, 2010, Appl Environ Microbiol	Target 251 (1)	Not determined	Not determined	Not determined	95%	Not determined	Not determined
M. intracellulare	16S rDNA (1)	1.91	10	10	100%	10 cells/L	1000 cells/swab

¶Amplification Efficiency = 10(1/-slope). If E = 2, the reaction is 100% efficient.
‡LOD = Limit of detection = lowest copy number/assay giving CT < 40 in 6/6 independent assays.
†LOQ = Limit of quantification = lowest copy number/assay yielding a coefficient of variation < 25%.
£Specificity = Number of target testing positive/total number targets tested x 100.
§ Sensitivity = lowest copy number detected when spiking serial dilutions of known cell quantities into actual tap water samples, processed as described in Beumer et al, 2010, Appl Environ Microbiol, 76:7367-70

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Benefits of qPCR Method

- Assays are specific for MA and MI (CCL)
- No CPC treatment
- Time to results = 3 days

Drawbacks of qPCR Method

- Assays are specific for MA , MI, and MAP only
- Cannot distinguish between live and dead organisms though studies have demonstrated that DNA contained within chlorine disinfected cells does not typically persist in water with a chlorine residual

  Page et al, 2010, Appl Environ Microbiol, 29:2946-2954

  Sen et al, 2010, Current Microbiol, 62:727-732

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National Occurrence Study using Culture

- Covert et al (1999) Appl Environ Microbiol, 65:2492-2496
- 139 samples
  - Drinking water (ground and surface water sources)
  - Bottled water
  - Cistern
  - Ice
  - Reservoir
- **Results**
  - 1/139 samples positive for MA (drinking water from surface water source)
  - 5/139 samples positive for MI (drinking water from surface water source)

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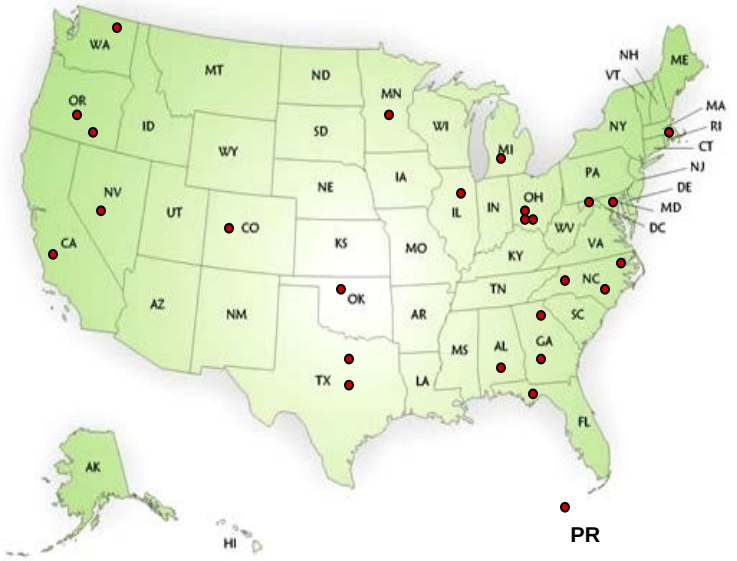
Occurrence Revisited
MJ Study: National Survey of MA and MI Occurrence using Culture and qPCR, 2009-2010

- 40 sites across the US
- 68 taps (1 to 2 taps/site)
  - ground + no treatment = 2
  - ground + chlorine = 6
  - ground + chloramine = 1
  - surface + no treatment = 0
  - surface + chlorine = 15
  - surface + chloramine = 12
  - mixed + chlorine = 2
  - mixed + chloramine = 2
- Samples collected 3 times in 2009, once in 2010



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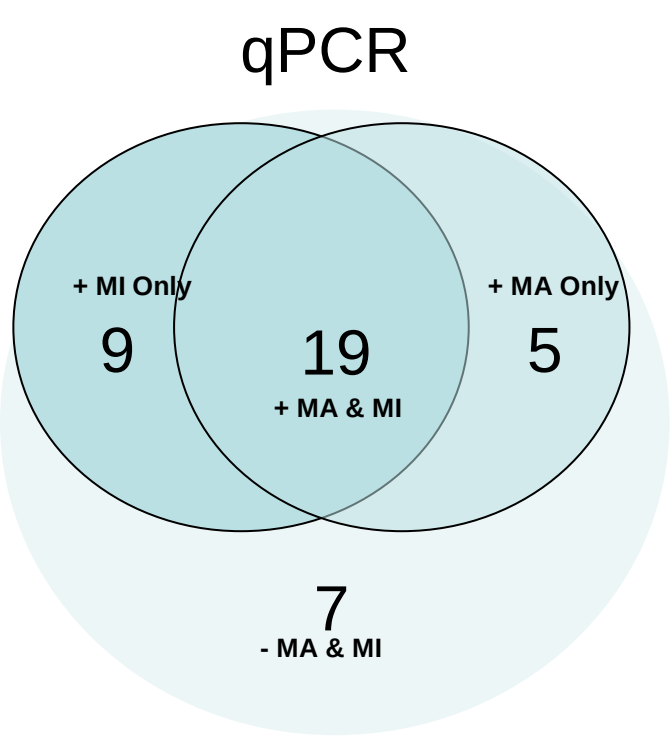
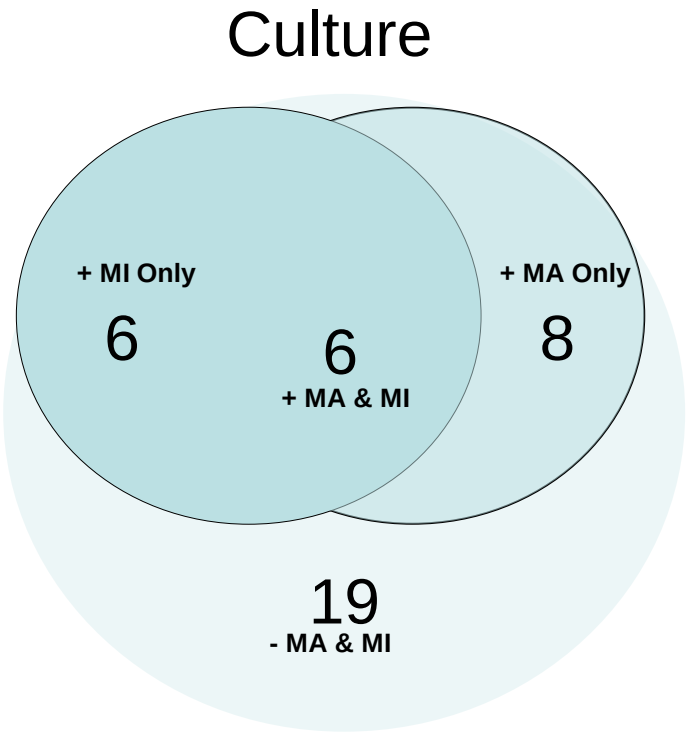
qPCR and Culture Results by Site



Number of Sites
N = 40



Number of Sites
N = 40

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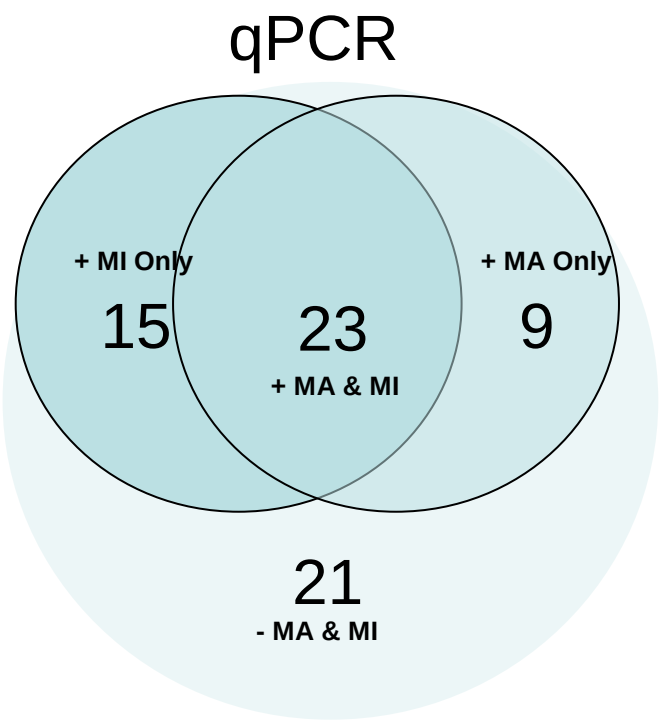
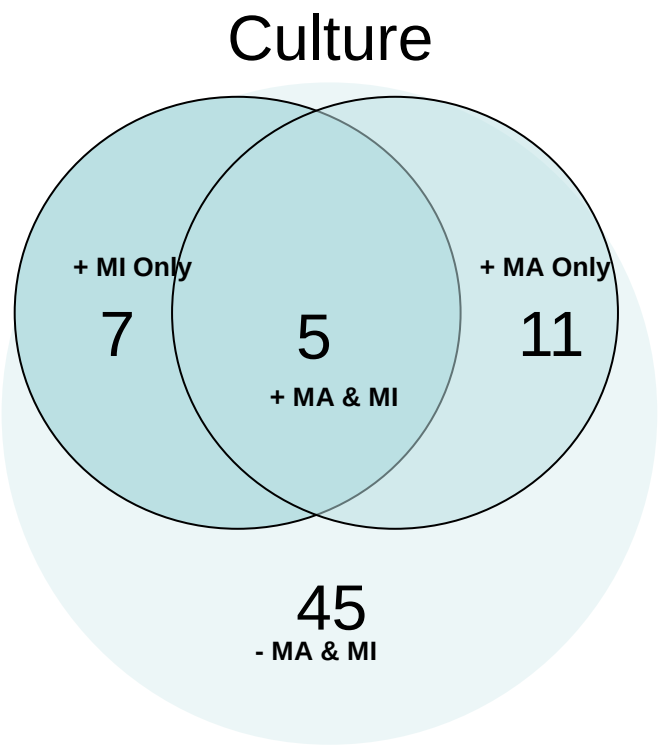
qPCR and Culture Results by Tap

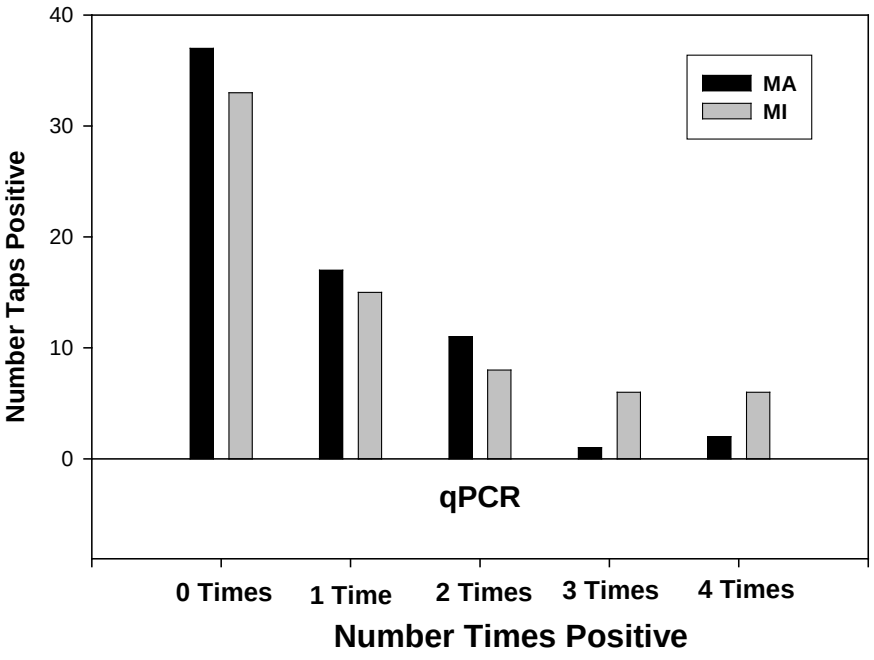


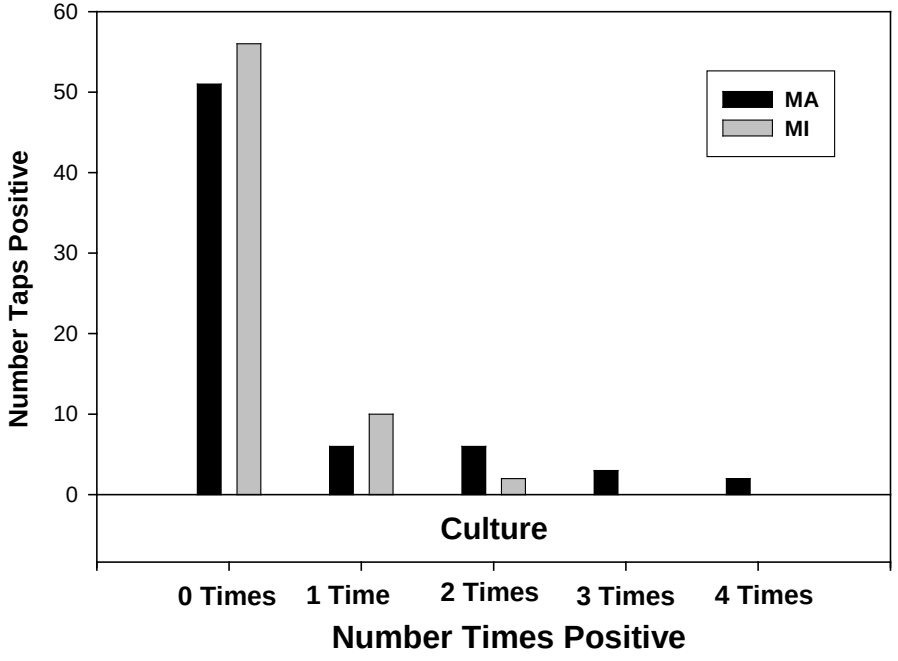
Number of Taps
N = 68



Number of Taps
N = 68

Persistence of MA and MI at taps





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Agreement Between Culture and qPCR

Target	No. Samples Positive Both Methods	Culture Positive Only	qPCR Positive Only
M. avium	12	16	37
M. intracellulare	11	4	67

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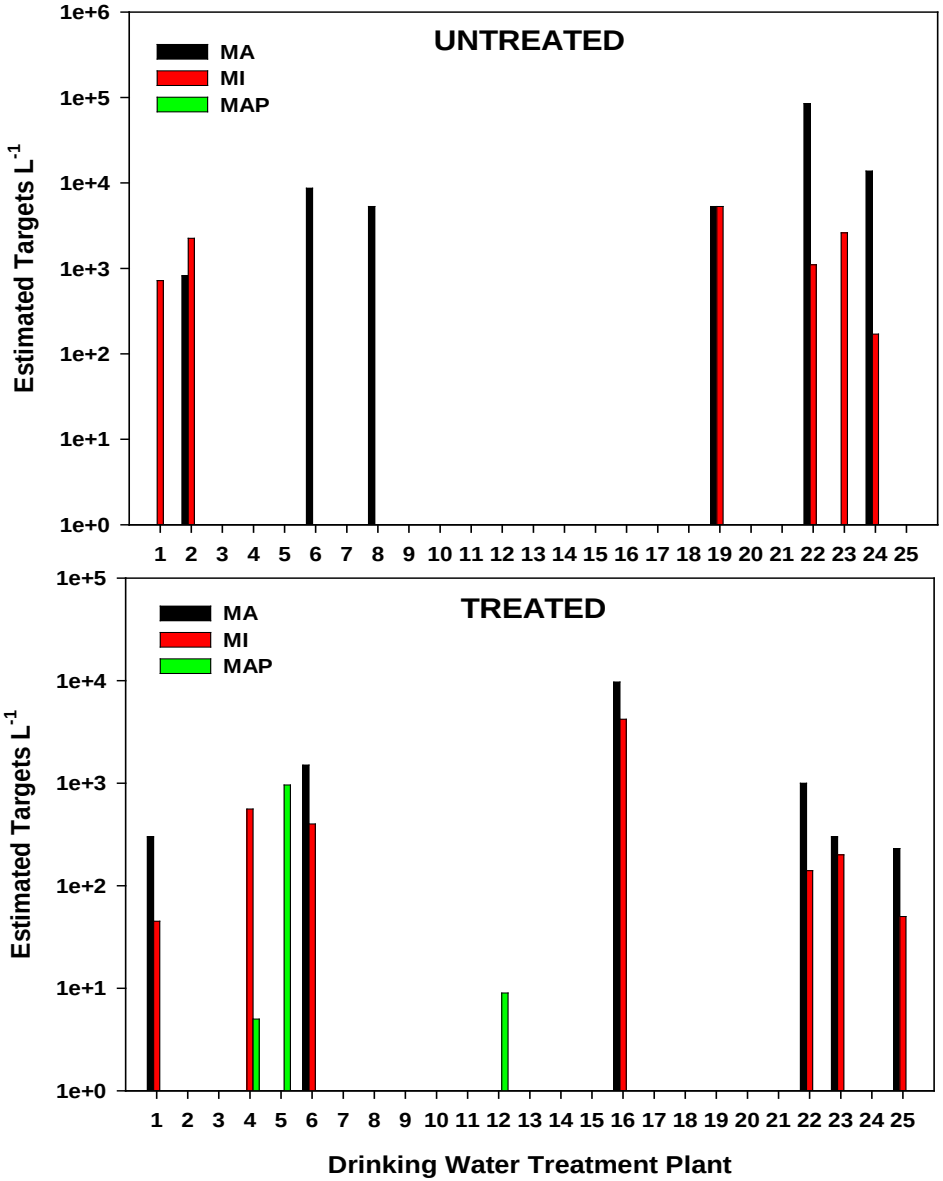
PHASE II Study Leads Susan Glassmeyer(USEPA), Ed Furlong (USGS), and Dana Kolpin(USGS): Occurrence of Contaminants of Emerging Concern at Drinking Water Treatment Plants (DWTPs)

EPA Region	Number of DWTPs
1	1
2	1
3	4
4	5
5	3
6	5
7	1
8	2
9	2
10	1

DWTPs positive by qPCR

- Twelve DWTPs were positive for MA, MI, and/or MAP by qPCR
- Estimated concentrations of MA, MI, and MAP are higher in untreated source water than finished treated water



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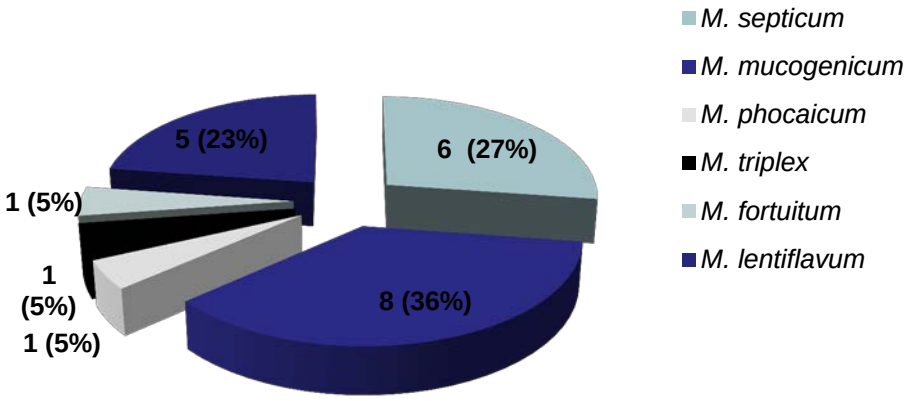
Description of qPCR Positive Samples

target	N	untreated	untreated	N	treated	treated
		geometric mean (target copies/L)	maximum (target copies/L)		geometric mean (target copies/L)	maximum (target copies/L)
M. avium	4	900	5200	2	240	300
M. intracellulare	3	1600	5300	3	40	50
M. avium subsp. *paratuberculosis*	0			3	20	300

Culture: NTM isolates from treated water at DWTPs

- No NTM isolated from untreated source water samples.
- All samples lost prior to 8 week minimum incubation period for MAC

- Thirty one isolates obtained from treated finished water samples from 6 DWTPs.
- None are MAC though 71% (22/31) have been putatively identified as other clinically relevant NTM species by 16S sequencing



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Method for *Mycobacterium* Detection for UCMR?

- Both, if possible, as both provide important information
- qPCR performs well regardless of sample matrix (water or biofilm)
- Culture does not perform well on microbiologically complex samples (contaminated water and biofilm) but does perform well on samples where microbiological water quality is good (i.g. treated water before distribution)

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- Ed Furlong (USGS)
- Dana Kolpin (USGS)