Concentration and quantification of somatic and F+ coliphages from recreational waters

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Abstract

Somatic and F+ coliphages are promising alternative fecal indicators, but current detection methods are hindered by lower levels of coliphages in surface waters compared to traditional bacterial fecal indicators. We evaluated the ability of dead-end hollow fiber ultrafiltration (D-HFUF) and single agar layer (SAL) procedure to concentrate and enumerate coliphages from 1L and 10L volumes of ambient surface waters (lake, river, marine), river water with varying turbidities (3.74–118.7 NTU), and a simulated combined sewer overflow (CSO) event. Percentage recoveries for surface waters were 40–79% (somatic) and 35–94% (F +). The method performed equally well in all three matrices at 1L volumes, but percent recoveries were significantly higher in marine waters at 10L volumes when compared to freshwater. Percent recoveries at 1L and 10L were similar, except in river water where recoveries were significantly lower at higher volume. In highly turbid waters, D-HFUF-SAL had a recovery range of 25–77% (somatic) and 21–80% (F +). The method produced detectable levels of coliphages in diluted wastewater and in unspiked surface waters, emphasizing its applicability to CSO events and highlighting its utility in recovery of low coliphage densities from surface waters. Thus D-HFUF-SAL is a good candidate method for routine water quality monitoring of coliphages.

Keywords

1. Introduction

Fecal contamination not only degrades recreational water quality but can also lead to potential public health risks due to the presence of enteric pathogens. Recent reports identified viral pathogens as leading contributors to waterborne disease outbreaks in

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recreational waters (Begier et al., 2008; Eftim et al., 2017; Jones et al., 2009; Yoder et al., 2008). However, direct measurements of viral pathogens are difficult because infectious virus detection methods (e.g. cell culture) are prohibitively expensive and time-consuming for routine testing, and molecular approaches (e.g. qPCR, RT-qPCR) fail to distinguish between infective and non-infective virions. Instead, fecal indicator bacteria (FIB) such as fecal coliforms, *Escherichia coli* and enterococci are routinely used to measure fecal contamination in recreational waters and signal potential presence of pathogens. However, it is well documented that survival and transport of FIB and viral pathogens differ, suggesting that viral indicators may be more suitable for recreational water quality applications (Ashbolt et al., 2001; Boehm et al., 2009a; Craig et al., 2003; Fujioka et al., 2015; Jofre et al., 2016).

Testing for somatic and F+ coliphages presents an attractive alternative to testing for viral pathogens, because they share similar morphologies overcoming some of the limitations with current fecal indicators (Ashbolt et al., 2001; United States Environmental Protection Agency, 2015; Vergara et al., 2015). For example, some somatic coliphages are morphologically similar to adenovirus, and F+ coliphages resemble enteroviruses, caliciviruses, astroviruses and some hepatotropic viruses (King et al., 2011). Coliphages are more environmentally stable than FIB (Brookes et al., 2004) and conditions required for its replication are rare under ambient conditions (Muniesa and Jofre, 2004). Additionally, coliphages are abundant in human fecal waste (Gantzer et al., 1998; Lucena et al., 2004; Zhang and Farahbakhsh, 2007), display a high degree of bacterial host specificity (Long et al., 2005), and require simple and inexpensive culture-based detection methods (International Organization for Standardization, 1995; International Organization for Standardization, 2000; United States Environmental Protection Agency, 2001a; United States Environmental Protection Agency, 2001b). While they have been the subject of research efforts for many years (Cole et al., 2003; Gerba, 1987; Havelaar, 1987; Palmateer et al., 1991; Simkova and Cervenka, 1981), coliphages recently became the focus for regulatory applications (Department of Environment and Conservation, 2012; North Carolina Environmental Quality, 2011; Queensland Government Environmental Protection Agency, 2005; United States Environmental Protection Agency, 2006). Due to the many potential advantages, the United States Environmental Protection Agency (US EPA) is considering the use of F-specific and somatic coliphages as possible viral indicators of fecal contamination in ambient waters (United States Environmental Protection Agency, 2015).

Previous epidemiology studies measuring coliphages have often reported high numbers of non-detects, potentially limiting the ability of the studies to fully characterize relationships between coliphages and public health risk (Abdelzaher et al., 2011; Boehm et al., 2009b; Colford et al., 2007; Medema et al., 1995; Viau et al., 2011; von Schirnding et al., 1992; Wade et al., 2010). Average coliphage concentrations are typically less than 1 $\log_{10}$ plaque forming units (PFU) per mL for F+ coliphages and 1 $\log_{10}$ PFU per 100 mL for somatic coliphages compared to 2 $\log_{10}$ colony forming units (CFU) per 100 mL of FIB in surface waters (Boehm et al., 2009b; McMinn et al., 2017; Ortega et al., 2009; Viau et al., 2011). Volumes used for standard phage enumeration methods (1–100 mL) (International Organization for Standardization, 1995; International Organization for Standardization, 2000; United States Environmental Protection Agency, 2001a; United

States Environmental Protection Agency, 2001b) are potentially insufficient for recreational water quality applications. As a result, the ability to efficiently concentrate larger volumes of environmental water may be useful to improve detection limits.

Hollow fiber ultrafiltration (HFUF) utilizes size exclusion rather than adsorption and (or) elution to concentrate viruses and, therefore can provide more consistent recoveries among viral targets in different water types, compared to the standard methods for virus concentration because it does not rely on highly variable isoelectric points of viral capsids (Gibson and Schwab, 2011; Mull and Hill, 2012; Rhodes et al., 2011; Smith and Hill, 2009). Moreover, HFUF has been shown to successfully recover a diverse range of microorganisms (viruses, bacteria and protozoans) from a variety of matrices such as drinking water (Hill et al., 2007; Hill et al., 2005; Hill et al., 2009; Rhodes et al., 2016), ground water (Morales-Morales et al., 2003; Olszewski et al., 2005), surface water (Kuhn and Oshima, 2001; Kuhn and Oshima, 2002; Leskinen et al., 2010; Leskinen et al., 2009; Rhodes et al., 2016) and wastewater (Asfahl and Savin, 2012; Gyawali et al., 2015).

In this study, we combine the concentration capability of HFUF with the standard somatic and F+ coliphage enumeration procedure (EPA Method 1602) to yield a simple, yet sensitive and robust method for recreational water quality monitoring. Method performance was evaluated with spiked preparations of tap, lake, river and marine water, at two different volumes (1L and 10L) and across a range of turbidities. Finally, somatic and F+ coliphage densities were measured in a simulated combined sewer overflow (CSO) event and in a series of environmental surface water samples collected from a variety of recreational waters.

2. Materials and methods

2.1. Preparation of wastewater derived somatic and F ± coliphage spikes

For spiked environmental water and turbidity experiments (Subsections 2.4.2 and 2.4.3), primary wastewater effluent was serially diluted using sterile 0.01 M phosphate buffer solution pH 7.4 (PBS) (Sigma-Aldrich, St. Louis, MO) and processed using double agar layer (DAL) as previously described (United States Environmental Protection Agency, 2001b). Plates that contained roughly 30 PFUs were selected for the subsequent enrichment steps. Approximately 15 plaques from each plate were picked for both coliphage types and transferred to 10 mL of tryptic soy broth (TSB) (Fisher Scientific, Waltham, MA) containing appropriate bacterial host in mid-log growth phase (CN-13 ATCC#700609 or F_{amp} ATCC#700891 for somatic and F-specific coliphages, respectively) and supplemented with 10 μL of appropriate antibiotic stock solution (100 μg/mL nalidixic acid for CN-13 and 15 μg/mL streptomycin/ampicillin for F_{amp}) (Fisher Scientific, Waltham, MA). The *E. coli* suspensions were incubated overnight at 37 °C. The following day suspensions were centrifuged at $3500 \times g$ for 5 min to remove bacterial cell debris, and the supernatant was filtered through a 0.22 μm syringe filter (Fisher Scientific, Waltham, MA). Coliphage levels were titered again using the DAL method (United States Environmental Protection Agency, 2001b). For the tap water experiments (Subsection 2.4.1) and CSO simulations (Subsection 2.4.4), different volumes (or dilutions) of primary wastewater were used as sources of coliphages.

2.2. Environmental water collection

Freshwater samples were collected from the William H. Harsha Lake (Clermont County, OH; 39°03′ 59″ N, 84°13′80″ W), Ohio River (Campbell County, KY; 39°09′85″ N, 84°49′49″ W), Lake Michigan (LaPorte County, IN; 41°72′ 87″ N, 86°90′68″ W) and Trail Creek (LaPorte County, IN; 41°72′ 67″ N, 86°90′98″ W). Marine water samples were collected from Morgan Beach Park (Pinellas County, FL; 25°51′16″ N, 81°41′29″ W). At the time of the experiments the water temperature ranged from 4 °C to 20 °C while average turbidity was 45 ± 2 NTU (marine water), 42 ± 18 NTU (lake water) and 95 ± 35 NTU (river water).

2.3. Concentration and enumeration of somatic and F ± coliphages

D-HFUF filtration apparatus consisted of Cole-Parmer Masterflex® L/S precision brushless drive (Cole Parmer, Vernon Hills, IL) equipped with an Easy-Load pump head, Masterflex® L/S 24 laboratory tubing (for the influent and elution lines), Masterflex®L/S-36 laboratory tubing (for the effluent line) and an ultrafilter (Asahi Kasei Rexeed® single-use high-flux dialyzer) (Dial Medical Supply, Chester Springs, PA). We utilized 15S and 25S ultrafilters, depending on the water volume processed. The Asahi Kasei ultrafilters have molecular cutoffs of 30 kDa, surface area of 1.5 m^2 (15S) or 2.5 m^2 (25S), and an inner fiber diameter of 185 μm.

Filtration set-up (Supplemental Fig. 1) involved connecting approximately 0.5 m of the influent line to the top port of the ultrafilter using custom-fitting DIN adapters (Molded Products Corporation, Harlan, IA) and threading it through the pump head. The other end of the influent line was connected to a sterile serological pipette (with cotton pulled out and tip broken off) and placed in a sterile vessel containing the bulk water sample to be filtered (2L or 20L). Approximately 1 m of effluent line was connected to the side effluent port located near the exit end of the filter and placed into a waste container. The filter was not pre-treated and the sample was passed through the filter using a setting of 300 rpm (approximately 850 mL/min). Average flow rate and pressure were 822 ± 45 mL/min and 7.6 ± 2.4 psi, respectively.

For the elution step (Supplemental Fig. 1), effluent line was removed and the side effluent port was capped, followed by connecting approximately 0.3 m of the elution line to the bottom port of the filter using a DIN adapter. The influent line remained attached. The ends of the influent and elution lines were placed in a clean, sterilized beaker containing either 200 mL (15S) or 400 mL (25S) of elution solution (0.01% Tween 80, 0.01% sodium hexametaphosphate, 0.001% Antifoam Y-30) (Sigma-Aldrich, St. Louis, MO). Elution of the filter was performed by setting the pump for either 1 min (15S) or 2 min (25S) to the clockwise, counterclockwise and finally clockwise direction. Depending on the sample volume processed, concentration factors per filter were 10-fold (15S) or 50-fold (25S). The possible effect of elution solution on coliphage was tested as a part of ancillary experiments by spiking primary wastewater effluent directly into either PBS or elution solution, followed by processing samples via SAL. There was no statistically significant difference ($P > 0.05$) in concentrations between the two solutions for either coliphage type (data not shown).

SAL procedure was performed as previously described (United States Environmental Protection Agency, 2001b). Briefly, concentrated samples were warmed to ~36 °C for 1–2 min in a water bath prior to commencement of the assay. In case of 15S filters, ~200 mL of elution solution was divided evenly between the two coliphage types, therefore the volume analyzed represented 1L per coliphage type. For the 25S filters, 100 mL of elution solution was aliquoted per coliphage type and the resulting plaque counts were multiplied by two in order to express results per 10L. One hundred mL of molten media (2X tryptic soy agar) (Fisher Scientific, Waltham, MA) was added to the sample along with the 10 mL of appropriate bacterial host in the mid-log growth phase, 0.5 mL of 4 M $MgCl_2$ (Sigma-Aldrich, St. Louis, MO) and 2 mL of the appropriate antibiotic stock solution. The mixture was inverted several times and poured evenly over five large petri plates (150 mm diameter). Plates were allowed to solidify then were inverted and incubated for 16–18 h at 37 °C. Characteristic somatic and F+ PFUs were enumerated using a light box, as previously described (United States Environmental Protection Agency, 2001b).

2.4. Performance of D-HFUF SAL method

2.4.1. Method performance metrics in tap water—In order to evaluate method performance metrics of D-HFUF-SAL, measured aliquots (and dilutions) of primary wastewater effluent were added to dechlorinated tap water to create series of ten-fold dilutions. Dilutions were created by adding 20 mL, 2 mL, 0.2 mL 0.02 mL and 0.002 mL of primary treated wastewater to 2L of tap water to create dilution range spanning 10^{-2} to 10^{-6}. Coliphage levels in undiluted primary wastewater effluent were 3.24 $\log_{10}$ PFU/mL and 2.27 $\log_{10}$ PFU/mL for somatic and F+ groups, respectively. The method performance metrics evaluated included limit of detection (LOD) and coefficient of variation across different dilutions. Here, LOD refers to the extent to which wastewater can be diluted and still be detected (defined as at least one out of nine replicates with countable plaques).

2.4.2. Percent recoveries from environmental water—In order to evaluate percent recoveries of the D-HFUF-SAL method, coliphages derived from wastewater were spiked into Ohio River, William H. Harsha lake and marine water samples. Spiked coliphage concentrations ranged from 1.65 to 3.70 $\log_{10}$ PFU and from 2.61 to 3.70 $\log_{10}$ PFU for somatic and F+ groups, respectively. In instances when background concentrations equaled or exceeded the target concentration, no wastewater derived spike was added. Instead, the autochthonous levels re-assayed on the day of the experiment using SAL without the D-HFUF were used to calculate percent recoveries.

2.4.3. Percent recoveries from turbidity experiments—The effect of turbidity on D-HFUF-SAL method was evaluated using sediment collected from a local river bank. Approximately 1 kg of sediment was dried at 60 °C in a heating oven overnight to remove autochthonous coliphages. To confirm that sediment did not contain viable coliphages, 5 g of sediment was added to 500 mL of sterile PBS, mixed, and assayed in ten replicate subsamples for each phage type using SAL (United States Environmental Protection Agency, 2001b). One liter of river water (per coliphage type) was spiked with waste-water derived coliphages (2.15 $\log_{10}$ and 2.44 $\log_{10}$ PFU/L for somatic and F+, respectively) and dried sediment was added in 0.4 g increments to create four turbidity levels in addition to

environmental turbidity (3.74 NTU), ranging from 38.4 to 118.7 NTU. Each turbidity level was replicated three times for a total of 15 samples (filters).

2.4.4. Simulated CSO event and detection of autochthonous coliphages from environmental waters—In order to mimic various scenarios for CSO events, different aliquots of primary wastewater effluent were added to river water to simulate 10^{-4}, 10^{-3} and 10^{-2} dilution. Dilutions were created by adding either 20 mL, 2 mL, 0.2 mL of primary treated wastewater to 2L of river water (1L experiments) or by adding 200 mL, 20 mL, 2 mL of primary treated wastewater to 20L river water (10L experiments). Coliphage levels in undiluted primary wastewater effluent for 1L experiments were 1.97 $\log_{10}$ PFU/mL and 1.65 $\log_{10}$ PFU/mL for somatic and F+ groups, respectively and 2.26 $\log_{10}$ PFU/mL (somatic) and 1.76 $\log_{10}$ PFU/mL (F +) for 10L experiments. The applicability of method for detection of autochthonous coliphages was assessed by applying D-HFUF-SAL to unspiked samples collected from two swimmable lakes (William H. Harsha Lake and Lake Michigan), two urban rivers (Ohio River and Trail Creek) and a marine beach (Morgan Park).

2.5. Data analyses

Statistical analyses were performed using PAST (Hammer et al., 2001), SigmaPlot, version 13.0 (Systat Software, San Jose, CA) and GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA). Briefly, concentration data (i.e. spiked tap water experiment and enumeration of autochthonous coliphage) was $\log_{10}$ transformed, while percent recovery data (i.e. spiked environmental water, turbidity experiment and CSO simulation) underwent arcsine square root transformation prior to statistical analyses. An analysis of covariance (AN-COVA) was used to assess whether diminishing levels of sewage inputs into the tap water reduced homogeneity between slopes for both phage types therefore negatively impacting method performance. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons and/or paired *t*-tests were used for to determine if matrix (lake vs. river water vs. marine), volume (1L vs 10L), turbidity level (3.74 NTU through 118.7 NTU), seed level or coliphage type (somatic vs F +) had a significant impact on recoveries. Paired *t*-tests were also used to determine whether a statistically significant difference existed between levels of autochthonous somatic and F+ coliphages in lake, river and marine waters.

3. Results

3.1. Method performance metrics in tap water

Mean concentrations of both somatic and F+ coliphages, as well as measured performance metrics are provided in Table 1. The highest dilution of primary wastewater effluent that could be detected in at least one replicate (corresponding to the LOD) was 10^{-6} for F+ coliphages (analogous to $\log_{10} 0.05 \pm 0.16$) and likely at least one to two orders of magnitude lower for the somatic group (Table 1). The precision of the method, or coefficient of variation (CV) in measuring varying concentrations of spiked coliphages ranged from 0.02 to 0.31 for somatic, and from 0.03 to 3.00 for F+ group and had an inverse relationship with the level of wastewater spike (the less wastewater input the larger the CV value observed) (Table 1). Slopes generated by ANCOVA were found to be indistinguishable ($P = 0.3567$, F = 0.8587) despite varying levels of coliphage inputs, demonstrating the consistent

and precise measurements of the D-HFUF method and suggesting that diminishing volume of sewage mixed with the tap water did not appear to impact the method performance.

3.2. Percent recoveries from environmental waters

The D-HFUF-SAL method was evaluated at two different volumes for coliphage recovery from three different ambient water sources: freshwater (river and lake), and marine water. Average and standard deviation for somatic coliphage recoveries from river were $79 \pm 14\%$ for 1L and $40 \pm 11\%$ for 10L samples (Fig. 1). Average recovery of F+ coliphages from river water was $62 \pm 15\%$ for 1L samples and $35 \pm 10\%$ for 10L samples (Fig. 1). Volume of river water filtered was found to significantly impact the somatic ($P < 0.0001$) and F+ ($P = 0.0168$) coliphage recovery with higher recoveries observed in lower volumes of water processed. Using the D-HFUF-SAL method in lake water resulted in $62 \pm 16\%$ average recovery of somatic coliphages from 1L samples and $47 \pm 6\%$ from 10L samples (Fig. 1). F+ coliphages were recovered at an average of $63 \pm 6\%$ from 1L samples and $45 \pm 6\%$ from 10L lake water samples (Fig. 1). Unlike river water, the volume of lake water filtered did not significantly impact recoveries of either phage type. In marine water, average recovery of somatic coliphages was $72 \pm 25\%$ from 1L volumes and $70 \pm 21\%$ from 10L volumes (Fig. 1). For F+ coliphages, average recoveries were $72 \pm 21\%$ and $94 \pm 27\%$ for 1L and 10L, respectively (Fig. 1). The volume of marine water filtered did not significantly impact recovery of either coliphage type. Comparisons between water types (freshwaters versus marine) indicated no significant difference in coliphage recoveries from 1L volumes, but a significantly higher recovery of both phage types in 10L of marine water compared to river water (P value range 0.0058– < 0.0001). For F+ coliphages, recoveries from marine water were also significantly higher when compared to the lake water ($P < 0.0001$). There was no statistically significant difference among recovery of two different coliphage types, irrespective of the volume or water type. Of note, turbidity in the river water was typically greater and more variable than the other two water types (lake and marine), possibly impacting percent recoveries especially at 10 L volumes.

3.3. Effect of turbidity

To assess the degree at which the D-HFUF-SAL method could be compromised by natural particulate matter, a series of experiments were designed to evaluate a range of realistic turbidities from unamended river water sample (3.74 NTU), to high levels of turbidity observed during periods of torrential rain events (119 NTU). Recovery of both somatic and F+ coliphages ranged from 25 to 77% and 21–80% respectively with mean concentrations of 1.42–2.40 $\log_{10}$ PFU/L (somatic) and 0.85–2.11 $\log_{10}$ PFU/L (F +) (Table 2). Comparisons between unamended samples (3.74 NTU) and those with the higher turbidity levels (96.2 NTU and 118.7 NTU) yielded statistically significant difference in recoveries for both somatic ($P = 0.0173$ and $P = 0.005$) and F+ coliphages ($P = 0.0036$ and $P = 0.0007$). In the case of F+ coliphages, a significant difference ($P = 0.0209$) was also observed when recoveries from unamended sample were compared to the sample with 68.7 NTU turbidity level. No statistically significant difference ($P > 0.05$) in recoveries was observed for any other comparisons among the different turbidity levels. Additionally, a paired *t*-test revealed no significant difference in recoveries between the two coliphage types at each corresponding turbidity level tested.

3.4. Simulated CSO event and detection of autochthonous coliphages from environmental waters

To evaluate a CSO event, river water was spiked with primary wastewater effluent in an attempt to simulate realistic levels of waste-water effluent released during such an event (Passerat et al., 2011). The highest dilution of primary wastewater effluent spiked equivalent to 10,000-fold dilution (and corresponding to 1.27 $\log_{10}$ PFU and 1.93 $\log_{10}$ PFU for somatic coliphages in 1L and 10L experiments, respectively and 0.11 $\log_{10}$ PFU and 1.80 $\log_{10}$ PFU for F+ coliphages in 1L and 10L samples, respectively) was reliably detected at both sample volumes. For 1L experiments, levels ranged from 1.27 to 2.96 $\log_{10}$ PFU/L (somatic) and from 0.11 to 2.83 $\log_{10}$ PFU/L (F +). For 10L experiments, levels ranged from 1.93 to 3.69 $\log_{10}$ PFU/L and from 1.80 to 3.20 $\log_{10}$ PFU/L for somatic and F+ coliphages, respectively. Average percent recoveries for the simulated CSO event ranged from 98 to 299% and from 31 to 154% for 1L sample volumes for somatic and F + coliphages, respectively (Fig. 2). The elevated and highly variable percent recovery of 299 (± 236%) was associated with the most dilute sample in which some of the replicates were at or below LOD of the assay. Ten liter volumes resulted in lower recoveries, ranging from 27 to 65% (somatic) and from 33 to 110% (F +) (Fig. 2). Within the two coliphage groups, there were no statistically significant differences in recoveries between different seed levels at either 1L or 10L volume. When different volumes (1L or 10L) were compared at the same seed level, the only significant observation ($P = 0.0273$) was for somatic coliphages at the lowest seed level where higher recoveries were observed at the lower volume. Comparison of percent recoveries between coliphage types matched by the volume yielded only one statistically significant observation at 1L volume and at the lowest seed level in which recoveries of somatic coliphages were significantly greater ($P = 0.0106$) compared to F+.

In order to demonstrate application of the method in a manner relevant for water quality management, we evaluated D-HFUF-SAL performance with unspiked field samples from two recreational lakes, two urban rivers, and a marine beach. Autochthonous somatic and F+ coliphages were detected in 100% and 75% of freshwater samples, respectively, but not in the marine beach samples. Average concentrations of somatic coliphages ranged from 1.48 ± 0.30 $\log_{10}$ PFU/L (Lake Michigan), 2.02 ± 0.11 $\log_{10}$ PFU/L (William H. Harsha Lake), and 2.55 ± 0.22 $\log_{10}$ PFU/L (Ohio River) to 3.30 ± 0.41 $\log_{10}$ PFU/L (Trail Creek) (Fig. 3). Average concentrations of F+ coliphages ranged from non-detectable to 0.36 ± 0.39 $\log_{10}$ PFU/L (William H. Harsha Lake), 0.42 ± 0.35 $\log_{10}$ PFU/L (Lake Michigan), 0.92 ± 1.09 $\log_{10}$ PFU/L (Trail Creek) to 0.99 ± 0.68 $\log_{10}$ PFU/L (Ohio River) (Fig. 3). In general, levels of somatic coliphages were at least two to four times higher ($P < 0.001$) than levels of F+ (Fig. 3) in the rivers and lakes tested. Levels of both coliphage types were higher in rivers as compared to lakes, although it was significant ($P = 0.0157$) only for somatic coliphages.

4. Discussion

In this study, we combined an ultrafiltration technique (D-HFUF) with a standardized single agar layer (SAL) procedure to concentrate and measure autochthonous F+ and somatic coliphages in both fresh and marine surface waters. The D-HFUF method was selected based on numerous studies suggesting its effectiveness in recovery of multiple

microbial targets (viruses, bacteria and protozoa), as well as its ability to handle large volumes of turbid water without substantial clogging issues (Leskinen et al., 2010; Mull and Hill, 2012; Rhodes et al., 2011). For a standardized coliphage assay, the SAL method was chosen (United States Environmental Protection Agency, 2001b) because it allows for the entire volume of filter eluate to be analyzed, thus increasing the likelihood of detecting low concentration coliphage targets. Furthermore, rather than using commercially available bacteriophage strains for sample spiking (e.g. T1, PP7, ΦX174 and MS2), we used coliphages autochthonous to municipal wastewaters. Considering the enormous diversity of the somatic coliphage group (*Myoviridae, Siphoviridae, Podoviridae* and *Microviridae* DNA phages) (Burbano-Rosero et al., 2011), this approach is likely to establish a more accurate understanding of D-HFUF-SAL method performance in dealing with the diversity of coliphage strains likely to be found in wastewater impacted environmental waters (Grose and Casjens, 2014).

Earlier studies have investigated the use of ultrafilters in recovery of singular bacteriophage strains spiked into surface freshwaters from volumes ranging between 10–50L (Kahler et al., 2015; Morales-Morales et al., 2003; Olszewski et al., 2005). Percent recoveries reported were $59 \pm 22\%$ (10L river) and $59 \pm 17\%$ (10L lake) for T1 phages (Morales-Morales et al., 2003), and for PP7 phages, $46 \pm 7\%$ (10L river) and $63 \pm 6\%$ (10L lake) (Olszewski et al., 2005). A similar study by Kahler et al., reported $58 \pm 16\%$ recovery of ΦX174 and $91 \pm 38\%$ of MS2 from 50L of river water and between 74 and 81% and 53–65% of ΦX174 and MS2 respectively from 50L of lake water samples (Kahler et al., 2015). The average recoveries we obtained from freshwater for 1L volumes were comparable to previously reported values. However, while the average recoveries for 10L samples were generally within previously reported ranges, they were somewhat lower than those obtained for 1L samples. This suggests that 1L sample volumes may be most suitable for recreational water quality testing applications in the dead-end ultrafiltration system tested.

Good performance of hollow-fiber ultrafiltration technology was reported concerning concentration of FIB from estuarine (Leskinen et al., 2010) and marine (Leskinen et al., 2009; Leskinen and Lim, 2008) waters, but analogous data for coliphages is lacking. To our knowledge, this study is the first to evaluate the performance of D-HFUF-SAL method for the detection and quantification of coliphages in marine waters. We found that the performance of D-HFUF-SAL method in marine waters was equivalent to or better than the freshwaters, suggesting that this method is sufficiently versatile to be employed in a wide variety of matrices.

The ultrafilter design allows processing of large volumes of turbid water, however, captured particulate matter can clog the filter fibers and interfere with filter elution processes potentially resulting in lower coliphage recoveries (Mull and Hill, 2012). In turbid waters, microbes as well as particulate matter can be trapped on filter surfaces potentially leading to loss of bacteriophages during filter elution steps. To date, the D-HFUF method has shown effectiveness in recovery of target microbes from environmental waters of low turbidity (Leskinen et al., 2009; Leskinen and Lim, 2008; Smith and Hill, 2009), however, the impact of suspended solids using the D-HFUF method needs further investigation for recreational water applications. In our turbidity experiments recoveries of both coliphage types were

negatively affected by higher turbidity levels (> 96 NTU). A similar study (Mull and Hill, 2012) included testing of three different turbidity levels (< 20, 50 and 100 NTU) and found significant effects of turbidity on recovery of F+ coliphages, but only between samples with the lowest and highest turbidity. Our study expands on these findings by assessing recoveries of both coliphage types at a broader range of artificially created turbidities. Both somatic and F+ coliphages were recovered at significantly lower levels in the most turbid samples (119 NTU) compared to samples with lower turbidity. The same pattern was observed in environmental waters with unamended ambient turbidity, in which less turbid waters yield higher recoveries, especially at larger sample volumes.

Due to the likelihood of containing human pathogens, wastewater entering recreational waterbodies can pose an elevated risk to human health (Jones et al., 2009; Sinclair et al., 2009). This scenario is especially true in cases of unregulated discharges, such as CSO events which are estimated to release upwards of 850 billion gallons of untreated wastewater and storm water in the U.S. per year (United States Environmental Protection Agency, 2004). In our simulated CSO release experiment, volumetric contribution of wastewater ranged from 0.01% to 1%. A recent study evaluating CSO composition estimated that the contribution of wastewater to rainwater runoff can range from 5% to 40% (Passerat et al., 2011), values far exceeding our experimental parameters. Accounting for seasonal dilution factors (CSO in the receiving waters) recently reported at ~340 in summer and ~6900 during snowmelt period (Madoux-Humery et al., 2013), emphasizes the applicability of D-HFUF-SAL as it reliably detected both coliphage types down to 10,000-fold dilution.

Earlier studies quantifying autochthonous coliphage levels in environmental waters often report a large percentage of samples with low to no detectable coliphage (Abdelzaher et al., 2011; Boehm et al., 2009b; Colford et al., 2007; McMinn et al., 2017; Medema et al., 1995; Viau et al., 2011; von Schirnding et al., 1992; Wade et al., 2010). This is routinely observed with F+ coliphages which are typically less abundant than the somatic group in wastewater (Contreras-Coll et al., 2002; Harwood et al., 2005; Lucena et al., 2003; McMinn et al., 2014; Zhang and Farahbakhsh, 2007), non-human fecal sources (Blanch et al., 2006; Hill and Sobsey, 1998; McMinn et al., 2014), as well as fresh (Contreras-Coll et al., 2002; Jiang et al., 2001; Lucena et al., 2003; Rezaeinejad et al., 2014; Viau et al., 2011) and marine waters (Boehm et al., 2009b; Contreras-Coll et al., 2002; Jiang et al., 2001; Rodriguez et al., 2012). Using the D-HFUF-SAL method, we were able to successfully quantify both somatic and F+ coliphages from unspiked lake and river water samples while observing a low level of non-detects (12.5% averaged for both coliphage groups). Enumeration of 100 mL or 1 mL samples typically recommended by standard methods (International Organization for Standardization, 1995; International Organization for Standardization, 2000; United States Environmental Protection Agency, 2001a; United States Environmental Protection Agency, 2001b) would have resulted in a considerably larger percentage of samples below LOD (37.5% and 62.5% averaged for both coliphage groups, respectively), suggesting that D-HFUF-SAL offers a substantial advantage over current standard procedures. Autochthonous coliphages were not detected in any of the marine water samples, which is not surprising considering that the area beaches have a long history of good water quality (defined as 0–35.4 enterococci per 100 mL) as reported by the Florida Department of Health (Florida Department of Health, 2017).

In this study, we paired two existing technologies (dead-end hollow fiber ultrafiltration and single agar layer) creating a novel, sensitive and robust method capable of greatly increasing the likelihood of detecting low levels of coliphages in environmental waters. Furthermore, we demonstrate consistent recovery of sewage-derived coliphages in fresh and marine waters and across a wide range of turbidity conditions. The ability to consistently measure small concentrations of coliphages in different types of surface waters provides the foundation for the potential inclusion of a viral indicator in future recreational water quality management applications. With a reliable procedure in place, future research efforts need to focus on field scale method performance evaluation and characterizing the potential relationship between the presence of coliphages and public health risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2017.08.006.
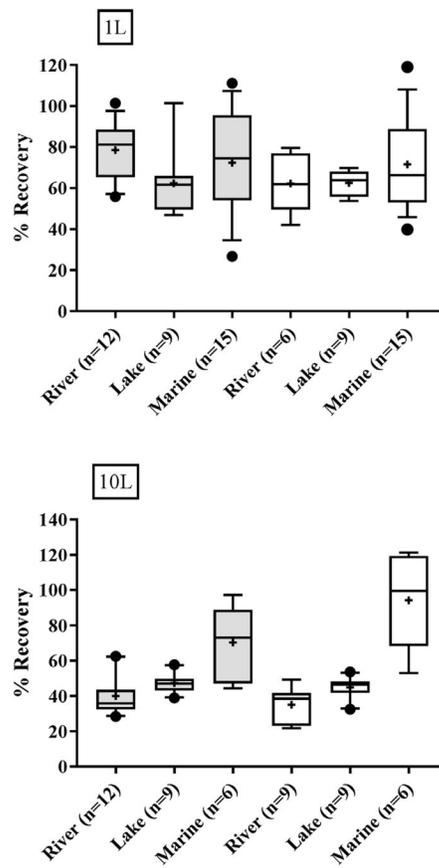
Fig. 1.
Percent recovery of somatic (grey bars) and F+ (white bars) coliphages from concentrated 1L and 10L samples of lake, river and marine water. Box is delimited by 25th and 75th percentiles, solid line within the box represents median and a plus sign represents mean. Whiskers are 10th and 90th percentile values. Values outside of the range are depicted as black dots.
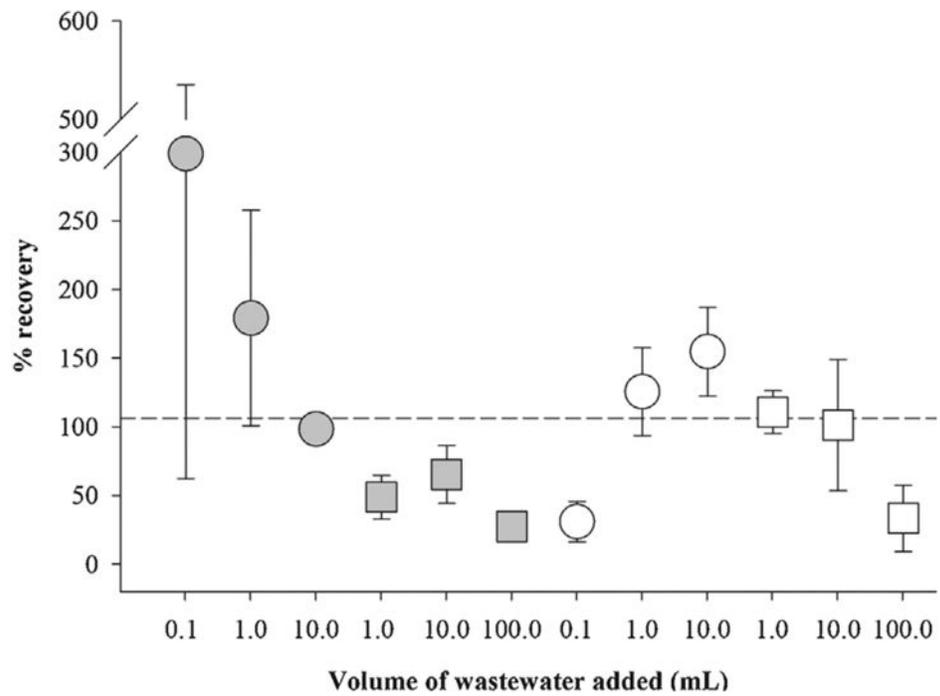
Fig. 2.
Percent recovery of somatic (grey symbols) and F+ (white symbols) coliphages in a simulated CSO event. Circles and squares represent 1L and 10L volumes of river water, respectively. Error bars represent standard deviation (n = 3). The dashed line represents average (106%) of all percent recoveries across both coliphage types and different volumes of river water/wastewater added.
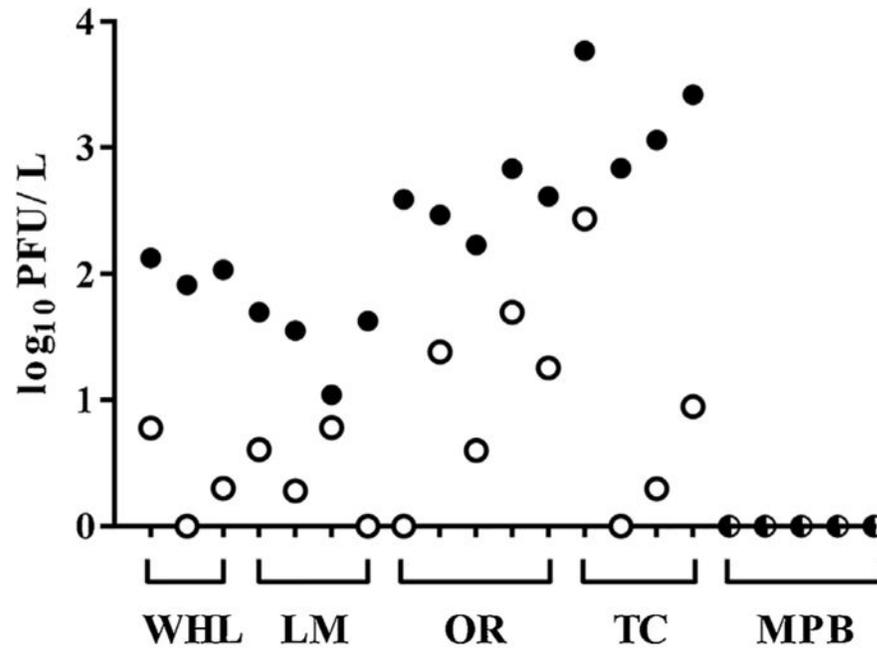
Fig. 3.
Field evaluation of method performance for quantifying autochthonous somatic (filled circles) and F+ (empty circles) coliphages from various recreational water sources. Samples were collected from: William H. Harsha Lake, OH (WHL, n = 3), Lake Michigan, IN (LM, n = 4), Ohio River, OH (OR, n = 5), Trail Creek, IN (TC, n = 4), Morgan Park Beach, FL (MPB, n = 5).

Table 1

Performance metrics of the D-HFUF-SAL method in tap water spiked with varying dilutions of primary treated wastewater.

Wastewater dilution	Concentration ± SD[a]	Coefficient of variation	Replicates with detectable plaques[b]
	Somatic coliphages		
10^{-2}	4.30 ± 0.08	0.02	9/9
10^{-3}	3.35 ± 0.04	0.01	9/9
10^{-4}	2.53 ± 0.05	0.02	9/9
10^{-5}	1.42 ± 0.10	0.07	8/8
10^{-6}	0.64 ± 0.20	0.31	9/9
	F+ coliphages		
10^{-2}	3.42 ± 0.10	0.03	9/9
10^{-3}	2.28 ± 0.11	0.05	9/9
10^{-4}	1.01 ± 0.34	0.34	9/9
10^{-5}	0.13 ± 0.28	2.08	4/8
10^{-6}	0.05 ± 0.16	3.00	1/9

[a]Data are average (± standard deviation) of $\log_{10}$ transformed concentrations recovered from (n = 9) replicate samples for each dilution tested, except for 10^{-5} dilution (n = 8) where one filter was discarded due to faulty elution technique.

[b]Number of replicates with at least one plaque.

Table 2

The effect of varying levels of turbidity on concentrations and percent recovery of somatic and F+ coliphages from 1L of environmental freshwater.

Turbidity (NTU)	Amount of dried sediment added (grams)	Percent recovery ± standard deviation[a]		Log$_{10}$ PFU per L ± SDa	
		Somatic	F+	Somatic	F+
3.74	0.0	76.9 ± 8.7	80.1 ± 15.4	2.32 ± 0.05	2.05 ± 0.09
38.4	0.4	57.4 ± 6.6	47.4 ± 12.1	2.19 ± 0.05	1.82 ± 0.11
68.7	0.8	39.5 ± 10.1	36.4 ± 16.9	2.02 ± 0.12	1.68 ± 0.23
96.2	1.2	30.0 ± 7.8	26.8 ± 7.4	1.90 ± 0.12	1.57 ± 0.13
118.7	1.6	24.8 ± 15.2	21.4 ± 20.5	1.76 ± 0.32	1.32 ± 0.48

[a]Data are average (± standard deviation) concentrations recovered from (n = 3) replicate samples for each turbidity level tested.