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Comparison of Ultrafiltration Techniques for Recovering Biothreat Agents in Water

Office of Research and Development
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Disclaimer

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Foreword

The National Homeland Security Research Center (NHSRC), part of the U.S. Environmental Protection Agency's (EPA's) Office of Research and Development, is focused on developing and delivering scientifically sound, reliable, and responsive products. These products are designed to address homeland security information gaps and research needs that support the Agency's mission of protecting public health and the environment. A portion of NHSRC's research is directed at decontamination of indoor surfaces, outdoor areas, and water infrastructure. This research is conducted as part of EPA's response to chemical, biological, and radiological contamination incidents. NHSRC has been charged with delivering tools and methodologies (e.g. sampling and analytical methods, sample collection protocols) that enable the rapid characterization of indoor and outdoor areas, and water systems following terrorist attacks, and more broadly, natural and manmade disasters.

NHSRC recently developed a field-portable ultrafiltration (UF) method and automated UF system. NHSRC funded, and collaborated with, the Centers for Disease Control and Prevention (CDC) to compare the performance of the EPA developed method and device with the established CDC Laboratory Response Network UF method for five suites of biothreat agents and/or their surrogates. This project determined if either method was associated with significantly higher recovery efficiencies for biothreat agents and microbial surrogates that had been seeded into 100-L samples of tap water. Having an understanding of the relative microbial recovery performance for the two methods may allow for potential interchangeability of the methods for use during a bioterrorism event.

This report represents a summary of methods and materials and results of the CDC and EPA UF method comparison.

Jonathan Herrmann,
Director, National Homeland Security Research Center

List of Acronyms

ANOVA	Analysis of Variance
ATCC™	American Type Culture Collection
BGMK	Buffalo Green Monkey Kidney
BSL	Biosafety Level
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming unit
CHAB-A	Cysteine Heart Agar with Chocolatized 9% Sheep Blood and Antibiotics
CIN	Cefsulodin-Irgasan Novobiocin
CT	Crossing threshold
CV	Coefficient of variation
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPD	Division of Parasitic Diseases
DQO	Data Quality Objectives
EMEM	Eagle's Minimum Essential Medium
EPA	Environmental Protection Agency
FA	Immunofluorescence assay
FBS	Fetal bovine serum
HPC	Heterotrophic plate count
IMS	Immunomagnetic Separation
kDa	Kilodaltons
LRN	Laboratory Response Network
LVS	Live vaccine strain
MWCO	Molecular weight cut-off
NCTC	National Collection of Type Cultures
NHSRC	National Homeland Security Research Center
NTU	Nephelometric turbidity units
ORD	Office of Research and Development
PBS	Phosphate buffered saline
PFU	Plaque-forming unit
PLET	Polymyxin, Lysozyme, EDTA, Thallous Acetate
PPE	Personal Protective Equipment
psig	Pound-force per square inch gauge
R2A	Reasoner's 2A
RPM	Revolutions per minute
SAM	*Standardized Analytical Methods for Environmental Restoration Following Homeland Security Events, Revision 5.0*
NaPP	Sodium polyphosphate
qPCR	Quantitative (real-time) Polymerase Chain Reaction
TOC	Total organic carbon
TSA	Trypticase soy agar
TSB	Trypticase soy broth
UF	Ultrafiltration
EPA	Environmental Protection Agency

WSC	Water sample concentrator

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Executive Summary

This is the final report for the U.S. Environmental Protection Agency (EPA) and Centers for Disease Control and Prevention (CDC) *Biological Sample Preparation Collaboration Project* to compare EPA and CDC ultrafiltration techniques for recovering biothreat agents in water. Hollow-fiber ultrafiltration (UF) is increasingly accepted as an effective sampling technique for simultaneous recovery of diverse microbes from water, including drinking water samples collected during water-related emergency response events. In this study, a laboratory-based UF method established by CDC for the Laboratory Response Network (LRN), a network of labs that can respond to biological and chemical terrorism, and other public health emergencies, was compared to a field-portable UF method developed by EPA for use with an automated UF system [the Water Sample Concentrator (WSC)]. Five suites of experiments were performed. For Suite 1 to 3 experiments, sodium polyphosphate (NaPP) was added as a sample amendment to water samples that were used for both the CDC and the EPA methods. For Suite 4 and 5 experiments, NaPP was added only to water samples processed with the CDC UF method. Suite 4 and 5 experiments were conducted to see if there was a measurable effect in adding NaPP to the water samples on the EPA method as had been done in Suite 1 – 3 experiments. Microbial recovery efficiencies were determined for the following microbes seeded into 100-L water samples which were then processed by each method:

Suite 1: *Bacillus anthracis* (Sterne) spores, *Yersinia pestis* (A1122), *Francisella tularensis* LVS (i.e., live vaccine strain), *Enterococcus faecalis*, and *Clostridium perfringens* spores
Suite 2: MS2 bacteriophage, phi X174 bacteriophage, echovirus type 1, high seed *Cryptosporidium parvum* oocysts, high seed *Giardia intestinalis* (aka *G. lamblia*) cysts, ColorSeed™ [containing 100 *C. parvum* and 100 *G. lamblia* fluorescent (oo)cysts]
Suite 3: *B. anthracis* (Sterne) spores, *Bacillus atrophaeus* subsp. *globigii*, *F. tularensis* LVS and *Brevundimonas diminuta*
Suite 4: *B. anthracis* (Sterne) spores, *B. atrophaeus* subsp. *globigii, Y. pestis* (A1122), *F. tularensis* LVS and *B. diminuta*
Suite 5: *E. faecalis*, MS2 bacteriophage, phi X174 bacteriophage, echovirus type 1, *C. parvum* oocysts, and *G. intestinalis* cysts

After performing the respective UF methods, samples were further concentrated and assayed using microbe-specific techniques, including membrane filtration and agar culture (for bacteria), microconcentrators and cell culture plaque assays (for viruses), and centrifugation and fluorescence microscopy (for parasites). In general, both the CDC and the EPA UF methods achieved greater than 50% recovery efficiencies during the Suite 1, 2 and 3 experiments:

Suite 1: *B. anthracis* spores (85 and 100%, respectively), *Y. pestis* (70 and 70%), *E. faecalis* (97 and 100%) and *C. perfringens* (100 and 110%)
Suite 2: MS2 (110 and 120%, respectively), phi X174 (100 and 95%), echovirus 1 (68 and 47%), *C. parvum* (82 and 73%) and *G. intestinalis* (99 and 85%)
Suite 3: *B. anthracis* spores (65 and 92%, respectively), *B. atrophaeus* subsp. *globigii* (57 and 99%) and *B. diminuta* (83 and 84%)

F. tularensis was the most challenging microbe to recover during the Suite 1, 2 and 3 experiments, with average recovery efficiencies of 13-17% for the CDC/LRN method and 25-29% for the EPA method. When UF concentrates were exposed to 1% ammonium chloride for 2 h before culture, *F. tularensis* culturability was significantly improved (and measured recovery efficiencies increased by 35-120%). While both methods were found to be similarly effective overall, statistical analysis indicated that the bacterial recoveries obtained using the EPA automated UF method were significantly higher ($\alpha < 0.05$) when Suite 1, 2, and 3 data were combined and analyzed. ColorSeed™ (BRF Precise Microbiology, Pittsburgh, PA) recoveries were similar for the EPA and CDC/LRN methods, with *C. parvum* oocyst recoveries of 30 and 38%, respectively, and *G. lamblia* recoveries of 44 and 42%, respectively.

In the Suite 4 and Suite 5 experiments, which included NaPP only in water samples processed using the CDC UF method, recovery efficiencies were also generally greater than 50% for both the CDC and EPA UF methods:

> Suite 4: *B. anthracis* spores (74 and 96%, respectively), *B. atrophaeus* subsp. *globigii* (47 and 89%), *Y. pestis* (100 and 76%), *B. diminuta* (82 and 78%)
> Suite 5: *E. faecalis* (100 and 63%, respectively), MS2 (99 and 69%), phi X174 (110 and 86%), echovirus 1 (79 and 37%), *C. parvum* (72 and 110%), and *G. intestinalis* (78 and 110%)

When Suite 4 and 5 microbial recovery data were combined and analyzed, no statistically significant difference between the EPA and CDC/LRN UF methods was observed. However, statistically significant different ($\alpha < 0.05$) recovery efficiencies were measured for a number of individual microbial analytes as follows. Higher recovery efficiencies were measured for the EPA UF method for *B. anthracis* spores, *B. atrophaeus* subsp. *globigii* spores, *C. parvum* oocysts, and *G. intestinalis* cysts while higher recovery efficiencies were measured for the CDC/LRN UF method for *E. faecalis*, MS2 bacteriophage, phi X174 bacteriophage, and echovirus 1 (See Table 5).

Operationally, filtrate rates for the WSC were slightly higher than for the CDC method. The higher filtrate rates and automation of the procedure resulted in the EPA procedure requiring approximately 20 fewer minutes to complete than the CDC/LRN UF procedure. Overall, despite physical, operational, and procedural differences between the two methods, the data from this study demonstrate that the EPA and CDC/LRN UF methods are highly efficient for recovering diverse microbes, including biothreat agent surrogates, and provide similar recovery performance.

1.0 Introduction

1.1 Background

Intentional contamination of drinking water supplies is a concern for water utilities, federal, state, and local agencies tasked with protecting human health and the environment. Because relatively low levels of biothreat agents can cause human health effects (1), sensitive detection of these agents in drinking water is needed. However, most rapid response analytical techniques [e.g., immunological "dipstick" methods, real-time polymerase chain reaction (qPCR)] assay small sample volumes or require high concentrations of analytes. Therefore, to enable sensitive detection of biothreat agents large volumes of water (on the order of 10-100 L) should be collected and concentrated. Alternative large-volume water sampling techniques have been published for viruses (e.g., various adsorption-elution techniques), bacteria (membrane filtration), and parasites (microfiltration cartridges), but the effectiveness of these methods are generally optimized for particular microbes types (i.e., viruses, bacteria or parasites). However, in the event of a biological attack on a drinking water system, the biothreat agent may not be known with certainty and deployment of multiple sampling techniques would be a logistical challenge and resource intensive. For this reason, the U.S. Environmental Protection Agency (EPA) and U.S. Centers for Disease Control and Prevention (CDC) have worked together to investigate methods to enable rapid and sensitive analysis of water samples for diverse, unidentified biothreat agents. This is the final report for the EPA and CDC *Biological Sample Preparation Collaboration Project* to compare EPA and CDC ultrafiltration techniques for recovering biothreat agents in water.

Homeland Security Presidential Directive 9 requires the development of a nationwide, interconnected network of federal and state laboratories that integrate resources and use standardized analytical procedures when supporting responses to homeland security incidents. The Laboratory Response Network was launched by CDC in 1999. Another key component of this directive is the *Standardized Analytical Methods for Environmental Restoration Following Homeland Security Events (SAM)* (2), which contains suggested assays for use by the LRN, the laboratories tasked with performing confirmatory analysis of environmental samples following a homeland security event (SAM is published by EPA's National Homeland Security Research Center (NHRSC) along with other EPA divisions and sister agencies). Though the manual details a variety of sample assays, it does not describe a method for sampling large volumes of water for an unidentified biothreat agent [e.g., viruses, bacteria, spores, parasite (oo)cysts, toxins]. Further, development of a field-deployable sampling method would make it unnecessary to manually collect large-volume water samples (e.g., in 20-L carboys) that would need to be shipped to an analytical laboratory at great expense and effort.

Ultrafiltration (UF) has become an established technique for co-concentrating diverse microbes (including viruses, bacteria and parasites) in large-volume water samples. Ultrafiltration has been of particular interest for bioterrorism preparedness because the technique can be used to capture unidentified biothreat agents. Since 2003, numerous studies have reported the effectiveness of UF for co-concentrating diverse microbes in water, including potential biothreat agents (3-9). In general, the ultrafiltration techniques reported within the last 10 years have used cross-flow

recirculation of water samples through hollow-fiber ultrafilters to concentrate 10- to 100-L water samples down to volumes on the order of 200-500 mL. While the recirculating flow UF technique can be performed in the field (10), it requires training and experience to perform effectively and consistently, and can be a challenge to set up under field conditions.

Since about 2003, EPA and CDC have been investigating UF methods for water-related bioterrorism preparedness. In 2006, CDC researchers developed an ultrafiltration method, and associated secondary sample processing protocols for the Laboratory Response Network (LRN). The LRN ultrafiltration and water processing procedure is a laboratory-based protocol. During the same time frame, EPA was also investigating recirculating flow UF methods, but with a focus on developing a field-portable and automated UF device. EPA and Idaho National Laboratory succeeded in developing an automated UF instrument, referred to as the water sample concentrator (WSC) in this report.

The CDC ultrafiltration method used in these experiments was established by the Laboratory Response Network in a document entitled "Filter Concentration for the Detection of Bioterrorism Threat Agents in Potable Water Samples" (11). The method involves the use of five, 20-L carboys, an ultrafilter, and a pump to filter a 100-L drinking water sample. The method is completely manual, with all steps performed by the technician. After 100 L is filtered, an elution step is performed to recover microbes that are adsorbed or otherwise retained in the filtration system; the final UF concentrate sample is then further processed and/or analyzed for the target microbe(s) using standard microbiological methods.

The WSC (approximate dimensions: 31 x 20 x 16 inches [795 x 518 x 393 mm])was developed as a field portable instrument to improve ease of use, safety, and consistency of the ultrafiltration concentration process. The device was controlled by software that was installed on a personal computer. As with the LRN method, the WSC used a hollow fiber filtration cartridge which was pre-treated prior to use. In addition the WSC, similar to the LRN method, used an elution procedure after filtration and prior to the final UF concentrate sample recovery and analysis for the target microbe(s) using standard microbiological methods.

Beyond the laboratory-based versus field-portable nature of the CDC/LRN and EPA UF methods, the two UF procedures differ in a few other potentially important ways (Table 1).

- The LRN method was developed using a Masterflex® L/S® peristaltic pump (≤ 2.9 L/min pumping rate) (Cole-Parmer Instrument Company, Vernon Hills, IL) versus the larger Masterflex® I/P® -sized pump (≤ 8 L/min pumping rate) used in the WSC. Thus, cross-flow rates and filtrate rates are higher for the WSC.
- The LRN method uses pre-treatment of water samples with NaPP, a dispersing agent. No sample pre-treatment is performed with the WSC procedure.
- Ultrafilters are blocked (i.e., pre-treated) with fetal bovine calf serum (FBS) in the LRN method, whereas in the EPA UF system the blocking is achieved by exposure of the filter to a solution containing Tween® 80 (ICS Americas, Foster, KY), Antifoam A, and NaPP.
- Both methods use elution to desorb microbes that may have become attached during the ultrafiltration procedure, but the LRN method uses an elution solution containing Tween®

80, Antifoam A (or Antifoam Y-30 Emulsion) and NaPP, whereas the WSC method uses a solution containing only Tween 80.

Table 1. Differential Characteristics Between the EPA and CDC/LRN Ultrafiltration Methods

Differentiating Characteristic	EPA Method	CDC/LRN Method
Sample Amendment	None	With 0.01% NaPP
Filter Blocking	With solution containing 0.055% Tween 80, 0.001% Antifoam A, 0.1% NaPP	With 5% FBS
Elution	With solution containing 0.001% Tween® 80	With solution containing 0.01% Tween® 80, 0.01% NaPP, 0.001% Y-30 Antifoam Emulsion
Set up	Field-portable	Laboratory method
Mode of control	Computer-controlled	Manual
Pump size	Masterflex I/P® (industrial/process scale)	Masterflex L/S® (lab scale)
Filter type	REXEED™ 25-S filter (for this study)	Fresenius F200NR filter (for this study)

Because the CDC/LRN and EPA UF methods were developed to achieve the same basic goal (rapid recovery of diverse biothreat agents in large-volume drinking water samples) it is important to understand the relative microbial recovery performance for the two methods. Such method comparison data will be useful to both the EPA and CDC for understanding the relative strengths of each method and the potential interchangeability of the methods if either—or both—are used during a bioterrorism response. In this study we compared the use of the laboratory-based LRN UF method to the EPA's field-portable WSC UF device to concentrate 100-L tap water samples for five suites of biothreat agents and/or their surrogates. Pathogens of concern in environmental matrices were selected from *SAM, 4.0* and are those that result in adverse human health effects upon infection or exposure.

1.2 Study Objectives

The primary objective of this project was to compare the CDC/LRN UF protocol and EPA UF device protocol to determine if either is associated with significantly higher recovery efficiencies for microbes seeded into 100-L samples of tap water. Pathogens and biothreat agent surrogates used in this study were *Bacillus anthracis* (Sterne) spores, *Yersinia pestis* (A1122), *Francisella tularensis* LVS, echovirus type 1, *Cryptosporidium parvum* oocysts, and *Giardia intestinalis* cysts. In addition, the following microbes were studied because they have been suggested as potential biothreat agent surrogates or UF method proficiency parameters: *Enterococcus faecalis*, *Bacillus*

atrophaeus subsp. *globigii*, *Brevundimonas diminuta*, *Clostridium perfringens* spores, MS2 bacteriophage, and phi X174 bacteriophage. Secondary goals of this study included:

- Comparing the recovery efficiencies of the two UF methods when water samples contained *C. parvum* and *G. intestinalis* at high [~10^5 (oo)cysts] and low [100 (oo)cysts] seed levels
- Evaluating use of 1% ammonium chloride for improving the culturability of *F. tularensis* in UF concentrates (measured as a change in recovery efficiency)
- Comparing average processing times associated with the two UF methods

For each experiment, physical and chemical water quality parameters were measured to enable evaluation of potential water quality influences on the performance of the UF procedures and analytical assays. The data quality objectives for this project included coefficient of variation (CV) goals for percent recovery efficiency data sets for each high seed microbe (CV ≤ 25% for each UF method) and for each low seed microbe (CV ≤50%, reflecting higher data variability associated with the multiple procedures [UF and secondary processing] employed for low-seed microbe).

2.0 Methods and Materials

2.1 Water Sample Preparation

Experiments were performed using five independent microbial "Suites". Experiments using microbial Suites 1, 2, and 3 were performed from May 2009 to April 2010 with tap water samples obtained from the CDC Waterborne Disease Prevention Branch Environmental Microbiology Laboratory on the CDC's "Chamblee Campus" (Table 2). In July, 2010 the WDPB Environmental Microbiology Laboratory moved to a new laboratory facility located on the CDC's "Roybal Campus." Experiments using microbial Suites 4 and 5 were performed using tap water from the laboratory on the Roybal Campus. Tap water samples were collected in sterile, 35-gallon high-density polyethylene tanks that were calibrated to 100-L using 10-L gradations. Prior to collecting each water sample, the tap was fully opened for 5 minutes to draw fresh water through the building distribution system. Two 100-L tap water samples were collected at the same time from two taps in the same laboratory room. To ensure that the same quality water was used to perform both the CDC/LRN and EPA methods, a third 35-gal tank was used to mix 50-L from each of the other tanks. Free chlorine was measured in each tank to assess initial chlorine residual using Hach® DPD (Division of Parasitic Diseases) Methods 8021 (Hach Companyl, Loveland, CO) and 8167, respectively (equivalent to Standard Method 4500-Cl G), and a Hach® DR/2400 spectrophotometer (12). A volume of 50 mL of 10% w/v stock of sodium thiosulfate solution was then added to each tank to quench the chlorine. Free chlorine was read again for each tank to confirm quenching. Additional sodium thiosulfate was added to each water sample, if necessary, until no free chlorine could be detected. A 500-mL sample of water was collected by obtaining 250 mL of water from each tank. For each experiment, this water sample was seeded with the same numbers of study microbes added to each 100-L sample and the sample was assayed in conjunction with the CDC/LRN and EPA UF concentrate samples at the end of the experiment. The data from this 500-mL "control sample" was used to quantify the microbe seeding levels for each experiment.

Table 2. Framework for Study Experiments

Site	Microbial Suites	CDC/LRN Method	EPA Method
Chamblee	1, 2, and 3	**With NaPP sample amendment** (per established protocol)	**With NaPP sample amendment** (not established protocol)
Roybal	4 and 5	**With NaPP sample amendment** (per established protocol)	**No NaPP sample amendment** (per established protocol)

NaPP, sodium phosphate

When the chlorine had been quenched in each tank, 75 mL of water was collected from each tank and combined for water quality analysis. All water samples were characterized using the following water quality parameters: specific conductance, temperature, pH, turbidity, total hardness, total organic carbon (TOC), and heterotrophic plate count (HPC) of bacteria. A 50-mL

portion of this sample was tested for specific conductance and temperature using an Oakton® CON 100 Conductivity/°C meter (Oakton Instruments, Vernon Hills, IL). This conductivity meter was calibrated weekly using vendor instructions for conductivity and temperature calibration. The pH of water samples was measured using a Fisher Scientific™ Accumet® Research AR25 Meter (Fisher Cat. No. 13-636-AR25A, Fisher Scientific, Pittsburgh, PA) and Accumet™ Standard Size Combination Electrode (Fisher Cat. No. 13-620-285). Turbidity was measured using a Hach Model 2100N Laboratory Turbidimeter (Cat. No. 4700000, Hach Company). All measurements were collected using the "Signal Averaging" function on the turbidimeter. Total hardness was measured using Hach Method 8213 with a Hach Hardness (Ca/Mg) Reagent Set (Cat. No. 24480-00, Hach Company) and Hach Model 16900 digital titrator (12). TOC was measured using Hach Method 10129 with a Hach Low Range TOC Reagent Set (Cat. No. 2760345) and the Hach DR/2400 Portable Spectrophotometer (12). HPC bacteria were measured in duplicate assays using a Standard Method (13). For the HPC tests, one 30-CFU (colony-forming unit) *E. coli* [NCTC 9001 (Pall Supor Acrodisc 11775)] Bioball® purchased from BTF Pty. Ltd. (Australia) was used as a positive control and 10 mL sterile wash phosphate buffer saline (PBS) was used as the negative control for the HPC count.

For Suite 1, 2 and 3 experiments, sodium polyphosphate (NaPP), a chemical dispersant, was added at a 0.01% w/v ratio to the 100-L water samples that were processed by the CDC and EPA UF methods. For Suite 4 and 5 experiments, NAPP was added only to the 100-L water samples processed with the CDC method (and not to the water sample processed using the EPA method). Suite 4 and 5 experiments were performed in a laboratory facility at the CDC's Roybal Campus, while Suite 1, 2 and 3 experiments were performed in a laboratory at CDC's Chamblee Campus, but both laboratory facilities were served by the same water treatment plant (Dekalb County Water and Sewer's Scott Candler Water Treatment Plant). The Scott Candler Water Treatment Plant produces drinking water that is conventionally treated before chlorine addition (to achieve disinfectant residual) and caustic soda (sodium hydroxide) addition for corrosion control in the distribution system. Tap water samples processed using the CDC/LRN UF method were pumped into 5, 20-L Cubitainers® to perform the method.

2.2 Microorganisms and Assays

2.2.1 Microbes and Seed Levels for Experiment Suites. In order to limit the number of microbes assayed for each experiment, five suites of microbes were used in separate experiments. After appropriate dilutions were made in diluent PBS (0.01M) containing 0.01% Tween 80, each bacterial stock used to create the seed spike for an experiment was filtered through a 5-µm Pall™ Supor Acrodisc™ syringe filter (Model No. 4650; Pall Corporation, Port Washington, NY).

Suite 1 consisted of 10 replicate experiments with water seeded with the following microbes:

B. anthracis (Sterne) spores – 3,600 ± 1,700 CFU
Y. pestis A1122 – 70,000 ± 16,000 CFU
F. tularensis LVS – 90,000 ± 100,000 CFU
E. faecalis (ATCC™ 29212 from BTF Multishot-550 BioBall®) – 1,100 CFU

C. perfringens spores (NCTC 8798 from HighDose-10K BioBall®, BTF Pty. Ltd., Australia) – 110 ± 56 CFU

Suite 2 included 11 replicate experiments with water seeded with the following microbes:

MS2 bacteriophage (ATCC 15597-B1) – 45,000 ± 29,000 PFU (plaque-forming unit)
phi X174 bacteriophage (ATCC 13706-B1) – 11,000 ± 2,500 PFU
echovirus 1 (Farouk strain, ATCC VR-1038) – 3,600 ± 1,600 PFU
C. parvum oocysts (Waterborne, Inc., New Orleans, LA) – 180,000 ± 100, 000 oocysts
G. intestinalis cysts (aka *Giardia lamblia*; Waterborne, Inc.) – 200,000 ± 110,000 cysts
ColorSeed™ [containing 100 (oo)cysts each of fluorescent *C. parvum* and *G. intestinalis*] (BTF Pty) – 1 vial containing 100 (oo)cysts each of fluorescent *C. parvum* and *G. intestinalis.*

Suite 3 consisted of 10 replicate experiments with water seeded with the following microbes:

B. anthracis (Sterne) spores – 6,600 ± 1,500 CFU
B. atrophaeus subsp. *globigii* spores – 9,300 ± 2,200 CFU
F. tularensis LVS – 81,000 ± 91,000 CFU
Brevundimonas diminuta – 42,000 ± 22,000 CFU

Suite 4 consisted of 9 replicate experiments with water seeded with the following microbes:

B. anthracis (Sterne) spores – 5,200 ± 690 CFU
B. atrophaeus subsp. *globigii* spores – 9,800 ± 3,700 CFU
Y. pestis – 5,100 ± 5,700 CFU
F. tularensis – 46,000 ± 44,000 CFU
B. diminuta – 5,100 ± 3,300 CFU

Suite 5 consisted of 8 replicate experiments with water seeded with the following microbes:

E. faecalis – 780 ± 72 CFU
MS2 bacteriophage – 110,000 ± 23,000 PFU
Phi X174 bacteriophage – 12,000 ± 2,000 PFU
Echovirus type 1 – 45,000 ± 14,000 PFU
C. parvum oocysts – 150,000 ± 24,000 oocysts
G. intestinalis cysts – 180,000 ± 46,000 cysts

2.2.2 Suite 1 and 2 Microbe Sources and Seeding Procedures

For each experiment, a seed stock was made that consisted of the study microbes for the experiment. One third of the stock was added to a 500-mL control sample that was drawn from the two 100-L water samples, one third was added to the 100-L "EPA Method" tap water sample, and one third was added to the "CDC/LRN Method" water sample (for this method an equal-volume aliquot of the microbial stock was added to each of the 20-L Cubitainers® [i.e., ~1/5th of

the stock volume was added to each of the 5, 20-L Cubitainers®]) (Hedwin Corporation, Baltimor, MD). Microbial seed stocks were prepared and added to water samples as described below.

Bacteria. *B. anthracis* spores were acquired from CDC Division of Healthcare Quality Promotion (Matt Arduino and Laura Rose) and were produced by culture and sporulation on AK Agar #2 (Sporulating Agar) (BD Diagnostics; Franklin Lakes, NJ) and purified by centrifugation through 58% Hypaque®-76 (Nycomed, Inc., Switzerland). *B. anthracis* spore stocks were stored at 4 °C in 40% (v/v) ethanol. *Y. pestis* stocks were acquired from CDC Division of Healthcare Quality Promotion (Matt Arduino and Laura Rose) and were produced on tryptic soy agar (TSA) containing 5% sheep blood (CDC Scientific Resources Program). *F. tularensis* stocks were acquired from CDC Division of Healthcare Quality Promotion (Matt Arduino and Laura Rose) and were produced on Chocolate II agar (CDC Scientific Resources Program). Six, 550-CFU *E. faecalis* BioBalls® (ATCC 29212) were used for each experiment (two for each 100-L water sample and two for the control sample). Although a BioBall® containing 10,000 CFU of *C. perfringens* spores was used for each experiment, when cultured on mCP agar these BioBalls® yielded an average of ~330 CFU. *C. perfringens* BioBalls® were processed following manufacturer's guidelines to disaggregate spores; they were vigorously shaken at 600 oscillations/min in diluent PBS for 30 min using a Pall Gelman laboratory shaker (Model No. 4821).

The total seeding amount for each bacterial analyte was determined by membrane filtration of appropriate volumes of the seeded 500-mL control sample and selective agar culture. These seed levels were selected to enable quantification of each microbe in control and UF concentrate samples at per-plate counts of 20-80 CFU when sample volumes of approximately 0.1-10 mL were assayed. Each bacterial stock used to create the seed spike for an experiment was filtered through a 5-µm Pall Supor Acrodisc (Model No. 4650) to remove bacterial aggregates before appropriate dilutions were made in diluent PBS (0.01M; CDC Scientific Resources Program) containing 0.01% Tween 80.

Viruses. Microbial seed dilutions of the stocks of MS2 and phi X174 bacteriophage were made in diluent 0.01M phosphate-buffered saline (PBS; Dulbecco's modification, pH 7.40), 0.01% (w/v) Tween 80 (Fisher), and 0.001% (w/v) Antifoam Y-30 emulsion (Sigma) to disperse viral particles. The stocks were vortexed vigorously for 30 seconds before making the dilutions and vortexed 10-15 seconds between dilutions. The bacteriophages and echovirus 1 were filtered through a 0.1-µm Acrodisc filter before seeding. A clone of echovirus 1 (Farouk strain) was prepared from a strain obtained from the American Type Culture Collection (ATCC, Manassas, VA) and propagated in BGMK (Buffalo Green Monkey Kidney) cells (Scientific Resources Program, CDC). Cell lines were maintained in either Eagle's Minimum Essential Medium (EMEM) or Dulbecco's Modified Eagle Medium (DMEM) as described previously (14).

Parasites. Before use in an experiment, *C. parvum* and *G. intestinalis* stocks from Waterborne, Inc. were diluted to achieve a diluted stock concentration of 100,000 (oo)cysts/mL. Three mL of each stock dilution were heat-treated for 10 min at 60 °C to inactivate the (oo)cysts. The stocks were then shaken on a Pall Gelman laboratory shaker for 30 min to disaggregate the (oo)cysts

before adding 1 mL of each stock to each 100-L water sample and the control sample for an experiment.

2.2.3 Suite 3, 4 and 5 Microbe Sources and Seeding Procedures

In Suite 3, 4, and 5 experiments, the microbes studied and seeding procedures used were the same as used in Suites 1 and 2 for *B. anthracis* spores, *Y. pestis*, *F. tularensis*, *E. faecalis*, MS2, phi X174, echovirus 1, *C. parvum*, and *G. intestinalis*. In Suite 3 and 4 experiments, water samples were seeded with *B. atrophaeus* subsp. *globigii* spores and *B. diminuta*. *B. atrophaeus* subsp. *globigii* spores were obtained from EPA (Cincinnati) and were propagated using Generic Spore Media as previously described (6). *B. atrophaeus* subsp. *globigii* spore stocks were stored at 4 °C in 40% (v/v) ethanol. A kanamycin-resistant isolate of *B. diminuta* was obtained from ATCC (#19146). *C. perfringens* spores and ColorSeed™ (oo)cysts were not studied in Suite 3, 4, and 5 experiments (ColorSeed™, BTF Precise Microbiology, Inc., Pittsburgh, PA) .

2.2.4 Post-Ultrafiltration Processing and Assays

Bacteria. UF concentrate samples and the input control sample for each experiment were assayed for each bacterial analyte using two or more sample volumes and duplicate assays for each sample volume. When each UF procedure was completed, UF concentrates were assayed immediately for *F. tularensis* by membrane filtration using 0.2-µm Supor® membranes (Pall Life Sciences, #66234) and culture of the filters on plates of Cysteine Heart Agar with Chocolatized 9% Sheep Blood and Antibiotics (CHAB-A) (15), which were prepared by CDC's Division of Scientific Resources. In addition, aliquots of the experiment control sample, CDC UF concentrate, and the EPA UF concentrate samples were also exposed to 1% ammonium chloride (final concentration with water sample added) for 2 h before membrane filtration and incubating on CHAB-A plates. CHAB-A plates were incubated for 4-7 days at 37ºC before inspecting for characteristic *F. tularensis* colonies (yellow, mucoid). Assays for *B. anthracis*, *Y. pestis*, *E. faecalis*, and *C. perfringens* were performed after culture assays for *F. tularensis* were completed. Membrane filtration was performed for each of these bacteria using 0.45-µm mixed-cellulose ester membrane filters. *B. anthracis* spores and *B. atrophaeus* subsp. *globigii* spores were cultured on plates of Polymyxin B-Lysozyme-EDTA-Thallous Acetate (PLET) agar (prepared by CDC's Division of Scientific Resources) incubated at 37ºC for 24 hours (16) and inspected for characteristic *B. anthracis* colonies (pink/cream) and *B. atrophaeus* subsp. *globigii* colonies (orange). *Y. pestis* was cultured on plates of (Cefsulodin-Irgasan -Novobiocin) CIN agar (prepared by CDC's Division of Scientific Resources) incubated at 27ºC in an environmental chamber for 2-3 d (17). *C. perfringens* spores were cultured on plates of mCP agar (Acumedia #7477A) incubated in an anaerobic jar at 41ºC for 18-24 h (18). Bacterial colonies on plates of mCP agar were exposed to ammonium hydroxide in fume hood and characteristic pink colonies were counted as *C. perfringens*. *E. faecalis* was cultured on plates of mEI agar (mE agar [Becton Dickinson #233320] with 0.075% [w/v] indoxyl β-D glucoside) incubated at 41 ºC for 24 h (19). *B. diminuta* was enumerated using R2A agar (Reasoner's 2A) (Remel #R454372) containing 0.4 µg/mL of kanamycin and 0.08 µg/mL of tetracycline (to minimize growth of background microbes) and incubated at 30°C for 48 h.

Viruses. UF concentrate samples and the input control sample for each experiment were assayed for each virus analyte using two or more sample volumes and duplicate assays for each sample volume. When each UF procedure was completed, MS2 and phi X174 were assayed in the experiment control sample and UF concentrates using the single agar plaque assay method using the *E. coli* CN-13 (ATCC 700609) and F_{amp} (ATCC 700891) host cells, respectively, according to EPA method 1602 (20). According to Method 1602, the appropriate bacterial host was inoculated into separate water sample aliquots and incubated briefly. The appropriate molten agar for each bacterial host was then added to each water sample, swirled to mix and then poured onto 150-mm Petri dishes. After cooling on a bench top for ~15 min, plates were then incubated at 37 °C for approximately 17 h.

Because echovirus 1 was seeded into water samples at a relatively low seed level, quantification of echovirus 1 recovery efficiencies required concentration of viruses in UF concentrates. UF concentrates produced by both the CDC/LRN and EPA methods were further processed for echovirus 1 analysis using Centricon Plus-70 microconcentrators. The manufacturer's procedure was followed with the exception that two 70-mL volumes of sample were processed for each UF method (140 mL total). Echovirus 1 was quantified in Centricon® (Millipore Corp., Billerica, MA) concentrates by plaque assay by inoculating 10-fold dilutions onto BGMK cell monolayers in 60 mm^2 dishes (9). After 1-h adsorption at 37 ºC and 5% CO_2, the infected cells were overlaid with 5 mL maintenance medium containing 0.5% agarose. Following a 2-day incubation, a second overlay containing 2% neutral red was added to visualize plaques within 4 h. For echovirus 1, 0.25 mL of a 10-fold dilution was assayed per plate.

Parasites. UF concentrate samples and the input control sample for each experiment were assayed for high seed *C. parvum and G. intestinalis* (oo)cysts in duplicate assays. Recovery efficiencies for *C. parvum and G. intestinalis* were based on direct fluorescence microscopy analysis of UF concentrates and the experiment control without immunomagnetic separation (IMS) processing. Microscopy slides were prepared with 300 µL of each sample using SuperStick™ slides (Waterborne, Inc, New Orleans, LA). Oocysts and cysts were stained using EasyStain™ (BTF, Australia) according to the manufacturer's instructions and observed using a fluorescence microscope at 400X magnification. In addition to adding *C. parvum* and *G. intestinalis* (oo)cysts at high seeding levels, low level seeding was also performed using ColorSeed™ (BTF) to enable comparative evaluation of the EPA and CDC/LRN water processing methods for a water-related biothreat agent present at a low concentration. To assay ColorSeed™ (oo)cysts, 250 mL from each UF concentrate sample was further concentrated by centrifugation according to the procedure of Lindquist et al. (4). ColorSeed™ (oo)cysts were recovered from the pellet using immunomagnetic separation (IMS) (Dynabeads® GC-Combo; Life Technologies/Invitrogen, Carlsbad, CA) according to the procedures in EPA Method 1623 (21) and counted on SuperStick™ slides by immunofluorescence assay microscopy. One immunofluorescence assay was performed for each UF concentrate sample (i.e., duplicate assays were not performed). For the ColorSeed™ sample, an initial control was not performed because ColorSeed™ is warranted by the manufacturer to contain 100 *C. parvum* oocysts and 100 *G. intestinalis* cysts. To calculate recovery efficiencies, microscopy counts were compared to this value and a percent recovery was determined.

2.3 CDC/LRN Ultrafiltration Set-Up

The CDC/LRN method was performed in accordance with the LRN protocol, *Filter Concentration and Detection of Bioterrorism Threat Agents in Potable Water Samples* (Rev 09/21/2007). The procedure was performed on a bench top in a BSL-2 (biosafety level 2) laboratory (no microbes were used that required a BSL-3 facility). A Cole-Parmer model 7550-30 Masterflex® L/S peristaltic pump and high performance, platinum-cured L/S 36 silicone tubing (Masterflex; Cole-Parmer Instrument Co., Vernon Hills, IL) were used to pump water from a 20-L Cubitainer® through the ultrafilter (Fig. 1; 1 of 5 Cubitainers® shown). The CDC UF method was performed using Fresenius F200NR polysulfone single-use dialysis filter (Fresenius Medical Care, Lexington, MA) because this is the filter that was used during LRN validation testing for the method. F200NR dialyzers have an approximate molecular weight cut-off (MWCO) of 30 kDa and surface area of 2.0 m^2. The CDC/LRN UF procedure included ultrafilter blocking (pre-treatment), sample amendment with NaPP, sample filtering, and a filter elution step.

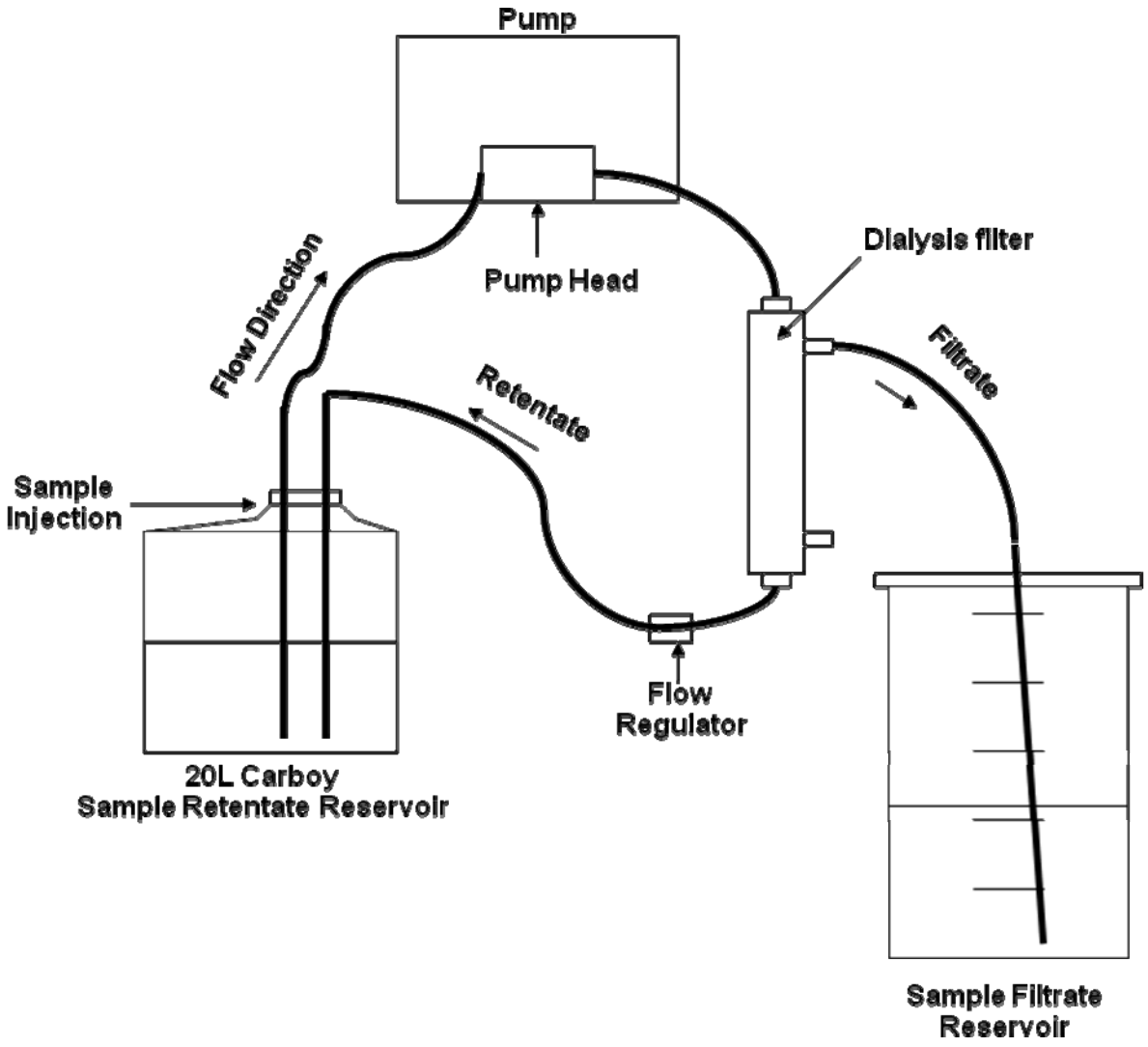

Figure 1. Schematic of CDC ultrafiltration set-up.

2.3.1 Ultrafilter Blocking. The ultrafilter was positioned vertically with a ring stand and clamp. A 50-mL syringe was then connected to the filter's inlet port using a piece of tubing approximately 6 inches in length and a DIN adapter. Using the syringe, approximately 150 mL of a 5% calf serum (Invitrogen catalog no. 16170-078) solution was injected into the ultrafilter. Both ends of the filter were capped and covered with Parafilm® (Pechiney, Stamford, CT) and both side ports were tightened to prevent leaks. The filter was placed on the rotisserie at room temperature for a period of at least 30 minutes. Immediately prior to performing an experiment, the blocking solution was flushed from the ultrafilter by pumping 1 L of a 0.01% NaPP (Sigma, catalogue #305553) (Sigma, St. Louis, MO) solution through the ultrafilter filtrate and retentate ports.

2.3.2 Sample Processing. As described in Section 2.1, the CDC/LRN UF method was performed using 100 L of tap water that had been dechlorinated, amended with NaPP to a concentration of 0.01% w/v, and distributed into 5, 20-L Cubitainers®. Per the LRN UF procedure, the target filtrate rate was 60% of the peristaltic pump flow with the balance of the pump flow exiting the filter through the retentate line and recycled back into the system. The average filtrate flow rate achieved for the CDC/LRN method during the study was 1,700 ± 180 mL/min (58% of the nominal pump flow rate of 2,900 mL/min). Sample water in each Cubitainer® was concentrated by ultrafiltration until a volume of ≤500 mL remained in the Cubitainer, at which point the pump was stopped. The outlet tubing was moved to a 1-L glass beaker and the retentate in the Cubitainer® was pumped into the beaker. The retentate in the beaker was then concentrated further until there was no retentate left in the beaker, at which point the pump was stopped and the tubing was moved to the next Cubitainer®. The beaker was then set aside and the next Cubitainer was processed. When processing the sample from the last Cubitainer (i.e., the 5th of 5 Cubitainers®), the retentate in the beaker was reduced to as low a volume as possible. Then the inlet tubing was removed from the sample with the flow regulator open to let the peristaltic pump run until all the sample from the filter was pushed out. At the end of the sample concentration procedure, retentate sample volumes were 260 ± 36 mL.

After the entire 100-L sample was processed and the retentate sample collected, the filter was then eluted using an elution solution containing 0.01% NaPP, 0.01% Tween 80, and 0.001 % Antifoam Y-30 Emulsion (Sigma). The inlet and the outlet tubing from the filter were placed in the 500 mL elution solution. The screw clamp was loosened and the pump flow rate was set to 2000 mL/min. The elution solution was recirculated until the system began to draw up air. The inlet tubing was then removed and eluent remaining in the ultrafilter and tubing was recovered in a glass beaker. The elution process was repeated until the volume was as close as possible to 250 mL. For the study, the final UF concentrate volumes (retentate + eluent) were 490 ± 38 mL. The time required to perform the filtration and elution procedures was measured using a watch. The time was noted when the filtration procedure was started and when the elution procedure was completed (using the same watch).

2.4 EPA Ultrafiltration Set-Up

The EPA UF procedure was performed on a bench top in a BSL-2 laboratory (no microbes were used that required a BSL-3 facility) (Figure 2). For each experiment, the EPA UF method was performed using the EPA-developed WSC and its associated UF operational protocols (Figure 3). At the time of this study, the WSC was not commercially available.

Figure 2. EPA water sample concentrator set-up at CDC laboratory facility.

The WSC primarily consisted of a modified peristaltic pump [Masterflex® I/P pump drive, (Cole Parmer model 77401-00) and I/P Easy-Load® pump head (Cole Parmer model 77601-00)], tubing pinch valves, sensors, DC power supplies for the valves and sensors, and data acquisition modules that facilitated communication between the computer and the various electrical components. Pre-made filtration assemblies were installed into the device prior to a concentration run. A filtration assembly consisted of a Rexeed™-25S single-use dialysis filter (Asahi Kasei Kuraray Medical Co. Ltd., Tokyo, Japan), sample bottle, tubing, fittings, clamps, and pressure sensor. REXEED-25S dialyzers have an approximate MWCO of 30,000 daltons and a surface area of 2.5 m^2. The pump tubing was Tygon® Lab tubing R-3603 [9.5 mm (3/8 in) ID x 16 mm (5/8 in) OD]. The pump tubing and the filter were connected by a coil of Tygon® Lab tubing [6.3 mm (1/4 in) x 13 mm (1/2 in)], in order to dampen pulsations from the pump. The remainder of the tubing was Tygon® silicone tubing 3350, of the following sizes, 6.3 mm (1/4 in) x 9.5 mm (3/8 in), 6.3 mm (1/4 in) x 11 mm (7/16 in) and 9.5 mm (3/8 in) x 16 mm (5/8 in).

A key design feature of the filtration assembly was that the parts that came into contact with the sample water were single use items (although for this study, some of these parts were disinfected and recycled into new assemblies to save supply costs). Thus the valves used in the device were solenoid pinch valves which resulted in only the tubing, and not the valve body, coming into contact with sample water. Similarly, the water level in the sample bottle was measured via a load cell, which the bottle rested on; this weight-based method allowed monitoring without a sensor contacting the sample water.

The computer software controlled the multi-step concentration process by directing the operation of the pump, valves, and by monitoring pressure, filtrate flow rate, and the amount of water in the sample bottle. The inlet pressure was set at 30 psig and the filtrate pressure was approximately at atmospheric pressure. If the inlet pressure exceeded 30 psig, the pump speed

would decrease which resulted in a decrease in pressure. Similarly if the pressure was below 30 psig, the pump speed would increase to increase pressure. The filtrate flow rate typically started off at ~2,800 mL/min but decreased as the run progressed as the peristaltic pump tubing was broken in. Likewise, an inlet pressure of 30 psig was maintained initially, but as the run progressed, (and pump flow decreased), the pressure eventually decreased to below 30 psig despite the pump running at maximum (650 RPM) speed. The average flow rate for a concentration run was ~1,700 mL/min; the average pressure was 25 psig. The retentate flow rate was not measured but in previous work under similar conditions had been measured to be ~4,000 mL/min on average over the course of a run.

2.4.1 Ultrafilter Blocking Solution. The REXEED-25S filters used in the EPA method were blocked according to the EPA protocol NHSRC 004 [Reagent Preparation – Filter Blocking Solution (0.055% Tween 80, 0.001% Antifoam A, 0.1 % NaPP)] prior to each experiment. This blocking solution is recirculated through the ultrafilter for 3 min, after which most the solution is removed from the system, but 250 mL is retained in the retentate bottle. Then the influent tubing is placed in the sample container to begin sample processing.

2.4.2 Sample Processing. The EPA method was performed by processing the 100-L tap water sample in the tank at an average system pressure of 25 psig and a flow rate of 1738 mL/minute. During each experiment, the water sample in the tank was manually stirred every 10 minutes. The process began with filter blocking and ended with the elution of the filter with an elution solution. One day prior to the experiment, fresh 1-L volumes of both the blocking and elution solutions were made. At the start of the run, a pre-made filtration assembly (provided by EPA) was installed into the WSC per instructions in the operator's manual (22) (Figure 3). After installation of the assembly, a volume of filter blocking solution was drawn up through the sample inlet port. After a 3 minute recirculation period, the blocking solution exited the system through the filtrate port. This was followed by the device drawing up and concentrating the water sample, and then by a drawing up multiple volumes of elution solution through the sample inlet port. During the UF process the software would prompt the user to perform simple steps such as placing the sample inlet into the filter block solution, water sample, and elution solution (0.001% Tween 80). The software also monitored operational parameters, including sample volume processed, system pressure, and filtrate flow rate (Figure 4). The final target volume for UF concentrates using the WSC was 450 ± 25 mL after elution. The time was noted when the filtration procedure was started and when the elution procedure was completed using the same watch.

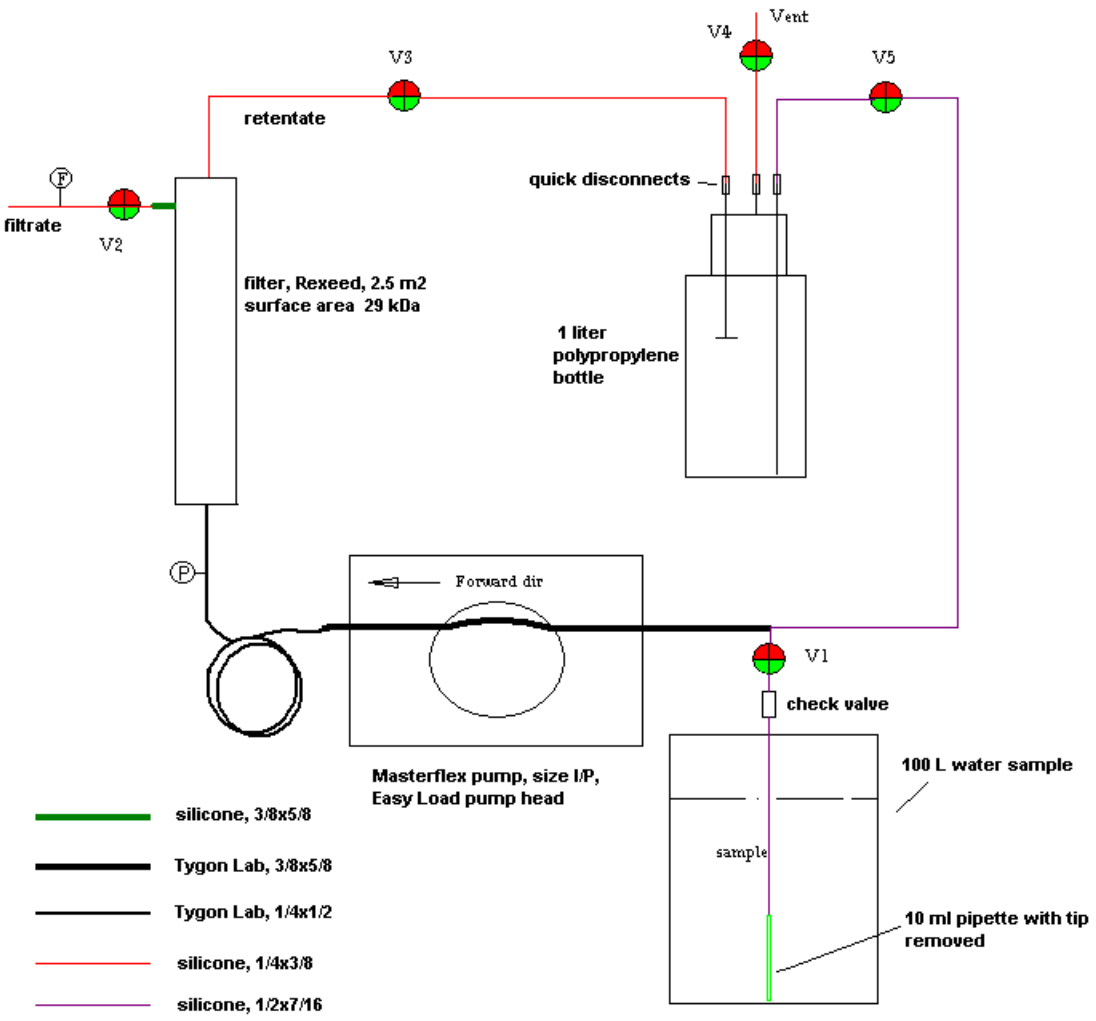

Figure 3. Schematic of ultrafiltration set-up for EPA method ("V" indicates valve location).

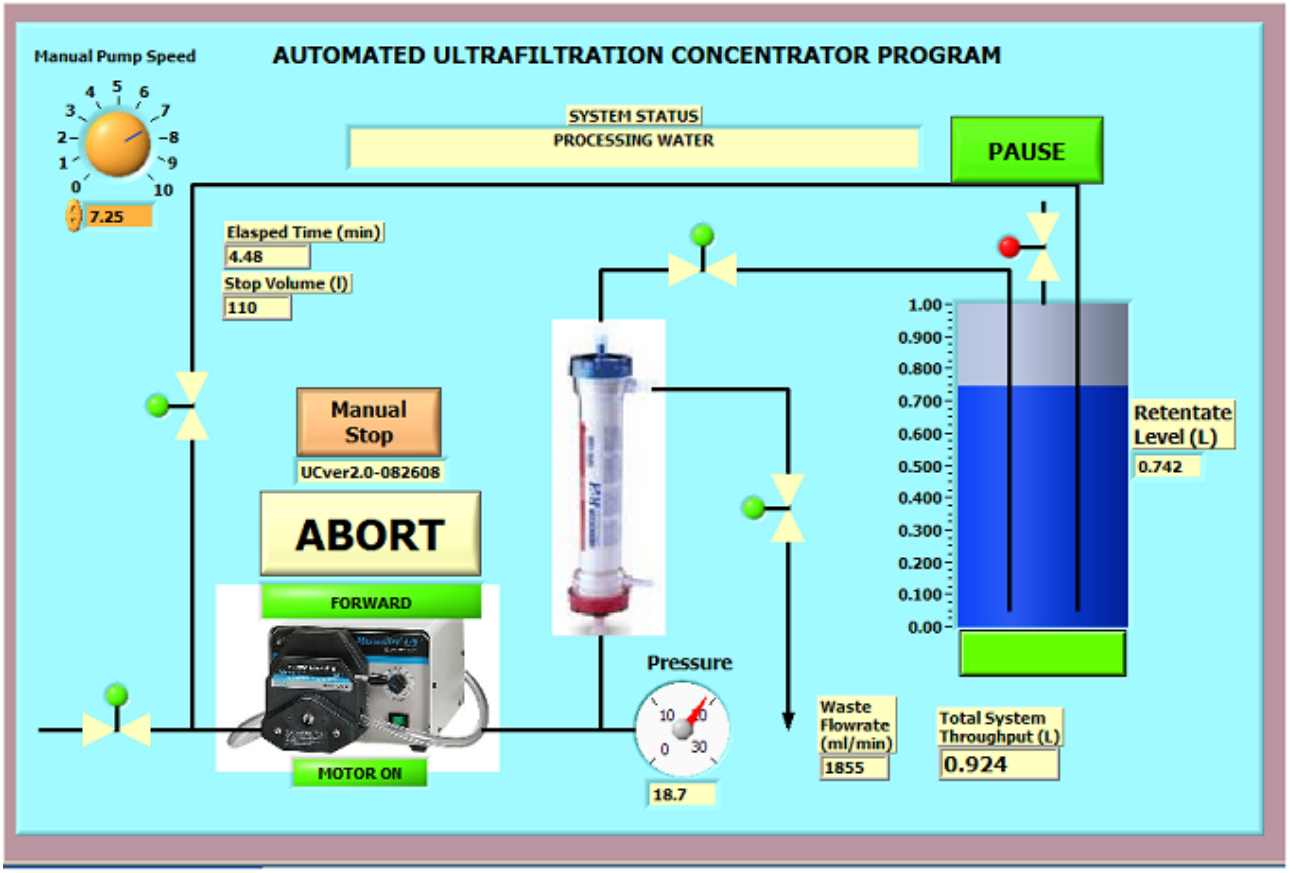

Figure 4. View of the water sample concentrator monitoring screen as seen during an ultrafiltration run.

2.5 Data Analysis

Calculation of microbial counts in each sample was performed using calculated concentration data and total sample volume data for input samples (i.e., non-concentrated, seeded 500-mL Control Samples) and output samples (i.e., UF concentrates, centrifuge concentrates for low-seed *Cryptosporidium* and *Giardia*). Concentration data were calculated on a per mL basis, using total microbial counts for a plate/slide [e.g., 20-60 CFU, 20-100 PFU, 20-100 (oo)cysts] per the sample volume assayed. Total counts of each microbe for each sample were calculated by multiplying the calculated concentration by the total sample volume.

Percent recovery efficiency was computed for each microbe using the following equation:

$$R = 100 \times (N/T)$$

Where: R = percent recovery, N = number of the microbe calculated to be in concentrated sample, T = number of the microbe calculated to be in the control sample (i.e., non-concentrated sample).

Comparative recovery efficiency data were statistically analyzed for Suite 1 to Suite 5 experiments using paired t-tests at an alpha level of 0.05. CDC and EPA method data were paired based on date of experiment. The difference between pairs was checked for normality using the Shapiro-Wilk W test (JMP 9.0.2, used for all statistical analyses). When the data was not normally distributed the Wilcoxon signed rank test was used instead. When comparing different suites for a single method, as well as water quality data from experiments performed at the CDC Chamblee and Roybal facilities, analysis of variance (ANOVA) was used. A two-sided F-test for variance was performed to determine the appropriate statistical procedure (t-test, ANOVA). The Bonferroni correction for multiple comparisons (n = 30) was used when performing the ANOVA test for difference in microbial recovery efficiencies between the EPA and CDC/LRN methods. The Wilcoxon rank sum test was used when comparing different suites for which data was not normally distributed.

2.6 Blanks and Controls

For every five UF experiments performed, one 100-L tap water procedural blank was processed for both the EPA and CDC/LRN UF methods. This quality control measure enabled evaluation for potential background contamination (e.g., from laboratory environment or from drinking water system).

Sample analyses were performed with an analytical positive control and negative control for each analytical parameter. Positive control data (e.g., number of CFU on a *B. anthracis* positive control plate) were compared against expected results to determine whether analytical conditions were appropriate.

3.0 Results

3.1 Water Quality

After collection of the 100-L water samples for an experiment, a suite of water quality tests were performed to characterize the water samples. These water quality data are summarized for the two laboratory facilities (Chamblee and Roybal campuses) in Table 3. Free chlorine residuals at both facilities were within normal ranges for drinking water. Post-dechlorination testing demonstrated that water samples contained no free chlorine when microbes were seeded into the water samples. Post-dechlorination free chlorine results were equal to or below the method detection limit for the analytical method (0.03 mg/L). The average pH of the water samples (8.8 - 9.0) reflected the higher pH employed by the water utility to control corrosion in the drinking water distribution system. Turbidity of the tap water varied from 0.078 nephelometric turbidity units (NTU) to 1.06 NTU, but average turbidity levels were similar at the two facilities. Total hardness and specific conductance data indicate that the water used in this study would be classified as soft with a low ionic strength. TOC concentrations in tap water at both facilities were also similar (average 3.1 and 2.7 mg/L). Heterotrophic plate count (HPC) bacteria levels were low in tap water from both facilities, but concentrations were more variable in tap water at the Chamblee campus. While it appears that tap water quality at the Chamblee and Roybal facilities was similar, data for the following parameters was found to be significantly different statistically: turbidity ($p = 0.03$), specific conductance ($p < 0.0001$), pH ($p = 0.0001$), and free chlorine ($p < 0.0001$).

Table 3. Water Quality Data for 100-L Tap Water Samples

Source		Free Chlorine (mg/L)	pH	Temp (ºC)	Turb (NTU)	SC (µS/cm)	T. Hardness (mg/L Ca as $CaCO_3$)	TOC (mg/L as C)	HPC (CFU/mL)
Chamblee	Avg	0.94	9.0	22	0.20	117	16	3.1	12
	SD	± 0.36	± 0.33	± 3.3	± 0.13	± 38.8	± 2.5	± 1.9	± 14
	n	40	41	41	43	42	20	34	40
Roybal	Avg	1.2	8.8	24	0.32	141	16	2.7	3.3
	SD	± 0.14	± 0.37	± 0.81	± 0.26	± 54.1	± 1.6	± 1.1	± 2.9
	n	19	19	9	19	19	19	18	17

CFU, colony forming units; HPC, heterotrophic plate count; SC, specific conductance; SD; standard deviation; T., total; TOC, total organic carbon; Turb, turbidity

3.2 Operations and Safety

During operation of the WSC instrument to perform the EPA UF method, the system software automatically monitored pressure and flow rate. For the CDC/LRN UF procedure, the filtrate rate was set by manually adjusting a tubing clamp on the return tubing at the start of an

experiment; system pressure monitoring was not performed. No evidence of filter clogging was observed (i.e., no increase in pressure or decrease in filtrate rate with cumulative increase in sample volume filtered) for either method/filter type (Table 4). Filtrate rates for the EPA method were slightly higher than for the CDC method; the higher filtrate rates and automation of the procedure resulted in the EPA procedure requiring approximately 20 fewer minutes to complete than the CDC/LRN UF procedure.

While some software and maintenance issues were encountered when operating the WSC instrument early in the project, these issues were readily resolved with improvements to the software and changes to the system components. During Suite 4 and 5 experiments, few operational problems were encountered when operating the WSC instrument. No explicit safety issues were encountered when performing either UF procedure, but it should be noted that the WSC instrument performs filtration in a contained system. Unless tubing connections are loose (risking sample leakage or spraying), there is no risk for sample exposure. The CDC/LRN method does not employ a completely contained system; tubing must be manually handled to switch-out carboys, retentate is collected in open beakers, and sample concentration is performed in open-mouth containers instead of a bottle enclosed with a vented cap. The potential risk for aerosol exposure with the CDC/LRN method must be controlled by performing the procedure in a laboratory having an appropriate biosafety level (BSL) and through the use of appropriate personal protective equipment (PPE) such as a powered air purifying respirator.

Table 4. Operational Data for EPA and CDC/LRN Ultrafiltration (UF) Methods for 100-L Water Samples

Method	Filter	System Pressure (psig)	Filtrate Flow Rate (mL/min)	UF Concentrate Volume (mL)	UF Processing Time (min)
EPA	REXEED 25SX	25 ± 2.0	1800 ± 250	420 ± 72	60 ± 10
CDC/LRN	Fresenius F200NR	NA	1700 ± 180	490 ± 38	80 ± 8

NA: Not analyzed

3.3 Microbial Recoveries

3.3.1 Bacterial Recovery Efficiencies

***B. anthracis* and *B. atrophaeus* subsp. *globigii*.** Recovery efficiencies for *B. anthracis* spores in Suite 1 and 3 experiments were significantly higher for the EPA method than for the CDC method ($p = 0.01$ and 0.001, respectively) (Table 5). The average recovery of *B. anthracis* spores by the EPA method was similar in the Suite 1 and Suite 3 experiments ($p = 0.07$). The average recovery of *B. anthracis* spores by the CDC method were significantly lower during Suite 3 experiments (65 ± 14%) than during Suite 1 experiments (85 ± 17%) ($p = 0.02$).

In Suite 3 experiments, *B. atrophaeus* subsp. *globigii* spore recovery was also significantly higher for the EPA method (99 ± 11%) than the CDC/LRN method (57 ± 15%) ($p<0.0001$). Suite 3 recovery efficiency data for both CDC/LRN and EPA methods indicated that *B. atrophaeus* subsp. *globigii* spores were effective surrogates for recovery of *B. anthracis* spores from tap water samples, based on no significant difference in recovery efficiencies between the two ($p = 0.07$ and $p = 0.18$ for CDC and EPA method data, respectively).

In Suite 4 experiments, for which NaPP was only added for filter blocking but was not added to the water sample processed using the EPA UF method, the EPA method resulted in significantly higher recoveries of *B. anthracis* spores and *B. atrophaeus* subsp. *globigii* spores than the CDC/LRN method ($p = 0.001$ and 0.0002, respectively). As observed for the Suite 3 experiments, Suite 4 recovery efficiency data for the EPA method indicated that *B. atrophaeus* subsp. *globigii* spores and *B. anthracis* spores were recovered from 100-L water samples at similar efficiencies ($p = 0.27$), but recovery efficiencies were significantly different when the CDC/LRN method was used ($p = 0.01$).

***C. perfringens* spores.** The percent recovery of the *C. perfringens* spores was similar for both the CDC (100 ± 22%) and the EPA (110 ± 27%) methods ($p = 0.33$). The high percent recoveries measured were likely due to disaggregation of cell aggregates during ultrafiltration, despite attempts to produce monodispersed *C. perfringens* spore seed stocks by vigorous mixing and filtration through 5-µm filters.

***E. faecalis*.** Percent recoveries of *E. faecalis* were similar for both the CDC/LRN method (97 ± 12%) and the EPA method (100 ± 12%) in Suite 1 experiments ($p = 0.4829$). In Suite 5 experiments, *E. faecalis* recovery efficiencies associated with the CDC/LRN procedure were similar to Suite 1 data ($p = 0.43$). *E. faecalis* recovery efficiencies for the EPA method were significantly lower in Suite 5 experiments than in Suite 1 experiments ($p = 0.0049$). Suite 5 *E. faecalis* recovery efficiencies were significantly higher for the CDC/LRN method than the EPA method ($p = 0.0001$). In addition to measuring lower *E. faecalis* recovery efficiencies for the EPA method in Suite 5 experiments, it was also observed that *E. faecalis* colony development was substantially slower for the EPA UF concentrate samples. When EPA sample plates were held an additional 24 h (i.e., 48 h incubation), 19% more colonies were counted on EPA agar plates but no additional colonies were counted on CDC/LRN plates.

Y. pestis. Percentage recoveries of *Y. pestis* were similar for both the CDC/LRN method (70 ± 16%) and the EPA method (70 ± 18%) in Suite 1 experiments ($p = 0.93$). In Suite 4 experiments, *Y. pestis* recovery efficiencies for the CDC/LRN method were higher (100 ± 38%), but statistical analysis versus Suite 1 data did not find a significant difference ($p = 0.15$), likely due to the higher variability in experimental results from the Suite 4 experiments. While Suite 4 *Y. pestis* recovery data for the EPA method (76 ± 22%) was also found to not be significantly different than in Suite 1 ($p = 0.53$), the difference in Suite 4 recovery efficiencies between the two UF methods was significantly different ($p = 0.03$).

Table 5. Average Microbial Recovery Efficiencies for the CDC and EPA Ultrafiltration Procedures

Microbe*	Methods									
	CDC Average Percent Recovery; CV (coefficient of variance)					EPA Average Percent Recovery; CV				
	Suite 1	Suite 2	Suite 3	Suite 4	Suite 5	Suite 1	Suite 2	Suite 3	Suite 4	Suite 5
***B. anthracis* spores**	85**; 20		65*; 21	74*; 35		100*; 12		92*; 11	96*; 35	
***B. atrophaeus* subsp. *globigii* spores**			57*; 26	47*; 48				99*; 11	89*; 34	
E. faecalis	97; 13				100*; 12	100; 10				63*; 33
Y. pestis	70; 23			100*; 37		70; 26			76*; 28	
***C. perfringens* spores**	100; 22					110; 24				
F. tularensis	17*; 41		13*; 67	29; 72		29*; 52		25*; 75	27; 61	
***F. tularensis* (1% NH_4Cl)**	23*; 38		29*; 41	62; 15		39*; 38		40*; 44	46; 48	
B. diminuta			83; 18	82; 19				84; 11	78; 16	
MS2		110; 34			98*; 5.7		120; 28			69*; 17
Phi X174		100*; 13			110*; 11		95*; 12			86*; 16
Echovirus 1		68*; 38			79*; 35		47*; 33			37*; 62
C. parvum		82; 36			72*; 14		73; 39			110*; 17
Color Seed *C. parvum*		38; 33					30; 72			
G. intestinalis		99*; 18			78*; 34		85*; 17			110*; 16
Color Seed *G. intestinalis*		42; 25					44; 53			

**Bacillus anthracis* (Sterne) spores, *Bacillus atrophaeus* subsp. *globigii*, *Enterococcus faecalis*, *Yersinia pestis* (A1122), *Clostridium perfringens* spores, *Francisella tularensis* LVS, *Brevundimonas diminuta*, MS2 bacteriophage, phi X174 bacteriophage, echovirus type 1, *Cryptosporidium parvum* oocysts, and *Giardia intestinalis* cysts

* *Significant differences between CDC and EPA methods for an organism in a particular suit

F. tularensis and B. diminuta. Recovery of culturable *F. tularensis* was challenging for both UF methods. Initially (for Suite 1), frozen stocks were used to seed water samples, but this procedure was associated with CDC/LRN method recoveries on the order of 1% and EPA method recoveries on the order of 10% (data not shown). After instituting use of an overnight culture of *F. tularensis* to seed water samples in Suite 1, recovery efficiencies were substantially higher for both UF methods. However, while use of an overnight culture improved the culturability of *F. tularensis* in UF concentrates this procedure also resulted in highly variable input seeding levels (which ranged from 3,000 to 290,000 CFU). Suite 1 *F. tularensis* recovery efficiencies using the EPA method (29 ± 15%) were significantly higher ($p = 0.0096$) than the CDC/LRN method (17 ± 7.0%). In Suite 3 experiments, *F. tularensis* recovery was investigated again, but in conjunction with a potential surrogate microbe, *B. diminuta*. For the Suite 3 experiments, *F. tularensis* recovery efficiencies using the EPA method (25 ± 19%) were significantly higher than the CDC method (13 ± 9%) ($p = 0.01$). *B. diminuta* recovery efficiencies were high for both the EPA method (84 ± 9%) and CDC/LRN method (83 ± 15%), and no significant difference was found in recovery efficiencies between the two methods ($p = 0.85$).

In Suite 4 experiments, *F. tularensis* recovery efficiencies for the CDC method were higher (29 ± 21%) than in Suite 1 and Suite 3 experiments, but the differences were not significant ($p = 0.052$). The use of NaPP only as a blocking agent in the Suite 4 experiments for the EPA method was not associated with a significant effect on *F. tularensis* recovery (27 ± 16% versus 29 ± 15% and 25 ± 19% in Suites 1 and 3, respectively) ($p = 0.98$). In Suite 4, *B. diminuta* recovery efficiencies were again very similar between the EPA and CDC/LRN UF methods ($p = 0.30$). *B. diminuta* recovery efficiencies were found to be significantly higher than *F. tularensis* recovery efficiencies for both the EPA and CDC/LRN methods when Suite 3 and 4 experiment data were combined for statistical analysis ($p = 0.001$ for CDC/LRN method and $p = 0.0012$ for EPA method).

In an attempt to improve the culturability of *F. tularensis* in UF concentrates, aliquots of UF concentrates produced using each UF method were exposed to 1% ammonium chloride for 2 h prior to membrane filtration and CHAB-A agar culture, as suggested by Valentine et al. (23). For the CDC/LRN method, in which water samples were always amended with NaPP, exposure to 1% ammonium chloride was associated with significantly higher recovery efficiencies ($p < 0.0001$). For EPA method experiments in which water samples were amended with NaPP (i.e., Suites 1 and 3), exposure to 1% ammonium chloride was also associated with a significant increase in recovery efficiencies ($p = 0.002$). In Suite 4 experiments, in which water samples processed using the EPA method were not amended with NaPP, exposure to 1% ammonium chloride appeared to be associated with higher *F. tularensis* recovery efficiencies, but the differences were not significant ($p = 0.07$). Incorporation of 1% ammonium chloride into the culture protocol did not impact relative *F. tularensis* recovery efficiency differences between the two UF methods in Suite 1 and 3 experiments, for which the EPA method recoveries were still significantly higher ($p = 0.0002$). In Suite 4 experiments, *F. tularensis* recovery efficiencies were higher for the CDC/LRN method (62 ± 9.4%) than the EPA method (46 ± 22%) when UF concentrates were exposed to 1% ammonium chloride prior to culture, but the differences were not significant ($p = 0.23$).

3.3.2 Viral Recovery Efficiencies

MS2. In Suite 2 experiments, recovery efficiencies for the CDC/LRN and EPA UF methods were high and variable (110 ± 38% and 120 ± 33%, respectively). No significant difference was found between the two methods ($p = 0.11$). In Suite 5 experiments, MS2 recovery efficiencies for the CDC/LRN method were similar to recovery efficiencies from Suite 2 experiments, but EPA method MS2 recovery efficiencies were significantly lower in Suite 5 than in Suite 1 ($p = 0.0004$). Suite 5 MS2 recovery efficiencies for the CDC/LRN method were significantly higher than the EPA method ($p = 0.003$).

Phi X174. As found for MS2, phi X174 recovery efficiencies in Suite 2 experiments were also high for both the CDC/LRN method (100 ± 13%) and EPA method (95 ± 11%), but variability in the data was much lower than for MS2. Consequently, while recovery efficiencies were similar between the two methods, the CDC/LRN recovery efficiencies were found to be significantly higher than for the EPA method ($p = 0.02$). In Suite 5 experiments, phi X174 recovery efficiencies associated with the CDC/LRN method remained high (110 ± 12%). Recovery efficiencies for the EPA method were lower (86 ± 14%), but were not significantly different than in Suite 2 experiments ($p = 0.13$). As determined for Suite 2, Suite 5 phi X174 recovery efficiencies were significantly higher for the CDC/LRN method than the EPA method ($p = 0.008$).

Echovirus 1. Echovirus 1 recovery data for this study reflect recovery of the virus after performing UF and secondary concentration using Centricon® Plus-70 microconcentrators. Recoveries of echovirus 1 were significantly higher in Suite 2 for the CDC UF method (68 ± 26%) than the EPA method (47 ± 15%) ($p=0.03$). In Suite 5, echovirus 1 recovery efficiencies remained high for the CDC/LRN method (79 ± 27%), but recovery efficiencies for the EPA method were slightly lower (37 ± 23%) than in Suite 2 ($p = 0.25$). As determined for the two bacteriophages, echovirus 1 recovery efficiencies for the CDC/LRN method were significantly higher than for the EPA method in Suite 5 experiments ($p = 0.0008$).

3.3.3 *C. parvum* and *G. intestinalis* Recovery Efficiencies

Average recoveries of high seed *C. parvum* oocysts were similar for the CDC/LRN (82 ± 29%) and EPA (73 ± 28%) UF methods in Suite 2 experiments ($p = 0.21$). Recoveries of high seed *G. intestinalis* cysts were also high (99 ± 18% and 85 ± 14% for the CDC/LRN and EPA methods, respectively), and the difference between the methods was significantly different ($p = 0.03$). In Suite 5 experiments, *C. parvum* and *G. intestinalis* (oo)cyst recovery efficiencies were significantly higher for the EPA method (0.0047 and 0.0043, respectively). The *C. parvum* oocyst recovery efficiencies for the EPA UF method were found to be significantly higher than the CDC/LRN UF method ($p = 0.0003$). *G. intestinalis* cyst recovery efficiencies were also significantly higher for the EPA UF method than the CDC/LRN UF method for Suite 5 ($p = 0.02$).

In Suite 2, ColorSeed™ *C. parvum* and *G. intestinalis* (*G. lamblia*) (oo)cysts were concentrated by UF, followed by centrifugation, and finally IMS before fluorescence microscopy analysis. ColorSeed™ *C. parvum* oocyst recoveries associated with the EPA method (30 ± 22%) were not significantly different than oocyst recoveries associated with the CDC/LRN method (38 ± 12%) ($p = 0.23$). ColorSeed™ *G. intestinalis* cyst recoveries associated with the EPA method (44 ± 24%)

were also not significantly different than cyst recoveries associated with the CDC/LRN method (42 ± 11%) ($p = 0.64$).

3.4 Project Data Quality Objectives and Overall Microbial Recovery Efficiencies for Each UF Method

In general, both the EPA and CDC/LRN UF methods recovered ≥50% of seeded microbes. To enable effective statistical comparisons of recovery efficiencies for the two methods, the goal for this project was to produce recovery efficiency data having coefficients of variation (CV) values less than 25%. This data quality objective was achieved for 23 of the 46 (50%) of the recovery efficiency percentages reported in Table 5 for high-seed microbial parameters. The most challenging microbe to recover for both methods was *Francisella tularensis*. CV values for *F. tularensis* recovery efficiencies were also generally above the target ceiling for the study. Other microbes for which recovery efficiency CV data was higher than data quality objectives were *B. anthracis* and *B. atrophaeus* subsp. *globigii* in Suite 4 experiments, *Y. pestis* in Suite 4, *E. faecalis* in Suite 5 (EPA method only), MS2 in Suite 2, *C. parvum* in Suite 2, and *G. intestinalis* in Suite 5 (CDC/LRN method only). Recovery efficiency CV data were also relatively high for echovirus 1 and ColorSeed™ (oo)cysts, but these relatively higher CV values were expected because echovirus 1 and ColorSeed™ required additional sample processing steps (having additional processing inefficiencies and variability).

ANOVA analysis (with Bonferroni correction) of microbial recovery data from Suite 1, 2 and 3 experiments indicated that the EPA method was associated with a significantly higher (5.4% higher) overall microbial recovery efficiency than the LRN method. This performance difference between the two methods was largely driven by differences in method performance for recovering *B. anthracis* spores and *B. atrophaeus* subsp. *globigii* spores. With data for these two microbes removed from the analysis, no significant difference in overall microbial recovery was observed between the two UF methods. For Suite 4 and 5 experiment data, including *B. anthracis* and *B. atrophaeus* subsp. *globigii* spore data, ANOVA analysis (with Bonferroni correction) found no significant difference between the EPA and CDC/LRN UF methods. When testing for the potential effect of performing Suite 4 and 5 experiments at a different laboratory facility, no significant difference in overall microbial recovery efficiency was found for the CDC/LRN method ($p = 0.45$). The same analysis for EPA method data found that combined Suite 1, 2 and 3 microbial recovery efficiencies were not significantly different than Suite 4 and 5 microbial recovery efficiencies ($p = 0.39$).

The change in laboratory facilities between Suite 1-3 and Suite 4-5 experiments was also associated with a change in laboratory protocol when NaPP was not used as a sample amendment for water samples processed using the EPA UF method. This change in lab location and NaPP protocol was not associated with significantly different recovery efficiencies for the EPA method when *B. anthracis* and *B. atrophaeus* subsp. *globigii* data was grouped together (98% combined recovery efficiency for Suites 1 and 3 versus 93% combined recovery efficiency for Suite 4) ($p = 0.20$). A similar analytical approach determined that EPA method recovery efficiencies for the virus parameters (MS2, phi X174 and echovirus 1 grouped together) were significantly higher in Suite 2 (grouped average = 87%) than in Suite 5 (grouped average = 64%) ($p = 0.01$). However, the opposite association was found for the parasite parameters. High seed (oo)cyst recoveries for the

EPA method were significantly lower in Suite 2 (grouped average = 79%) than in Suite 5 (grouped average = 109%) ($p < 0.0001$).

4.0 Discussion

The data from this study demonstrate that the EPA and CDC/LRN UF methods were effective at recovering diverse microbes in 100-L drinking water samples. Despite significant differences between the EPA and CDC/LRN UF methods (e.g., blocking solution composition and method, pump size and recirculation flow rate, ultrafilter type, elution solution and method, operating pressure) both UF methods were able to recover >50% of seeded bacteria (with the possible exception of *F. tularensis*), viruses, and parasites. For echovirus 1 and ColorSeed™ (oo)cysts, total method recoveries (including secondary processing steps after UF) were generally above 30%. Although NaPP was added to water samples processed by both methods in Suite 1, 2 and 3 experiments (resulting in a concentration of 0.01% NaPP in the water samples), the EPA UF method for the WSC does not typically include sample amendment with NaPP. Suite 4 and 5 experiments were more exemplary of EPA's likely methodology for the WSC, as NaPP was not added to water samples processed using the EPA method (but NaPP was added to water samples processed by the CDC/LRN UF method in Suite 4 and 5 experiments). The filter blocking method used in the EPA protocol did result in some residual NaPP remaining in the system after the blocking procedure was completed, but the corresponding amount of NaPP (≤ 0.00025%) was at least 40-fold lower than the concentration of NaPP resulting from the sample amendment procedure. The presence of NaPP at a level of 0.001% has been suggested in previous research as being relatively ineffective for recovering microbes (e.g., *E. coli*) in water samples using tangential-flow UF (9).

While both methods were found to be similarly effective overall, there were microbial recovery performance differences between the methods for certain analytes. In particular, the data from this study demonstrate that the EPA WSC UF method was more effective at recovering *B. anthracis* spores than the CDC/LRN UF method. *B. atrophaeus* subsp. *globigii* spores were found to be good surrogates for *B. anthracis* spores for both UF methods and were also determined to be more effectively recovered using the EPA UF method versus the CDC/LRN UF method. In a 2007 study report in which the EPA method was performed manually (before the WSC device was developed), *B. atrophaeus* subsp. *globigii* spore recoveries were also found to be similar to *B. anthracis* spore recoveries, although recoveries of both microbes (average = 26-32%) were lower than achieved during the present study (4). The data from the present study support EPA's ongoing initiative to develop quality control (QC) criteria for *B. atrophaeus* subsp. *globigii* for determining UF method performance proficiency (24). Another study performed using a manual version of the EPA UF method reported higher *B. anthracis* spore recoveries (80 ± 44%) when a Fresenius F200NR ultrafilter was used (3). For *Y. pestis*, no performance difference between the methods was found, except in the Suite 4 experiments when recovery efficiencies for the CDC/LRN UF method were substantially higher than in Suite 1 and were found to be significantly higher than paired recovery efficiencies for the EPA method. *Yersinia pestis* recovery efficiencies for the EPA UF method in the present study (average = 70-76%) were similar to *Y. pestis* recovery efficiencies reported previously for a similar EPA UF method (84 ± 38%) when Fresenius F200NR filters were used (3). For *F. tularensis*, it was found that recovery efficiencies were significantly higher for the EPA UF method than the CDC/LRN method when NaPP was used as a sample amendment for both methods. When NaPP was not used as a sample amendment for water samples processed using the EPA UF method, there was no significant difference between the two UF methods, but this was due to higher recovery efficiencies for the CDC/LRN method in Suite 4 experiments than were obtained in Suite

1 and 3 experiments. *F. tularensis* recovery efficiencies for the EPA UF method in the present study were within the range of average recovery efficiencies (18-103%) reported for a previous EPA UF method when using Fresenius F200NR ultrafilters to process seeded water samples from three US cities (10). In all *F. tularensis* experiments from the present study, it was found that average recovery efficiencies for both the EPA and CDC/LRN methods were higher when UF concentrates were exposed to 1% ammonium chloride for 2 h prior to culturing. These data indicate that protocols for culturing *F. tularensis* from water samples should include this ammonium chloride exposure technique. A recent report from Pacific Northwest National Laboratory researchers indicated that exposure to 1% ammonium chloride was of significant benefit in preserving forensic analysis specimens for *F. tularensis* testing (23).

For the non-biothreat agent bacterial parameters investigated in this study, significant differences were found between the EPA and CDC/LRN UF methods for some of the analytes, but there were no consistent overall trends. No significant differences were found between the EPA and CDC/LRN UF methods for recovering *C. perfringens* spores or *B. diminuta*. *B. diminuta* was of interest in this study because it is small and similar in size to *F. tularensis* (0.2-0.3 μm). However, recovery/culture efficiencies were significantly higher for *B. diminuta* than *F. tularensis*, which raises the issue of whether it is a useful indicator of the effectiveness of a UF procedure for recovering/culturing *F. tularensis*. The other bacterial parameter in this study, *E. faecalis*, was included because it has been proposed as a QC parameter for establishing proficiency for the CDC/LRN UF method (24). In the present study, no significant difference was found between the EPA and CDC/LRN UF methods for recovering *E. faecalis* when NaPP was used as a sample amendment for both methods. But when NaPP was not used as a sample amendment for water samples processed using the EPA method, *E. faecalis* recovery efficiencies were significantly lower for the EPA method than the CDC/LRN method. *E. faecalis* recovery efficiencies for the CDC/LRN UF method in the present study were similar to *E. faecalis* recovery efficiencies reported by Hill et al. for a similar UF method applied to tap water (8) and by EPA for a multi-laboratory study of *E. faecalis* recovery as a QC parameter for the CDC/LRN UF method (24).

Bacteriophages MS2 and phi X174 were used as model enteric viruses in the present study based on prior research recommending them as useful models for water sampling methods based on morphological and surface charge characteristics (25). MS2 was recovered at a high level by both UF methods. In Suite 5 experiments, MS2 recovery efficiencies were significantly lower for the EPA UF method than in Suite 1 and were significantly lower than MS2 recovery efficiencies for the CDC/LRN method. This apparent association of higher MS2 recovery efficiencies with the use of NaPP as a sample amendment has been reported previously (6, 9). The CDC/LRN MS2 recovery efficiencies were similar to MS2 recovery efficiencies previously reported by Hill et al for a similar UF method (120 ± 22%) and were greater than MS2 recovery efficiencies reported by 13 laboratories (67 ± 8.3%) for a QC study for the CDC/LRN UF method (8, 24). Average MS2 recovery efficiencies for the EPA UF method (using the REXEED-25S filter, without NaPP sample amendment) in the present study (69 ± 12%) were slightly greater than average MS2 recovery efficiencies reported previously for a manual version of this method (52 ± 34%) (3). Phi X174 was also recovered at a high level by both UF methods, but recoveries were found to be significantly higher for the CDC/LRN method. The CDC/LRN phi X174 recovery efficiencies were slightly higher than phi X174 recovery efficiencies previously reported by Hill et al for a similar UF method (86 ± 13%) (8). Average phi X174 recovery efficiencies for the EPA UF method (using a

REXEED-25S filter, without NaPP sample amendment) in the present study (86 ± 14%) were greater than average phi X174 recovery efficiencies reported previously for a manual version of this method (57 ± 34%) (3).

The third viral parameter included in this study, echovirus 1, was seeded at a relatively low level (approximately 1,000 PFU) that only enabled determination of recovery efficiencies after secondary concentration. Reported echovirus 1 recovery efficiencies for each UF method in Table 3 were lower than for MS2 and phi X174, but these relatively lower recovery efficiencies for echovirus 1 reflect additional sample processing losses associated with the Centricon procedure. Total method recovery efficiencies for echovirus 1 from the present study (average = 37-47% for EPA method, 68-79% for CDC/LRN method) were similar to or higher than echovirus 1 recovery efficiencies reported in other UF studies (6, 26). The factors associated with the significantly higher echovirus 1 recoveries for the CDC/LRN method versus the EPA method are not clear. Higher recoveries for MS2 and phi X174 were also observed for the CDC/LRN method.

Recovery of parasite (oo)cysts by tangential flow UF have been studied extensively. In the present study, *C. parvum* and *G. intestinalis* (oo)cyst recoveries were similar between the two UF methods, and were generally above 70%. Similar UF recovery efficiencies for large-volume water samples have been previously reported (4, 8, 6, 26). ColorSeed™ (oo)cyst recovery efficiencies in the present study were also similar to low-seed *C. parvum* oocyst recoveries reported by Holowecky et al. (3), and were slightly lower than *C. parvum* and *G. intestinalis* recovery efficiencies reported by Hill et al. (26). ColorSeed™ recovery efficiencies were lower than for high seed *C. parvum* and *G. intestinalis* recoveries, but this was expected because of (oo)cyst losses inherent in additional sample processing (centrifugation, IMS) that was required for enumeration of ColorSeed™ (oo)cysts in water sample concentrates. In the present study, when NaPP was used as a sample amendment the recovery efficiencies for the EPA and CDC methods were similar for the high seed and ColorSeed™ (oo)cysts. In Suite 5 experiments, when NaPP was not used as a sample amendment for water samples processed by the EPA UF method, (oo)cyst recoveries were significantly higher than in Suite 1 and were significantly higher than for the CDC/LRN UF method. The apparent association of NaPP sample amendment with lower *C. parvum* oocyst recoveries for tangential flow UF was not expected based on previous published research results indicating that NaPP and associated polyphosphates are effective in dispersing *Cryptosporidium* oocysts, which should enable more efficient recovery during UF. Previous studies have reported that higher *C. parvum* oocyst recovery efficiencies were obtained during tangential flow UF when NaPP was used as a water sample amendment (6, 9). Other researchers have reported using NaPP to disperse *Cryptosporidium* oocysts prior to flow cytometry (27).

The results of this study demonstrate that the EPA and CDC/LRN UF procedures are effective at recovering diverse microbes from 100-L drinking water samples. When recovery data for all the microbial analytes were combined, a statistically significant difference between the two methods was observed for Suite 1, 2 and 3 experiments, indicating that the EPA UF method obtained higher recovery efficiencies for these experiments than the CDC/LRN UF method. This apparent difference in method performance was driven by consistently higher recoveries of *B. anthracis* spores and *B. atrophaeus* subsp. *globigii* spores. No significant difference between the EPA and CDC/LRN methods was observed when Suite 4 and 5 data were statistically analyzed. Recovery and culturability of *F. tularensis* was challenging for both UF methods, but exposure of UF

concentrates to 1% ammonium chloride for 2 h was found to consistently increase the culturability of *F. tularensis* by approximately 35-120%. The move to a new laboratory facility for Suite 4 and 5 experiments did not appear to affect experimental results based on potential water quality effects. Although some significant differences in water quality were measured, the significant differences were considered to be more reflective of low water quality variability rather than reflecting biologically or chemically plausible differences that could cause differential recovery efficiencies for UF. The move to a new laboratory facility was also associated with a change in protocol; NaPP was not used as a sample amendment for water samples processed by the EPA UF method in Suite 4 and 5 experiments. The NaPP experimental variable was not associated with consistent trends in microbial recovery efficiencies for the EPA UF method. When NaPP was used as a sample amendment, higher recovery efficiencies were measured for *E. faecalis* and MS2, but lower recovery efficiencies were measured for *C. parvum* and *G. intestinalis*.

5.0 Conclusions

1. Data from this study indicate that the CDC and the EPA UF methods can be similarly effective for the recovery of diverse biothreat agents in large-volume drinking water samples and thus validation of the EPA method for use during a response event is recommended.

2. UF recovery efficiencies were >50% for both methods for *B. anthracis* spores, *Y. pestis*, *E. faecalis*, *C. perfringens* spores, *B. diminuta*, MS2 bacteriophage, phi X174 bacteriophage, *C. parvum*, and *G. intestinalis*.

3. The lowest UF recovery efficiencies obtained in this study were for *F. tularensis*, but the use of 1% ammonium chloride was found to significantly increase the culturability of *F. tularensis* in UF concentrates.

4. ColorSeed™ recoveries were similar for the EPA and CDC/LRN methods, with *C. parvum* oocyst recoveries of 30 and 38%, respectively, and *G. lamblia* recoveries of 44 and 42%, respectively.

5. In general, data quality objectives (DQOs) for this project were met, including generating recovery efficiency data for high seed microbes with standard deviations ≤ 20% and CV values ≤ 25%; standard deviation and CV value DQOs for low seed microbes were ≤ 25% and ≤ 50%, respectively. Microbial data of note that did not meet DQOs were: *B. anthracis* and *B. atrophaeus* subsp. *globigii* (Suite 4, both methods), *F. tularensis* (throughout the study), *E. faecalis* (Suite 5, EPA method), *Y. pestis* (Suite 4, EPA method), MS2 (Suite 2, both methods), *C. parvum* (Suite 2, both methods), and *G. intestinalis* (Suite 5, CDC/LRN method).

6. No significant difference in overall microbial recovery efficiency was observed between the EPA and CDC/LRN UF recovery methods in Suite 4 and 5 experiments. However, differences for individual microbial parameters were observed.

7. Significantly higher recovery efficiencies for the EPA UF method were found for *B. anthracis* spores, *B. atrophaeus* subsp. *globigii* spores, *C. parvum*, and *G. intestinalis*.

8. Significantly higher recovery efficiencies for the CDC/LRN UF method were found for *E. faecalis*, MS2, phi X174, and echovirus 1.

9. The use of NaPP did not appear to be associated with a consistent trend in microbial recovery efficiency, but effects on recovery of individual analytes was indicated. When NaPP was used as a sample amendment, higher recovery efficiencies were measured for *E. faecalis* and MS2, but lower recovery efficiencies were measured for *C. parvum* and *G. intestinalis*. Use of NaPP as a sample amendment when operating the WSC under field conditions does not appear to be warranted considering the additional sample process complexity associated with adding NaPP in-line as a water sample is being processed.

10. The time required to concentrate 100 L of tap water using the CDC method (average = 80 min for filtration and elution) was approximately 20 min longer than the time required by the EPA method (average = 60 min for filtration and elution).

6.0 Presentations and Other Activities

Results from this project were presented in part as a poster at the 2010 American Society for Microbiology Biodefense and Emerging Diseases Research Meeting, Baltimore, MD. The poster presentation was entitled "Comparative Performance of Hollow-Fiber Ultrafiltration Procedures for Recovery of Biothreat Agents from 100-L Tap Water Samples" and was co-authored by S. Pai, T. Lusk, V. Gallardo, S. Shah, H.D.A. Lindquist, and V.R. Hill.

Funds for this project were also used for travel by the Principal Investigator, Vincent Hill, to Virginia to participate in a workshop on persistence of biothreat agents in the environment. Dr. Hill led the discussion on persistence of viruses.

7.0 References

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Appendix A: Quality Assurance/Quality Control

Data Quality Objectives	QA/QC Implemented
Data for comparison of the Environmental Protection Agency (EPA) and Centers for Disease Control and Prevention (CDC)/Laboratory Response Network (LRN) Ultrafiltration (UF) method performance should be obtained in paired experiments for statistical analysis.	Comparative recovery efficiency data was statistically analyzed for Suite 1 to Suite 5 experiments using paired t-tests at an alpha level of 0.05. CDC and EPA method data were paired based on date of experiment. The difference between pairs was checked for normality using the Shapiro-Wilk W test. When the data was not normally distributed the Wilcoxon signed rank test was used instead.
Percent recovery efficiency data for each microbe studied should have a standard deviation $\leq 20\%$ for each UF method. Coefficients of variation (CV) values for percent recovery efficiency data sets for each microbe should be $\leq 25\%$, with the exception of *F. tularensis* (for which highly variable recovery efficiency data is anticipated).	In general, data quality objectives (DQOs) for this project were met. For high seed microbes recovery efficiency data had standard deviations $\leq 20\%$ and CV values $\leq 25\%$. For low seed microbes, standard deviation and CV value DQOs were $\leq 25\%$ and $\leq 50\%$, respectively. Microbial data of note that did not meet DQOs were: B. anthracis and B. atrophaeus subsp. globigii (Suite 4, both methods), F. tularensis (throughout the study), E. faecalis (Suite 5, EPA method), Y. pestis (Suite 4, EPA method), MS2 (Suite 2, both methods), C. parvum (Suite 2, both methods), and G. intestinalis (Suite 5, CDC/LRN method). See Section 3.4 for more information.
Real-time polymerase chain reaction (RT-PCR) triplicate data for an assay should have interassay CV values (standard deviation divided by the mean) of $\leq 4\%$. Mean cycle threshold (CT) values should be below a value of 40 facilitate reproducibility and statistical analysis (positive RT-PCR results are generally limited to CT values of ≤ 42).	RT-PCR was performed early in the project to evaluate whether this technique could be effective as an additional measure for characterizing performance differences between the CDC/LRN and EPA methods. This DQO was met for F. tularensis, but not Y. pestis or B. anthracis (for which seed levels were too low for consistent detections below CT = 40. It was determined that this technique was not useful or needed for this project. Description of this real-time PCR work was not incorporated into the main body of the report in order to maintain clarity for readers by focusing on culture- and microscopy-based data.
Procedural blanks and negative controls should ensure that background or introduced contamination does not affect experimental data used for method comparison analysis.	For every five UF experiments performed, one 100-L tap water procedural blank was processed for both the EPA and CDC/LRN UF methods. This quality control measure enabled evaluation for potential background contamination (e.g., from laboratory environment or from drinking water system). Sample analyses were performed with an analytical positive control and negative control for each analytical parameter.
Positive control data should indicate that analytical assay conditions met performance expectations.	Sample analyses were performed with an analytical positive control and negative control for each analytical parameter. Positive control data (e.g., number of colony forming units on a B. anthracis positive control plate) were compared against expected results to determine whether analytical conditions were appropriate.

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