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The utility of flow cytometry for potable reuse

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Protecting public health from pathogens is critical when treating wastewater to drinking water standards (i.e., planned water reuse). Viruses are a principal concern, yet real-time monitoring strategies do not currently measure virus removal through reuse processes. Flow cytometry (FCM) has enabled rapid and sensitive bacteria monitoring in water treatment applications, but methods for virus and protozoa monitoring remain immature. We discuss recent advances in the FCM field and FCM applications for quantifying microorganisms in water. We focus on flow virometry (FVM) developments, as virus enumeration methods show promise for water reuse applications. Ultimately, we propose FVM for near real-time monitoring across treatment to more accurately validate virus particle removal and for pilot studies to characterize removal through understudied unit processes.

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Introduction

Wastewater is increasingly used as an alternative water source to meet potable needs [1–3], giving rise to new challenges in assuring public health. Pathogenic microorganisms are of principal concern in wastewater reuse due to the acute health risks they pose to consumers. Virus removal, in particular, is a major driver in the regulation and design of planned potable water reuse because they are present in high concentrations in wastewater [1,4–6], and their small size (20 nm to over 200 nm)

makes them difficult to remove [7]. Depending on the intended application and project location, reuse regulations and guidelines for virus removal range from 8- to 13-logs or more from raw or treated wastewater to finished water [4,5,8].

Ideally, pathogens would be monitored directly in finished drinking water to demonstrate the water is safe; however, this is infeasible due to the extremely low pathogen concentrations in safe finished water (e.g., 10^{-7} enteric viruses/l [1]). Instead, individual unit processes in the treatment train are allotted log removal credits for groups of pathogens, and the credits are summed across the treatment train. To maintain removal credits, the proper functioning of a unit process is ensured in real-time or near real-time by monitoring an easy-to-measure surrogate parameter, such as turbidity or electrical conductivity. These surrogate parameters often underestimate actual microorganism removal. Virus removal credits, in particular, are very conservative. Consequently, potable water reuse treatment trains may be over engineered for pathogen removal because utilities cannot demonstrate the actual log reductions for common unit processes (e.g., biofiltration, ultrafiltration, reverse osmosis).

The water treatment field in general, and the wastewater reuse field in particular, would greatly benefit from technologies that accurately depict microorganism concentrations in real-time or near real-time and demonstrate their reductions through specific unit processes. We believe flow cytometry (FCM), a high-throughput technique that uses light scattering and fluorescence for particle detection [9], can fill some of these needs for microbe monitoring and will be increasingly applied for wastewater reuse monitoring. The main advantage of FCM over currently used surrogate parameters is that it directly detects microorganisms. The main advantages of FCM for reuse applications over other microbial detection techniques are that it is high-throughput, reproducible, and can concurrently enumerate different microorganism groups based on size and fluorescence properties. In this perspective, we review recent applications and advances in FCM for environmental monitoring. We discuss the three main pathogen groups but focus on virus detection using FCM, coined flow virometry (FVM), as we see this as an area ripe for advancement in coming years. In accordance with the demonstrated capabilities of FCM and FVM, we propose three-specific applications in potable water reuse.

Recent applications and advances in the use of FCM for bacteria and protozoa monitoring

Bacteria enumeration via FCM is far more advanced than protozoa or virus monitoring in terms of experience, automation, and proof-of-concept research [10,11]. Bacteria in drinking and surface water matrices can effectively be monitored in real-time [12,13,14**] using flow cytometers with automated modules that routinely sample, stain, and enumerate bacteria with 15-min resolution [15]. Online bacteria monitoring via FCM in full-scale water treatment systems offers improved resolution, reproducibility, and statistical power over traditional bacteria monitoring methods such as heterotrophic plate counts [11]. Bacteria staining techniques aimed at assessing viability are now commonly applied to distinguish intact from membrane compromised bacteria [10]. Total and viable bacteria levels have been enumerated via FCM in various water types (Table 1). Total bacteria reductions of about two-logs have been reported across conventional wastewater treatment [16,17], whereas a micro-filtration unit process in a water reclamation facility can remove over five-logs [18*].

Unlike bacteria monitoring with FCM, measuring total protozoa populations has not been a focused area of research. This may be due to the presence of algae or other detrital material of similar size or fluorescent intensity [19,20]. Instead, protozoa FCM research has centered on quantifying the pathogens *Cryptosporidium* spp. and *Giardia* spp. in water because of their health and regulatory relevance. Depending on the sample matrix, significant concentration steps are required before FCM analysis to detect them [21,22]. Future work to address these limitations would help make protozoa monitoring using FCM more realistic as a real-time reuse monitoring strategy.

Recent applications and advances in the use of FVM for virus detection

Advances in FVM

Improvements in sample preparation and FVM instrumentation are enabling quantification of total virus populations as well as specific viral strains. Most flow cytometers are unable to differentiate biological particles below approximately 300 nm from the background signal (i.e., noise) of the instrument-based solely on light scattering properties [23,24]. As a result, virus particles are commonly tagged with fluorescent dyes via antibodies, fluorescent proteins, or nucleic acid stains to facilitate detection. Even when stained or tagged, however, virus particle signals are at or near the background signals of some flow cytometers. The background signal arises from the optical, fluidic, and electronic components of the flow cytometer. Increased laser wattage, use of photo-multiplier tubes (PMTs) or digital focusing systems (DFSs) in place of photodiode detectors, filtration of sheath fluid used during sample analysis, decreased internal chamber size, and continual instrument cleaning are all strategies to help reduce background signals for improved nanoparticle detection [25**,26]. The difficulty in distinguishing a single virus particle from multiple virus particles in one FVM event [27] can be addressed via sample dilution [28], slower flow rates (<1000 events per second) [29], or smaller internal chamber size [25**]. Building on these advances, the field of medical virology has conclusively demonstrated the utility of FVM to detect-specific virus particles, including HIV-1 [30,31], T4 and lambda phage [32], HSV-1 [33*], Junin virus [34**], and filoviruses [35]. These studies have used specialized flow cytometers with stringent controls to ensure accuracy in distinguishing viral populations.

Application of FVM to environmental samples

Applications of FVM in medical virology tend to concentrate on the detection and characterization of targeted virus-species. Antibody-based fluorescent tagging, therefore, provides advantages in these applications due to its

Table 1

Microorganism concentrations measured by FCM/FVM in water samples that are relevant for potable reuse

Sample type	Bacteria		Viruses (counts/ml)
	Total (counts/ml)	Viable (counts/ml)	
Surface water	10^6 [12,50]	10^6 [51]	No data
Groundwater	1×10^3 to 5×10^6 [12,14**,52,53]	10^5 [54]	No data
Raw wastewater	1.74 to 2×10^8 [16,17]	8.4×10^7 [17]	3.72×10^8 [16]
Primary treated wastewater	10^8 [16,17,18*]	8.4×10^7 [17]	10^8 [16,18*]
Activated sludge	2.24 to 3.3×10^9 [16,17]	1.24 to 2.3×10^9 [16,17,55]	10^8 to 7.33×10^9 [16,41]
Secondary treated wastewater	2.2×10^6 to 3.87×10^8 [16,17,18*]	1.7×10^6 to 10^8 [16,17]	10^8 [16,18*]
Disinfected wastewater effluent	10^6 [16,18*]	10^6 [16]	10^8 [16,18*]
Microfiltration effluent	$\leq 10^2$ [18*]	No data	7.3×10^8 [42]
Reverse osmosis effluent	$\leq 10^2$ [18*]	No data	$\leq 10^4$ [18*]
Finished drinking water	10^5 [56]	10^5 [51]	No data

specificity. Environmental FVM studies, in contrast, have typically focused on the enumeration of total virus particles. Here, nucleic acid staining is more applicable than antibody tagging because it theoretically targets all viruses in the sample. In reality, FVM fluorescence signals observed following nucleic acid staining are not consistent among viruses with variable genome types, genome sizes, and capsid structures.

In terms of environmental measurements, FVM has been used most extensively in marine biology for the enumeration of native marine virus populations stained with nucleic acid dyes [28,36–38]. FVM research in the marine biology setting has almost exclusively relied on dyes from the SYBR family. These are newer dyes with lower intrinsic fluorescence and improved nucleic acid signals compared to older dyes (e.g., DAPI; Table 2). SYTO, TOTO, and YOYO dyes, also newer dyes commonly employed by the medical virology field, are avoided in marine biology because they lose their binding affinity in samples with high ionic strength [39,40]. These dyes have yet to be explored with viruses in freshwater samples. Before analysis, marine virus samples are often pretreated with fixation, heat, and flash-freezing to improve virus particle fluorescence signals.

FVM for water quality monitoring

Water quality and water treatment researchers have drawn from procedures used in marine biology to enumerate total

virus populations in wastewater and reclaimed water samples [16,18*,41*,42]. Overall, various combinations of SYBR Gold, SYBR Green I, and SYBR Green II have been employed, and pretreatments include sample flash-freezing, heating and incubation, and fixation [16,41*,42]. In complex samples such as wastewater, an additional virus disaggregation step, such as Tween 80 and sodium pyrophosphate pretreatment can improve virus enumeration [41*]. An ultrasonication pretreatment step did not improve enumeration in activated sludge samples [16,41*] but did improve virus particle counts in settled wastewater samples [16].

FVM has been used to measure virus concentrations and removal rates for a range of treatment technologies in wastewater and water reuse systems (Table 1). No significant reduction in virus concentrations were observed through traditional wastewater processes via FVM [16,18*]. Of note, total detectable virus concentrations were reduced by over four-logs through the microfiltration process of a reclamation facility in a recent study employing FVM [18*]. Reductions through the subsequent reverse osmosis unit processes were not measurable because the detection limit of the method had been reached [18*]. In the same study, over four-logs of total detectable virus particles were removed through a membrane bioreactor process. At this point, infective and non-infective viral fractions have not been differentiated with FVM.

Table 2

Properties of nucleic acid stains and reference FCM studies that have used the specified stains for different analyses

Fluorescence dye	Quantum yield	Fluorescence absorption/ emission maxima (nm)	Examples of use in FCM
Traditional dyes			
Ethidium bromide (EB)	0.14 (DNA) [57]	518/605 [58]	Bacteria and enumeration [59]
4',6-Diamidino-2-phenylindole (DAPI)	0.34 (DNA) [58]	358/461 [58]	Bacteria enumeration [60]
Hoechst family			
Hoechst 33342	0.38 (DNA) [58]	350/461 [58]	Bacteria enumeration [60]
Enhanced dyes			
PicoGreen	0.53 (dsDNA), 0.42 (RNA) [61]	500/523 [61]	Virus enumeration [39]
SYBR family			
SYBR Gold	0.7 (DNA, RNA) [62]	495/537 [62]	Virus enumeration [37] Virus/bacteria enumeration [18*] Virus enumeration [28,63]
SYBR Green I (SGI)	0.8 (DNA), 0.4 (RNA) [40]	494/521 [40]	Bacteria enumeration [17,55,64*] Virus/bacteria enumeration [16] Virus enumeration [41]
SYBR Green II (SGII)	0.36 (DNA), 0.54 (RNA) [40]	494/521 [40]	Virus enumeration [41]
SYTO family			
SYTO 9	0.6 (DNA), 0.2 (RNA) [40]	480/500 [40]	Bacteria sorting [65] Bacteria enumeration [66]
SYTO 13	0.4 (DNA), 0.4 (RNA) [40]	488–491/509–514 [40]	Bacteria sorting [65,67] Virus sorting [33,68]
TOTO family			
TOTO-1	0.34 (DNA) [58]	514/533 [58]	Bacteria diversity [69]
YOYO family			
YOYO-1	0.52 (DNA) [58]	491/509 [58]	Virus sorting [34] Virus enumeration [39]

Methodological challenges in FVM for water quality monitoring

A number of challenges must be addressed before the utilization of FVM for water quality monitoring can be fully realized. One primary challenge is in confirming that all or most virus particles are actually being measured by FVM (i.e., avoiding false negatives). This is particularly difficult when enumerating virus particles with small genomes or single stranded genomes (e.g., ssRNA or ssDNA), which tend to emit smaller fluorescence signals. Studies often use transmission electron microscopy and/or epifluorescence microscopy to confirm total virus particle counts obtained by FVM [16,41,42]. Spike additions of pure virus stocks into sample matrices are also critical to verify that the FVM method can effectively quantify the virus populations of interest. For example, Brown *et al.* [41] measured total virus particle counts in samples with and without spike additions of T4 coliphage to assess recovery in activated sludge samples. Realizing the diversity of potential virus targets, we propose future studies spike virus cocktails, consisting of several different viruses, into samples to more accurately characterize the impacts of genome and structure type and size on recoveries.

Another significant challenge is minimizing false positives. These can be caused by cytometer background noise [28], particles that autofluoresce (e.g., colloids) [43], and biological particles that fluoresce when stained (e.g., microvesicles, gene transfer agents, or extracellular DNA) [44]. To address background noise of the machine, filtered and autoclaved samples are typically run through the cytometer and subtracted from stained samples [41,42]. Measuring the same sample before and after staining can help identify particles that are not virus particles. For biological samples, DNase treatments have been used with limited success to reduce the likelihood of detecting free DNA [41]. Chloroform treatment of samples before the addition of DNase could also prove beneficial by releasing membrane-associated nucleic acids from biological particles that may otherwise result in false positives (e.g., microvesicles, gene-transfer agents).

Our vision of FCM in wastewater reuse applications

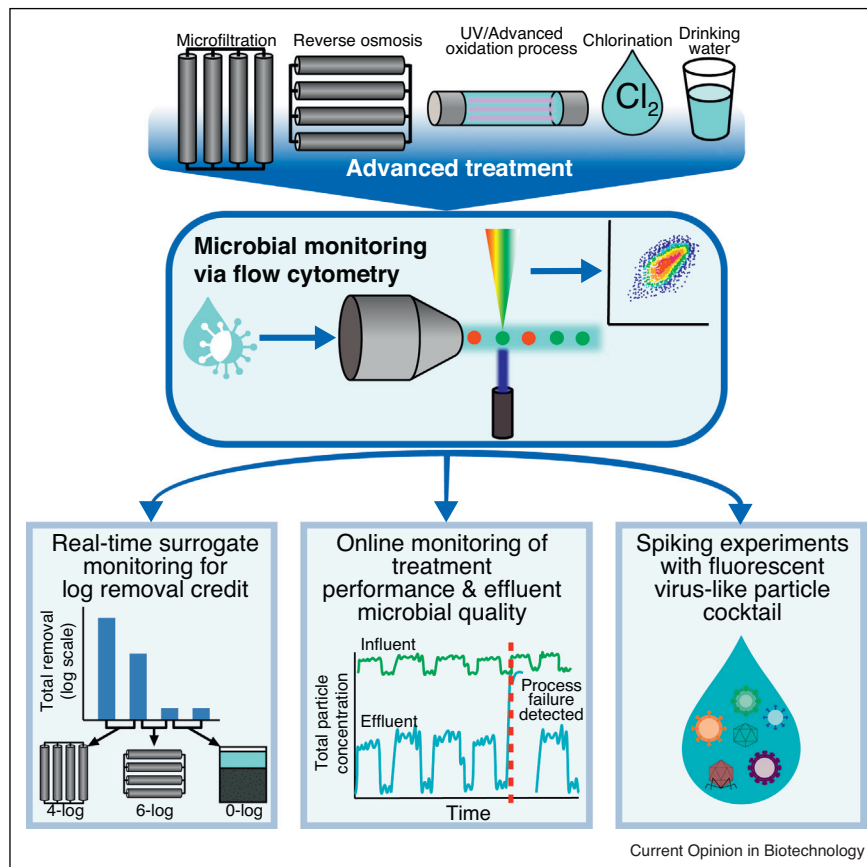
Based on previous work in FCM for water monitoring, we envision at least three major applications of flow cytometry in the water reuse setting (Figure 1). First, we believe FCM will become an important near-real time surrogate measurement for validating log reduction values through physical treatment processes (e.g., filtration, sedimentation). Specifically, reductions of groups of particles with certain fluorescence properties could be used to represent the removal of microorganisms with the same FCM properties. For example, if flow cytometer measurements show a 99% reduction in

detectable virus-like particles across a unit process, then two-log virus reduction will be granted for enteric viruses. Before this is feasible, research will need to establish whether reductions measured with FCM correlate with actual virus removal. As an example of our proposed approach, the four-log total virus removal measured by Huang *et al.* [18] through microfiltration with FVM is similar to virus removal that has been achieved through microfiltration [45], although microfiltration virus removal has been highly variable (i.e., 0 to >5-log removal [46]). Where this approach can be applied in the reuse scheme will depend on detection methods for the particular cytometer and native virus particle concentrations. A wide range of FVM detection limits have been reported in various matrices, from 80 to 10⁴ particles/ml [16,18,31]. Beyond viruses, we imagine similar approaches could be made for bacteria and protozoa reductions across unit processes.

We also envision using FCM to continuously monitor particles of a certain size or fluorescence to help inform operators of changes in treatment plant influent or effluent quality. For example, potable reuse effluent could be continuously measured with FCM and trends in particle size distributions, fluorescence characteristics, or particle concentrations could be correlated with overall system performance. Aberrations in the FCM data would thus serve as an immediate warning for failures in the treatment train. This is similar to using turbidity measurements to detect changes in water quality, but FCM would provide more relevant and extensive information related to microbial water quality. Future research at actual plants should study how variations in FCM ‘fingerprints’ correlate with other indices used to assess influent water quality or overall treatment train performance.

Finally, we see FCM as a powerful tool for improved virus removal studies at the bench-scale and pilot-scale level. Currently, bench-scale and pilot-scale assessments of unit processes involve spiking in one or two surrogate viruses and measuring removal with culture-based methods. These studies are not only time consuming, but the selected surrogate viruses do not represent the behavior of all viruses of interest in water [47]. An alternative approach measures the reduction in spiked fluorescent latex bead concentrations [46], but these particles have little in common with virus particles. Instead, we propose using cocktails of bacteriophages of various sizes and genome types with stained nucleic acids that are readily detected by FVM for bench-scale and pilot-scale assessment. Alternatively, lab-synthesized virus-like particles identical to a variety of human pathogens in structure but containing nontoxic fluorescent tags instead of nucleic acids can be used as a cocktail for spiking experiments [48]. In either manner, the virus cocktails could be utilized to directly and rapidly measure virus log removals in pilot scale systems with FVM. An exciting application

Figure 1



Three potential applications of FCM in an example advanced water treatment scheme. In the first application, log removal credits are maintained through unit processes by real-time particle monitoring. In the second application, online influent and effluent FCM monitoring detects aberrations in water quality and system performance. In the third application, cocktails of noninfective fluorescent viruses are added to water samples before unit processes to characterize virus removal.

of the virus-like particle spike cocktail is in assessing pathogenic virus reductions through biological treatment processes, which often also involve physical particle removal. Biological treatment likely propagates bacteriophage and thus increases total virus concentrations in treated water while pathogenic virus concentrations are concurrently decreasing. Therefore, measuring background total virus concentrations before and after biological processes by FVM would not provide an accurate assessment of pathogenic virus removal. Adding fluorescent virus particles that do not replicate could alleviate these issues and enable the accurate measurement of physical virus removal in unit processes where virus propagation occurs.

Conclusions and future implications

We envision that FCM will revolutionize how microbial monitoring is conducted through potable reuse, especially for virus detection. To bring this vision toward reality, research should compare instrument performance with

different virus particle sizes of variable genome types (i.e., ssDNA, dsDNA, ssRNA, dsRNA) and assess fluorescence stains already employed in other applications. This research should be conducted in real waters with a range of characteristics, spanning from untreated municipal wastewater to finished reclaimed drinking water. Techniques should be developed that differentiate infective and noninfective virus particles with FVM, particularly as virus particles are inactivated through disinfection unit processes. A potential method for distinguishing infective virus particles could include the use of enzymatic pretreatment [49] to eliminate fluorescence from virus particles with degraded capsids. Research is also necessary to establish relationships between total particle concentrations measured with FCM and pathogenic microorganism concentrations. Finally, FVM monitoring should be studied through bench-scale unit processes, followed by testing at the pilot-scale, and should ultimately be applied in full-scale systems with automated monitoring.

Conflict of interest statement

Nothing declared.

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