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Simple SARS-CoV-2 concentration methods for wastewater surveillance in low resource settings

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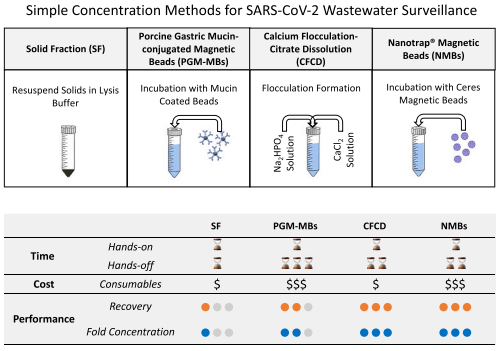
HIGHLIGHTS

- Application of WBE in low-resource settings is limited due to the complex and expensive equipment and processing techniques.
- Four simple and inexpensive virus concentration methods were compared to recover SARS-CoV-2 from wastewater.
- Calcium Flocculation-Citrate Dissolution and Nanotrap® Magnetic Beads showed the highest recovery efficiency of SARS-CoV-2.
- Nanotrap® Beads offer electricity-free use, but Calcium Flocculation-Citrate Dissolution had the lowest time and cost burden.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater-based epidemiology (WBE) measures pathogens in wastewater to monitor infectious disease prevalence in communities. Due to the high dilution of pathogens in sewage, a concentration method is often required to achieve reliable biomarker signals. However, most of the current concentration methods rely on expensive equipment and labor-intensive processes, which limits the application of WBE in low-resource settings. Here, we compared the performance of four inexpensive and simple concentration methods to detect SARS-CoV-2 in wastewater samples: Solid Fraction, Porcine Gastric Mucin-conjugated Magnetic Beads, Calcium Flocculation-Citrate Dissolution (CFCD), and Nanotrap® Magnetic Beads (NMBs). The NMBs and CFCD methods yielded the highest concentration performance for SARS-CoV-2 (~16-fold concentration and ~ 41 % recovery) and require <45 min processing time. CFCD has a relatively low consumable cost (<$2 per four sample replicates). All methods can be performed with basic laboratory equipment and minimal electricity usage which enables further application of WBE in remote areas and low resource settings.

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1. Introduction

In devastating disease outbreaks like the COVID-19 pandemic, reliable data on infection trends is critical to public health authorities to make informed and timely decisions. Frequent clinical testing of large populations is a logistical challenge and requires access to massive healthcare infrastructure and testing resources. The problem is further exacerbated in areas with socio-economic disparities and limited access to the healthcare system (Ondoa et al., 2020). A potential solution is to utilize wastewater-based epidemiology (WBE), which measures pathogens in wastewater to monitor the disease trends at community levels (Sims and Kasprzyk-Hordern, 2020). In the case of COVID-19, symptomatic and asymptomatic patients shed the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in their stool (Schmitz et al., 2021), leading to viral particles in the community sewage. Routine sampling from a sewage system and testing for SARS-CoV-2 can provide a rapid and inexpensive alternative to determining COVID-19 infection rates in the community (Ai et al., 2021). Therefore, since the advent of the COVID-19 pandemic, there has been a growing interest in applying SARS-CoV-2 WBE in various communities such as college dormitories (Scott et al., 2021), nursing homes (Davó et al., 2021; Keck et al., 2023), and urban neighborhoods (Graham et al., 2021). The application of SARS-CoV-2 WBE is particularly impactful in rural and remote areas (Medina et al., 2022; Street et al., 2020) with limited resources, as WBE can serve as a bridge towards more equitable health. However, the widespread adoption of WBE in low resource settings is hindered by technical and logistical barriers such as complex sample processing, expensive equipment (Michael-Kordatou et al., 2020), and cold chain sample transportation (Parra-Arroyo et al., 2023).

One of the major technical challenges in WBE is the dilution of wastewater due to rainfall, agricultural run-off, commercial or industrial wastewater, and other sources of residential wastewater such as bathing and cooking (D. Lu et al., 2020). This results in extremely low and even undetectable concentrations of the pathogen of interest in wastewater samples. Therefore, a concentration step is recommended to achieve reliable viral load signals. The most common concentration methods include polyethylene glycol (PEG) (Farkas et al., 2021) and aluminum hydroxide precipitation (Barril et al., 2021), ultrafiltration (Kaya et al., 2022), electronegative filtration (Ahmed et al., 2023b), and ultracentrifugation (Ahmed et al., 2020). These methods are widely used in many advanced laboratories, with a wide range of viral recovery rates (Zheng et al., 2022). However, these concentration methods often require long processing times, expensive equipment such as ultracentrifugation systems and vacuum pumps, and specialty consumables such as filters, spin columns, and ultracentrifugation bottles. These technical requirements have resulted in advanced laboratories undertaking the bulk of WBE, of which few exist in low resource and rural settings. These settings encompass much of the world, including known emerging pathogen hot spots (e.g., Sub-Saharan Africa). Therefore, there is a need for viral concentration methods that are adaptable for low resource settings and ideally field applications, supporting a decentralized approach to environmental surveillance.

To enable viral concentration in more basic laboratory settings, the concentration method must be *simple* to carry out. There are four main attributes we considered in defining *simple* concentration methods. First, the method should need only simple and affordable equipment and not necessitate the use of large and costly equipment like ultracentrifuges. Second, consumables should be cheap and locally available or easily shipped (e.g., no cold chain or no biohazard concerns), which discourages the use of expensive items such as ultrafilters. Third, the method should be time efficient. And fourth, the method should be resilient to electricity limitations/interruptions which can be a challenge in low resource settings. We identified four *simple* methods to concentrate SARS-CoV-2 from wastewater samples: enrichment with Nanotrap® Magnetic Beads (NMBs), concentration using Porcine Gastric Mucin-conjugated Magnetic Beads (PGM-MBs), Calcium flocculation Citrate dissolution (CFCD), and Solid Fraction (SF) separation (Fig. 1). While the main focus of our discussion is on SARS-CoV-2, we have measured CrAssphage, Influenza A Virus (IAV), Respiratory Syncytial Virus A (RSV A), and Clostridioides Difficile (C. Diff), to study the differential performance of varying targets across methods. The findings from this study can help stakeholders to choose a concentration method for the implementation of WBE in their region.

2. Methods and materials

2.1. Wastewater sample collection

Wastewater was obtained in November and December of 2022 from nine wastewater treatment plants (WWTPs) in eastern Kentucky, USA. These WWTPs serve populations ranging from 2500 to 22,000 individuals (Table S1). An automated sampler drew composite wastewater samples from the inlet stream over a 24-h period. From the autosampler at each WWTP, 250 mL of raw wastewater was collected from the sampler reservoir, immediately placed on ice, and transferred to the laboratory for storage at 4 °C until processing. While most of the samples were processed within 24 h, a few were processed within 72 h. To ensure consistency, all concentration methods were performed simultaneously to account for the potential degradation of SARS-CoV-2 in wastewater (Babler et al., 2023; Torabi et al., 2023).

2.2. Wastewater characterization

We measured the pH, total suspended solids (TSS), turbidity, and spectral absorption at 500 and 800 nm of all samples to investigate the impact of wastewater characteristics on virus recovery using different concentration methods. A wireless pH meter (Hanna Instruments, HI10532) was used to measure the pH. The instrument was calibrated using provided reagents each time before use. We used a filtration method to determine the total suspended solids (TSS). First, mixed cellulose esters membranes with a pore size of 0.65 μm and a diameter of 47 mm (MilliporeSigma, DAWP04700) were weighed prior to sample filtration. Subsequently, 25 mL of untreated wastewater was filtered through each membrane using a disposable vacuum filtration unit (MilliporeSigma, MCFLX4710) attached to a vacuum storage bottle. The filters were then carefully placed in an oven and dried at 60 °C for 16 h. Next, we measured the change in filter weight to calculate the total suspended solids in mg/L of wastewater. Our experiments show that the change in the filter's own weight after drying out is negligible (<1 % of the weight change). 25 mL of distilled water was processed using the same method as a negative control. We used an Orion™ AQUAfast AQ3010 Turbidity Meter (ThermoFisher Scientific, AQ3010) to measure the turbidity according to the manufacturer's instructions. Turbidity is assessed through the dispersion of light caused by particles present in the water and is expressed using Nephelometric Turbidity Units (NTU). Moreover, the cuvette chamber on the SpectraMax® M2 Multimode microplate reader (VWR, 89429–532) was used for spectral absorption measurement. Specifically, 3 mL of thoroughly mixed wastewater was transferred to each cuvette and directly placed inside the cuvette chamber of the reader, with spectral absorption measured at 500 and 800 nm. All measurements of wastewater characteristics were conducted in triplicate.

2.3. Wastewater sample concentration

In this study, we selected four concentration methods, including enrichment with Nanotrap® magnetic beads (NMBs), concentration using porcine gastric mucin-conjugated magnetic beads (PGM-MBs), Calcium Flocculation-Citrate Dissolution (CFCD), Solid Fraction (SF) separation, and a control with no concentration. These methods have short hands-on processing times (<15 min), use inexpensive reagents, do not require ultra-low temperature storage, and can be conducted with

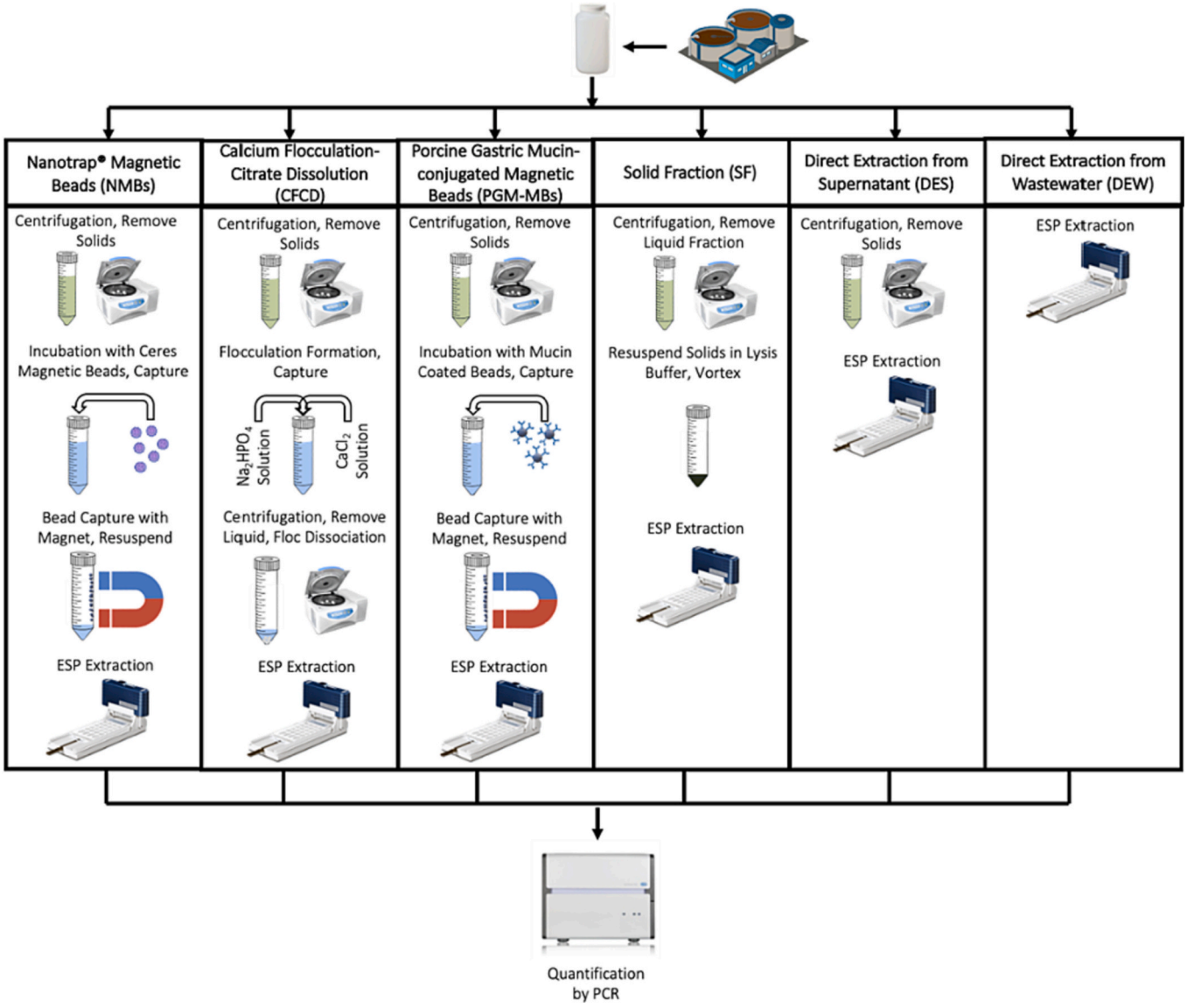

Fig. 1. Schematic of the concentration methods. Split wastewater samples from WWTPs were processed using different concentration methods followed by ESP extraction and the extracted viral RNA was quantified by RT-qPCR.

minimal lab equipment. It should be noted that a low-cost or manual centrifuge can perform the centrifuge step in all the methods. Ultimately, two of the methods were tested in an electricity-free viral concentration protocol, which could be used near the sample collection point in the field. All the methods were performed in parallel on 20 different wastewater samples with varying characteristics. Following concentration, RNA was extracted using a method (termed Exclusion-based Sample Preparation (ESP)) that has previously been shown to be both simple and effective (Strike et al., 2022). We carried out all concentration and extraction procedures concurrently for each wastewater sample and in four replicates. Fig. 1 shows a schematic of all the methods.

2.3.1. *Nanotrap® Magnetic Beads (NMBs)*

The Ceres Nanotrap® Magnetic Beads are effective in capturing a wide range of viruses in various sample matrices, such as blood (Ii et al., 2021), nasal swabs (Shafagati et al., 2016), and wastewater (Karthikeyan et al., 2021). Different manual and automated protocols have been reported for these beads (CERES Protocols, n.d.). In this work, the manual protocol for the Nanotrap® Magnetic Beads was slightly modified to make it compatible with our nucleic acid extraction method. For this protocol, 40 mL of wastewater was aliquoted into a 50 mL conical tube (ThermoFisher, 339,652) and then centrifuged for 2 min at 1500 xg using a swinging bucket centrifuge (Southwest Science, SCL636) to remove solid particles. The pellet was discarded, and the concentration was performed on the supernatant of the wastewater. In the next step, 400 μL of Nanotrap® Enhancement Reagent 2 (ER2) (Ceres Nanosciences, SKU #10112–10) was added to the sample and mixed by manually inverting the tube multiple times. Then, 600 μL of Nanotrap® Microbiome A Particles (Ceres Nanosciences, SKU #44202) were added to the sample and mixed at room temperature for 10 min using a tube rotator (ThermoFisher, 88–861-051). The tube was then placed on a magnetic ring (K&J Magnetics, RY0X04) for 10 min to collect the magnetic beads. The supernatant was carefully discarded, and the bead pellet was resuspended in 2.5 mL of lysis buffer containing 4 M Guanidine Thiocyanate (GTC) (ThermoFisher, AM9422) and 10 mM 4-morpholinethanesulfonic acid (MES) sodium salt (Sigma Aldrich, M3671) dissolved in 1:1 *v*/v absolute ethanol and water. The resultant sample containing Nanotrap® Magnetic Beads was aliquoted into four 1.5 mL centrifuge tubes, yielding 625 μL of sample for nucleic acid extraction.

2.3.2. Calcium flocculation-citrate dissolution (CFCD)

The CFCD is a flocculation protocol based on the formation of Calcium Hydrogen Phosphate (CaHPO4), which is insoluble in water. Liu et al. (Liu et al., 2007) used this method to concentrate noroviruses in drinking water. The flocculants are formed by addition of two inorganic salts solutions, calcium chloride and disodium phosphate, into the sample. The calcium chloride solution was prepared by addition of 147 mg of calcium chloride (CaCl2) (Sigma Aldrich, C4901) into 1 mL of nuclease-free water (Growcells, NUPW100012), resulting in a 1 M CaCl2 solution. To prepare the 1 M disodium phosphate solution, 142 mg of disodium phosphate (Na2HPO4) (Sigma Aldrich, S0876) was dissolved in 1 mL of nuclease-free water. A citrate buffer is used to dissolve flocculants and release viruses. We prepared the 0.3 M citrate buffer by introducing 770 mg sodium citrate dihydrate (Sigma Aldrich, 567,446) and 75 mg of citric acid monohydrate (Sigma Aldrich, C7129) into 10 mL of nuclease-free water. All the prepared solutions were shelf stable and stored at room temperature.

For the CFCD protocol, wastewater solids were removed after centrifugation at 1500 x*g* for 2 min. Then 100 μL of 1 M CaCl2 was added to 40 mL of the wastewater supernatant and briefly vortexed, followed by the addition of 100 μL of 1 M Na2HPO4. The sample was then tumbled for 10 min on a tube rotator (ThermoFisher, 88–861-051) to allow for formation of flocculants. In the next step, to pellet the calcium flocculants, the sample was centrifuged for 10 min at 1500 x*g* using a swinging bucket centrifuge (Southwest Science, SCL636). The supernatant was removed and discarded without disturbing the calcium flocculants. Then, we added 1 mL of citrate buffer to the pellet and pipetted up and down to ensure citrate dissolution and breaking of the flocculants. Next, 1.5 mL of lysis buffer was added to release the nucleic acids. Finally, the solution was divided into four 625 μL aliquots and used for nucleic acid extraction.

2.3.3. Porcine gastric mucin-conjugated magnetic beads (PGM-MBs)

We prepared the porcine gastric mucin-conjugated magnetic beads as described by Oh et al. (Oh et al., 2022), who used the PGM-MBs method to concentrate human and animal viruses from wastewater. To prepare the PGM-MBs, 1 mL of MagnaBind carboxyl-derivatized beads (ThermoFisher, 21,353) was transferred to a 1.5 mL centrifuge tube and washed three times using phosphate buffered saline (PBS) (ThermoFisher, 70,011,044) and a magnet. Two separate 1 mL aliquots of MES-NaCl solution were prepared by dissolving 19.5 mg MES (Sigma Aldrich, M3671) and 9.0 mg NaCl (Sigma Aldrich, S9625) in 1 mL of nuclease-free water (Growcells, NUPW100012), resulting in 0.1 M MES and 0.9 % NaCl solution. Ten mg of EDC (ThermoFisher, 22,980) was dissolved in one of the MES-NaCl aliquots to make MES-NaCl-EDC solution. Ten mg of mucin from porcine stomach (Sigma Aldrich, M1778) was dissolved in the other MES-NaCl aliquots to make MES-NaCl-Mucin solution. Next, 1 mL of MES-NaCl-Mucin solution and 100 μL of MES-NaCl-EDC solution were added to the washed beads and vortexed for 30 min at 300 rpm to ensure conjugation of porcine gastric mucin to the magnetic beads. In the last step, the magnetic beads were washed three times using PBS and a magnet and resuspended in 1 mL PBS and kept at 4 °C until further use in the concentration process.

The protocol for PGM-MBs concentration started with removing solid particles from wastewater by centrifugation, as described before. Then, 2.1 mL of 1 M MgCl2 (VWR, 97062–848) was added to 40 mL of the wastewater sample for a final concentration of 50 mM with a brief vortex. Next, 100 μL of PGM-MBs were added to the sample and mixed at room temperature for 20 min. A ring magnet (K&J Magnetics, RY0X04) pelleted the magnetic beads for 10 min. The supernatant was discarded without disturbing the pellet and the magnetic beads were resuspended in 2.5 mL lysis buffer (described before) and divided into four aliquots (each 625 μL) for nucleic acid extraction.

2.3.4. Solid fraction extraction (SF)

A commonly used and simple method for wastewater-based epidemiology (WBE) is the extraction of viruses from solid particles in the wastewater (Kitamura et al., 2021). To do this, a 40 mL sample of wastewater was centrifuged for 2 min at 1500 x*g* using a swinging bucket centrifuge (Southwest Science, SCL636) to separate the solid fraction. Next, 2.5 mL of lysis buffer were added to the pelleted solids and vortexed for 3 min. The tube was allowed to settle for 5 min without any movement to ensure that larger solid particles settled to the bottom. Using the top liquid layer, four 625 μL aliquots were transferred to 1.5 mL centrifuge tubes for nucleic acid extraction.

2.3.5. Direct extraction from wastewater (DEW)

For direct extraction, 250 μL of untreated wastewater was aliquoted into a 1.5 mL centrifuge tube and 375 μL of lysis buffer was added. The resulting lysed sample (625 μL) was used for nucleic acid extraction.

2.3.6. Direct extraction from supernatant (DES)

After removing the solid particles by centrifugation as described previously, 250 μL of wastewater supernatant was carefully transferred to a 1.5 mL centrifuge tube. Then, 375 μL of lysis buffer was added to the sample. The solution was then used for nucleic acid extraction as described in the next section.

2.4. Nucleic acid extraction

Concentrated samples were extracted using Exclusion-based Sample Preparation (ESP) technology described previously (Strike et al., 2022). ESP nucleic acid extraction is fast, simple, and has high extraction efficiency. In brief, immediately after adding the lysis buffer to the beads/flocculant/pellet, 10 μL of each of two different types of paramagnetic particles (PMPs) (Cytiva, Serasil-Mag™ #29357369 and #29357374), were added to the sample and incubated at 50 °C for 20 min with brief vortex every 5 min. In the next step, the tubes were tumbled for 20 min using a tube rotator (ThermoFisher, 88–861-051) to ensure conjugation of nucleic acids to the PMPs. Next, to remove the contaminants and increase the purity, the PMPs were washed in two wash buffers. Wash buffer 1 contained 1 M Guanidine Thiocyanate (GTC) (ThermoFisher, AM9422), 10 mM Tris buffer pH 8 (ThermoFisher, AM9855G), and 1 % *v*/v Tween 20 solution (Sigma Aldrich, P1379) in distilled water. Wash buffer 2 contained 10 mM Tris buffer pH 8 dissolved in absolute ethanol. The washing step of the beads were performed using the ESP technology described in the next paragraph. The beads are then resuspended in 100 μL of nuclease-free water in a new 1.5 mL centrifuge tube and incubated at 70 °C for 20 min to ensure the elution of nucleic acids from the magnetic beads. In the final step, the beads are separated using a magnet and the purified sample is transferred to a clean 1.5 mL centrifuge tube.

ESP exploits the hydrophobic surfaces and magnets to displace and wash magnetic beads (Pezzi et al., 2018). In brief, the sample, along with wash buffer 1, wash buffer 2, and elution buffer (nuclease-free water), were loaded into the wells of a polypropylene Extractman plate (Gilson, 22,100,008). The Extractman plate was placed onto the Extractman device (Gilson, 22,100,000), which consists of a base with a magnet capable of linear movement and a head that holds a magnet capable of vertical movement. As shown in Fig. 2, once the Extractman plate is placed on the Extractman device, the head slides over the sample well and magnetically collects the beads on a hydrophobic strip (Gilson, 22100007). Immediately after collection of the beads, the head slides over the next well which contains wash buffer 1. The beads are dropped into the wash buffer 1 using the magnet in the base, which is controlled manually by the operator. The movement of the base magnet results in the washing of magnetic beads. Again, the head magnets collect the beads to move them to the next well. In total, beads are washed two

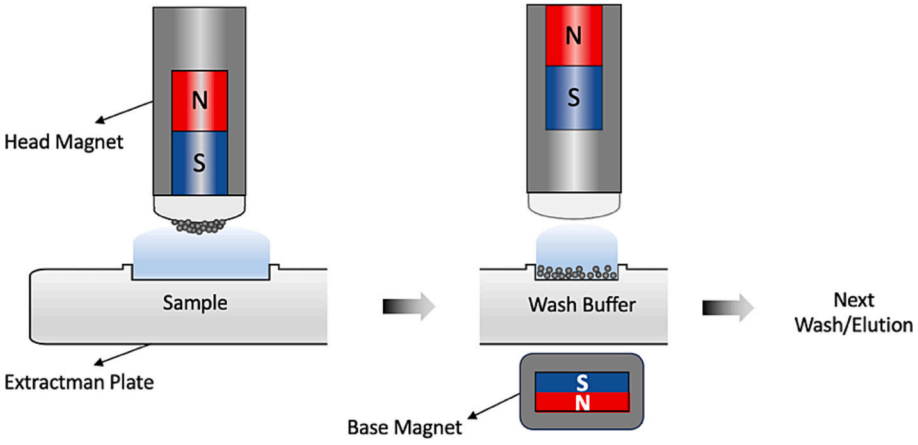

Fig. 2. Schematic of exclusion-based sample preparation (ESP). The beads are collected on a hydrophobic strip using the head magnet. With a slide of the head, the beads are positioned on the next well (wash or elution buffer). The beads are dropped in the well using the base magnet.

times in wash buffer 1 (250 μL and 110 μL) and two times in wash buffer 2 (110 μL and 110 μL), until the head slides over to the elution well, in which the beads are released inside 100 μL of nuclease-free water.

2.5. RT-qPCR analysis

While we anticipate using less resource intensive endpoints in a low resource setting (e.g., loop mediated isothermal amplification (LAMP)), reverse transcription qPCR (RT-qPCR) remains the gold standard for quantitation. Thus, we performed RT-qPCR on all RNA extracts to detect and quantify SARS-CoV-2, CrAssphage. A subsection of the samples is tested for Influenza A Virus (IAV), Respiratory Syncytial Virus (RSV A), and Clostridioides difficile (C. Diff). The SARS-CoV-2 assay used the N1 gene with a primer and probe listed in Table S2, as suggested by the CDC (Centers of Disease Control and Prevention, 2020). The primer and probe sequences for CrAssphage (Stachler et al., 2017), IAV (Whiley and Sloots, 2005), RSV A (Wang et al., 2019), and C. Diff (Kubota et al., 2014) are listed in Table S2. For SARS-CoV-2, CrAssphage, and IAV assays, the probes include a FAM fluorophore and MGB quencher. For the RSV A assay, the probes include a FAM fluorophore and BHQ1 quencher. The C. Diff assay uses a probe that includes a HEX fluorophore and MGB quencher. The one step RT-qPCR reaction was carried out on a LightCycler 480 II (Roche Diagnostics, 05015278001). Both assays were performed in 20 μL of reaction mixture, consisting of 10 μL sample, 5 μL TaqMan 4× Fast Virus 1-Step Master Mix (Applied Biosysyems, 4,444,434), 1 μL primer and probes (at 20× concentration), and 4 μL nuclease-free water (Growcells, NUPW100012). The primer and probes are synthesized by ThermoFisher at a final concentration of 60×, where the final 1× concentration in the reaction is equal to 900 nM of each primer and 250 nM of the probe. Thermocycling conditions included reverse transcription at 50 °C for 5 min and a hot start of 95 °C for 20 s, followed by 50 cycles of 60 °C for 1 min and 95 °C for 20 s, while measuring the real-time FAM fluorescence signal.

For the SARS-CoV-2 assay, the positive control included SARS-CoV-2 genomic RNA (NR-52508, Isolate USA-CA4/2020, BEI Resources) in one of the RT-qPCR reaction wells. The RT-qPCR reaction for CrAssphage was validated on each plate using previously extracted samples known to contain CrAssphage. The positive controls for IAV, RSV A, and C. Diff are genomic RNA from Influenza A Virus (NR-10046, Puerto Rico/8/1934 (H3N2), BEI Resources), Quantitative Genomic RNA from human respiratory syncytial virus strain Long (VR-26DQ, ATCC), and synthesized gBlock (IDT), respectively. The no template control (NTC) for both assays was 10 μL of nuclease-free water. All positive controls in each run successfully amplified and there was no amplification in any of the negative controls throughout the experiments. The threshold cycle of quantitation (Cq) is measured by identifying the cycle number at which the amplification curve of the PCR meets a predetermined mathematical threshold. Wastewater samples with a Cq higher than 40 in all replicates are called negative. To convert SARS-CoV-2 Cq to cp/mL, a standard curve was created by spiking serial 1:10 dilutions of heat-inactivated SARS-CoV-2 virus (NR52350, Isolate USA-WA1/2020, BEI Resources) in nuclease free water. BEI resources quantified the stock concentration of this standard using droplet digital PCR (ddPCR) and reported a concentration of 3.4E8 genome equivalents per mL. The standard curve exhibited a slope ranging from −3.22 and − 3.38 and a y-intercept between 39.52 and 40.24 (Fig. S1). The correlation coefficient (r^2) was between 0.998 and 0.999 and the amplification efficiency was between 97.5 % and 104.6 %, which is in accordance with the MIQE guidelines (Bustin et al., 2009). The standard curve was developed in triplicate to ensure repeatability. In the case of the four concentration methods (NMBs, CFCD, PGM-MBs, and SF), each replicate began with 10 mL of sample and the elution volume was 100 μL, resulting in a concentration factor of 100×. As for the two extraction methods (DEW and DES), the starting volume was 250 μL, and the elution volume was 100 μL, resulting in a concentration factor of 2.5×. These numbers were used to convert copies per reaction to cp/mL of wastewater. Since there was no available control for CrAssphage and C. Diff assays to create the standard curve, Eq. (1) was used to estimate the Cq to CrAssphage and C. Diff cp/mL conversion. As a result, the recovery rate and fold concentration were determined using the values calculated by Eq. (1).

$$Concentration = Dilution\ factor \times 2^{(40-Cq)} \quad (1)$$

To assess the effect of each concentration method in co-concentrating inhibitory molecules, 1:3 dilutions of the extracted nucleic acid were amplified in parallel with the undiluted sample on the PCR plate. In a sample with no inhibition, a 1:3 dilution should result in an increase of two PCR threshold cycles. However, wastewater is a complex sample containing various substances that can inhibit the PCR reaction (Bayati et al., 2022). Moreover, some variation in the quantification is expected due to subsampling variability. As described by Ahmed et al. (Ahmed et al., 2021), the diluted sample should be within 2 Cq values of the reference undiluted sample. As a result, if the Cq value of the diluted sample (1:3 diluted) is equal or higher than the reference undiluted sample, the PCR reaction is considered inhibited.

2.6. Data analysis

Recovery efficiency is an important control parameter to assess different concentration methods. In the case of a wastewater sample with a known spiked concentration of virus, the recovery efficiency can be easily calculated using the concentrations in the extracted sample and the original spike concentration. However, there are some implications

when comparing concentration methods using the spiked process. First, surrogate viruses such as Bovine coronavirus (BCoV) are usually used (LaTurner et al., 2021), which may behave differently than the target virus of interest. Moreover, due to safety issues, the spike-in viruses are inactivated prior to experiments, which can alter the virus morphology and even lyse them and release the nucleic acids. Experiments have shown that RNA is not stable in wastewater owing to the high presence of RNase (Torabi et al., 2023). As a result, the spike-in method can lead to under- or overestimation of the recovery efficiency. Even though spike-in experiments usually include a long incubation and shaking time to homogenize the wastewater, the presence of SARS-CoV-2 in the solid and liquid fractions can be greatly different in each wastewater sample, introducing further artifacts. Since our direct nucleic acid extraction has a consistently high recovery rate (average 89 %) (Strike et al., 2022), we used the virus concentration from the DEW method as a fair estimate of the concentration of virus in the wastewater. As a result, the relative recovery efficiency is calculated based on the following equation (Zheng et al., 2023):

$$Relative\ Recovery\ Efficiency = \frac{Cp/mL\ recovered\ from\ concentration}{Cp/mL\ calculated\ from\ direct\ extraction\ (DEW)} \quad (2)$$

In which, the direct extraction (DEW) is performed on 250 μL of wastewater, and the resultant Cq is converted to cp/mL of wastewater. In this study, 20 raw wastewater samples were studied, of which 19 samples were positive using the direct extraction method (DEW). We used Kendall's Tau correlation analysis, a non-parametric test, to measure the correlations between different concentration methods.

2.7. Time and cost analysis

Equipment (included as startup cost) and the materials (included as consumable cost) used in this study were priced from corresponding vendors at the time of preparing the manuscript in early 2023. Hands-off times (including centrifugation, heating, and mixing wait times) were measured for each step using a bench timer. We acknowledge that the hands-on times may vary between personnel, therefore we used an estimation of 1 min per manual handling of 4 sample replicates. The manual handling is defined as each laboratory task that needs personnel involvement such as pipetting, manual vortexing, and moving sample tubes between racks and equipment.

3. Results and discussion

3.1. Wastewater sample characteristics

The composition of wastewater is complex, comprising a mixture of solid and liquid components, and the amount of solids present can significantly differ among samples, as shown by other studies (Ahmed et al., 2023a). Table S3 demonstrated that total suspended solids (TSS) in wastewater samples varied from 55 to 737 mg/L, averaging 284 mg/L. Additionally, the turbidity of the samples ranged from 17.8 to 249 NTU, with an average of 97.7 NTU. The TSS and turbidity measurements differed between wastewater treatment plants and for wastewater sourced from similar locations on different dates. As an example, within a three-week period, the turbidity ranged from 17.8 to 92.4 and 99 to 249 NTU for WWTP B and C, respectively. As shown in Fig. S2, the TSS and turbidity were highly correlated ($r^2 > 0.96$). Moreover, the turbidity was highly correlated with absorbance at both 500 and 800 nm ($r^2 > 0.94$ and $r^2 > 0.95$, respectively). The wastewater pH ranged from 7.2 to 8.1 (Table S3) and the average wastewater temperature during the pH measurement was 7.9 °C (4–10 °C).

3.2. Extraction of SARS-CoV-2 from liquid and solid fractions

For most concentration methods, the presence of solid particles can affect sample processing; for example, solid particles can clog filters when performing ultrafiltration for virus concentration (Forés et al., 2021). As a result, solid particles are often discarded using filtration or centrifugation. However, several studies have shown that the concentration of SARS-CoV-2 in wastewater samples is higher in the solid fractions than in the liquid fractions (Kim et al., 2022; Kitamura et al., 2021). In this regard, the isolation of the solid fraction and extraction from this fraction can be used as a simple method to concentrate SARS-CoV-2. In this study, nucleic acids were directly extracted from raw wastewater (DEW method), directly from liquid supernatant (DES method), and from solid fractions (SF method). The SF method detected the SARS-CoV-2 N1 gene in all the samples (100 %, $n = 20$), while the DEW and DES methods resulted in a positive signal in 90 % and 85 % of samples ($n = 20$), respectively. In terms of genome quantification, as shown in Fig. 4a, there was not a significant difference between the DEW and DES methods. The SF method resulted in an average of 6.65-fold SARS-CoV-2 concentration increase in the final elution compared to the DEW method. However, the inhibition assay (Fig. S3) suggested that the RNA extracted from solid fraction contained more PCR inhibitors than RNA extracted from raw wastewater or the liquid fraction. PCR amplification inhibition was observed in 6 out of 20 RNA extracts from the SF method, while it was only seen in 2 and 1 out of 20 RNA extracts from DEW and DES methods, respectively. Therefore, we recommend assessing PCR inhibition when using the SF method.

While the liquid fraction contained less SARS-CoV-2 per volume, it accounts for most of the total influent wastewater mass and can be used to concentrate the virus. Using liquid fractions minimizes inhibition associated with solid particles. Therefore, we started all liquid concentration methods discussed in this work with a solid separation step using low-speed centrifugation. We minimized the time and equipment required for solids separation by choosing a relatively low centrifugation speed (1500 x*g*) and time (2 min), which can be performed by slow or manual centrifuges. Further simplification of this step is discussed later in this article.

3.3. Concentration of SARS-CoV-2

We evaluated six methods for concentration of SARS-CoV-2 RNA in 20 wastewater samples from 9 different WWTPs. The DEW and DES methods detected SARS-CoV-2 in 18 and 17 of the 20 samples, while the SF, PGM-MBs, CFCD, and NMBs processes detected SARS-CoV-2 in all the samples. Fig. 3 compares the average SARS-CoV-2 copies per PCR reaction for each method with the DEW results for each sample. The x-axis shows the DEW copies per reaction, and each point represents a unique wastewater sample (average of four replicates). The points' positions relative to the dashed equity line indicate the degree of SARS-CoV-2 concentration relative to the unconcentrated samples. A data point on or above the equity line suggests that the concentration method concentrated the SARS-CoV-2 RNA, whereas a point below the line indicates that the method did not concentrate SARS-CoV-2 RNA. DES showed comparable values to DEW, which justifies the selection of liquid fraction for further concentration. Also, CFCD, NMB, and PGM-MB methods showed successful concentration of SARS-CoV-2 in most samples. As shown by two points on the y-axis of Figs. 3b-3e, for two of the samples the DEW tested negative, while the SF, PGM-MB, CFCD, and NMB methods resulted in detectable concentrations of SARS-CoV-2 in the final elution.

The SARS-CoV-2 concentration performance of each method is shown by the relative recovery efficiency (calculated as described in the Methods and materials section) and fold concentration compared to the

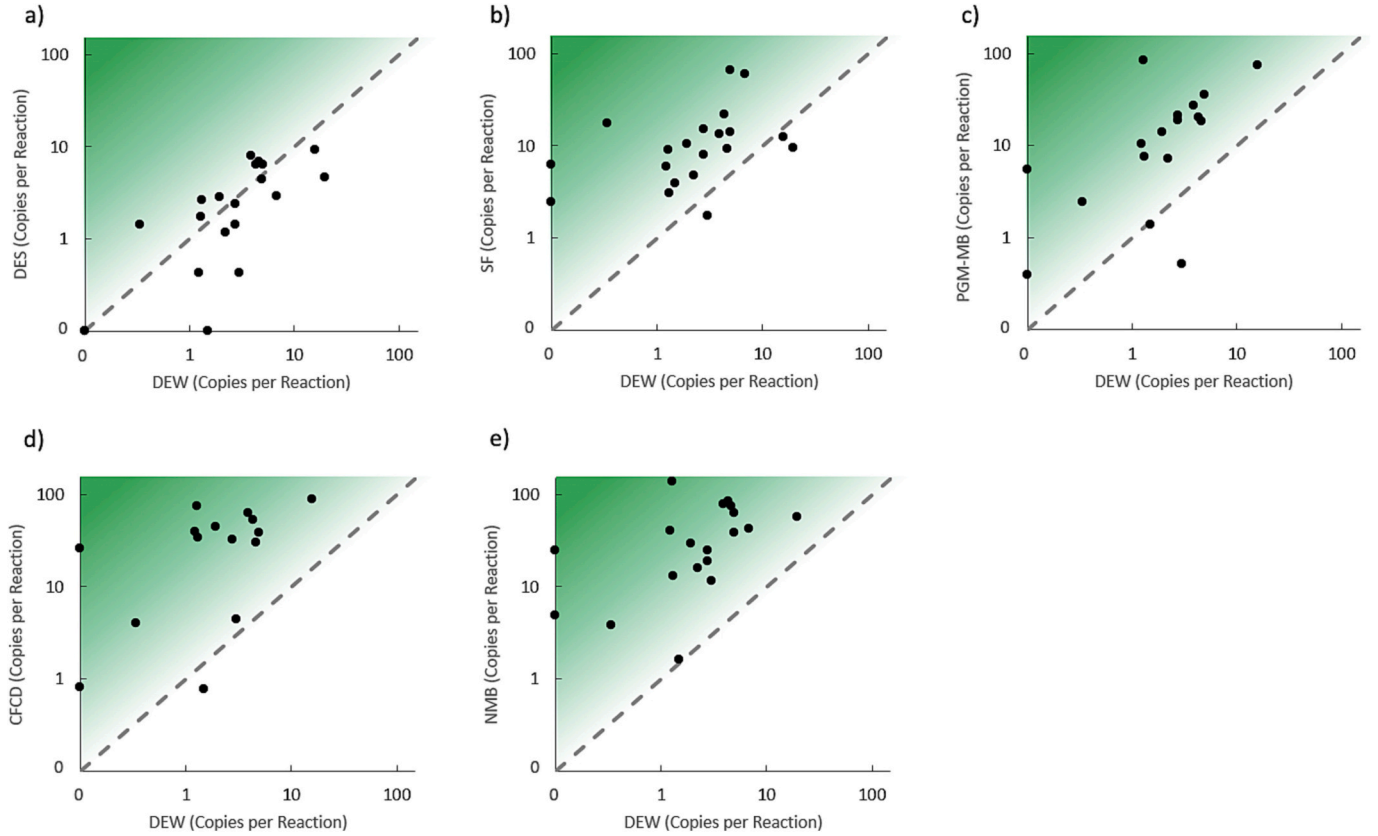

Fig. 3. SARS-CoV-2 copies in the final PCR reaction from a) DES, b) SF, c) PGM-MB, d) CFCD, e) NMB vs direct extraction from wastewater (DEW). Each data point represents a unique wastewater sample, with x and y-axis values showing the DEW and the corresponding concentration method, respectively. Dashed line represents the equity line. The points' positions relative to the dashed equity line indicate the degree of SARS-CoV-2 concentration relative to the unconcentrated samples. The closer the data points are to the upper left of each graph (the darker shade), the higher the concentration efficiency.

DEW method (Fig. 4). The dots in Fig. 4a and b represent each sample that was processed by all methods. The results from each method are normalized to the DEW result for that sample and presented as fold concentration. The dashed lines are the average fold concentration of each method. The average fold concentration for the DES method is 1.09, which suggests that the average concentration resulting from the DEW and DES methods are the same. The box plots in Fig. 4c and d show the range, average, and median relative recovery efficiencies from the 20 wastewater samples. The NMBs method was the most efficient, with an average fold concentration of 16.54 and a recovery rate of 41.3 %. The CFCD method was the second most effective, with an average fold concentration of 16.24 and a recovery rate of 40.6 %. The PGM-MBs and SF methods had fold concentrations of 9.32 and 6.65, respectively, and exhibited recovery rates of 23.3 % and 16.6 %.

The NMBs method has been widely employed for concentrating viruses from wastewater through both manual (Brighton et al., 2024) and automated workflows (Karthikeyan et al., 2021). Previous comparative studies have indicated that the NMBs method yields comparable results to the Adsorption-Extraction method in recovering SARS-CoV-2, known for its high recovery rates (Ahmed et al., 2023a; Ahmed et al., 2023c). The results of our study suggest that NMBs exhibits the highest recovery rates among various methods, consistent with other's findings. Our CFCD protocol originates from a study aimed at concentrating noroviruses in drinking water (Liu et al., 2007). However, this study did not report on the recovery rate, concentration factor, or any other comparable measures. As a result, it is impossible for us to compare our CFCD results with previous studies. Compared to other flocculation protocols (skim milk flocculation and Aluminum polychloride flocculation), the CFCD method has a higher recovery efficiency (Barril et al., 2021; Philo et al., 2021; Salvo et al., 2021). In an earlier investigation involving the PGM-MBs method (Oh et al., 2022), recovery efficiency was reported between 1.3 % and 64 % across five different virus strains. Our reported recovery efficiency using the PGM-MBs method (23.3 %) falls in this range. The variations in recovery efficiencies stem from concentrating various virus strains. While several studies indicate a higher concentration of SARS-CoV-2 in the solid fraction compared to the liquid fraction (Kim et al., 2022; Kitamura et al., 2021; Westhaus et al., 2021), only a limited number of studies have addressed the recovery efficiency of this approach. A study reports a 15 % recovery rate which is close to our finding for SF method (recovery efficiency of 16.6 %) (Street et al., 2021).

The effects of wastewater characteristics (i.e., turbidity and pH) on each extraction method are shown in Table S4. Moreover, the effect of SARS-CoV-2 unprocessed sample concentration (derived from DEW) on each method is shown in Table S4. These effects are assessed by correlating wastewater characteristics with the recovery of each method. None of the mentioned characteristics (i.e., turbidity, TSS, spectral absorption, and pH) had a significant effect on the methods ($r < 0.7$ for all). However, some weak and moderate positive and negative correlations were observed. Sample turbidity had a weak positive correlation with the DES and SF recovery rates, while the PGM-MBs, CFCD, and NMBs method had a weak negative correlation with sample turbidity. The higher efficiency of the SF method for higher turbidity samples, compared to other concentration methods that primarily rely on the liquid fraction, can be partially attributed to the preferential attachment of SARS-CoV-2 to solid particles. On the other hand, the pH had a weak to moderate positive correlation with the PGM-MBs, CFCD, and NMBs methods. Since altering the pH will change the charge, these methods are possibly charge-dependent. The inhibition study (Fig. S3) suggested that the SF method had the highest inhibition rate (6 out of 20 samples),

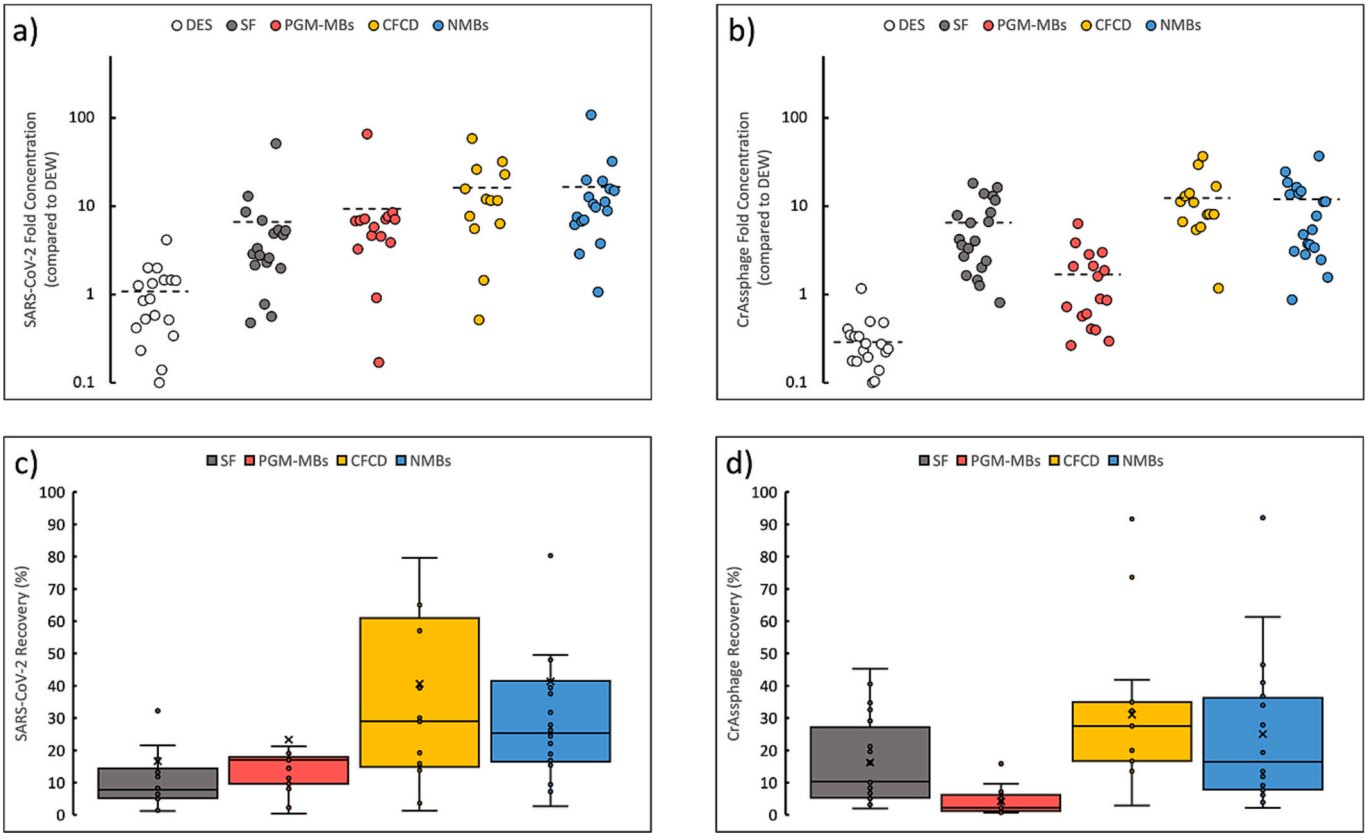

Fig. 4. Performance comparison of each method a) SARS-CoV-2 concentration compared to the DEW method b) CrAssphage fold concentration c) SARS-CoV-2 recovery efficiency of each method d) CrAssphage recovery efficiency. The dashed line in a and b represents the average fold concentration. Box plots show the range, median, and average (showed by x) of recovery efficiency.

while the PGM-MBs and NMBs methods resulted in the inhibition of 2 and 1 out of 20 samples, respectively. The CFCD method did not show inhibition in any of the samples.

3.4. Concentration of CrAssphage, IAV, RSV A, and C. Diff

CrAssphage is a fecal indicator that exists in wastewater at high levels. Along with Pepper mild mottle virus (PMMOV), this bacteriophage is routinely used as a positive control for the presence of human fecal material in wastewater-based epidemiology (Ai et al., 2021; Holm et al., 2022). Moreover, some studies have used CrAssphage to normalize the SARS-CoV-2 signal in wastewater (E. Lu et al., 2022; Sangsanont et al., 2022). While CrAssphage is mostly associated with the solid particles in the wastewater (Wilder et al., 2021), it is also present at high concentrations in the liquid fraction. Across all methods, CrAssphage was positive in all replicates, with the lowest concentrations resulting from the DES method, shown in Fig. 4b. As expected, extraction from the sol7id fraction yielded one of the highest concentrations of CrAssphage. Interestingly, due to the low recovery efficiency of the PGM-MBs method for CrAssphage (Oh et al., 2022), the PGM-MBs did not concentrate CrAssphage, and there was no significant difference between the CrAssphage copies of DEW and PGM-MBs. Although the CFCD and NMBs methods use the liquid fraction, they concentrated CrAssphage to higher levels than the SF method.

As show in Fig. S4a, C. Diff (a bacterial target) behaves similarly to CrAssphage. As a result, SF, CFCD, and NMBs had the best performance, followed by the PGM-MBs. The DES method failed to recover C. Diff efficiently. The IAV and RSV A were not highly prevalent in our sampling period in eastern Kentucky, and the majority of samples were negative. As a result, instead of concentration factor, each method's positivity rate is reported for these two targets (Fig. S4b). Similar to SARS-CoV-2, the direct extraction methods (DEW and DES) had the lowest positivity rates, while concentration methods (SF, PGM-MBs, CFCD, and NMBs) had higher positivity rates. The NMBs method had the best performance for IAV, and RSV A.

3.5. Effect of wastewater processing technique on measuring the SARS-CoV-2 viral load

Some wastewater-based epidemiology assays rely on direct extraction of nucleic acids from wastewater samples (Kantor et al., 2022; Whitney et al., 2021). However, it is common that the concentrations of viral pathogens are close to or below the limit of detection of the quantification method (e.g., RT-qPCR), potentially resulting in false negative assay results. Therefore, a concentration step can enhance WBE assay sensitivity in highly diluted samples. To evaluate whether there is a correlation between different methods in terms of SARS-CoV-2 quantification, we performed a Kendall's Tau statistical analysis. The correlation is measured between SARS-CoV-2 from each paired method using 20 wastewater samples. As shown in Table 1, almost all methods are significantly correlated, except for CFCD which has moderate and weak correlations with DEW and SF methods, respectively. Interestingly, the PGM-MBs, CFCD, and NMBs methods have stronger correlations (higher Kendall's Tau) with DES, compared to the DEW method. This may be because these methods start with the supernatant of wastewater. The highest correlation is between the CFCD and NMBs methods, which are the methods with the highest recovery rate.

We next examined the impact of concentration methods on determining the SARS-CoV-2 viral loads trend in wastewater from two wastewater treatment plants (WWTP B and WWTP C) over a three-week period, as shown in Fig. 5. Six samples (two times per week) were collected from each WWTP, and the viral load was measured using

Table 1
Paired Kendall's Tau correlation among methods. The yellow boxes indicate significant correlation (p-value<0.05).

	DEW	DES	SF	PGM-MBs	CFCD	NMBs
DEW		0.57	0.38	0.47	0.33	0.39
DES			0.42	0.57	0.58	0.61
SF				0.46	0.28	0.36
PGM-MBs					0.71	0.69
CFCD						0.77
NMBs						

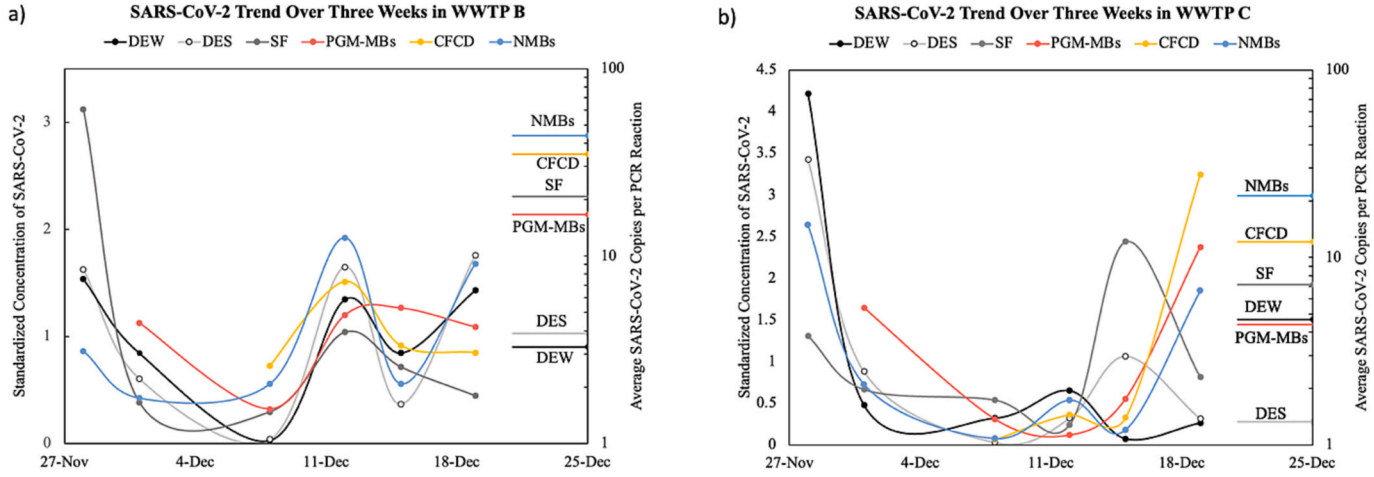

Fig. 5. SARS-CoV-2 viral load trend in wastewater samples from a) WWTP B and b) WWTP C using different concentration methods.

different methods. Due to protocol optimization, data for the PGM-MBs and CFCD methods is missing for some of the first timepoints. The SARS-CoV-2 concentrations were normalized using the average concentration (across all timepoints) of each method. Also, the average SARS-CoV-2 copies per final PCR reaction is shown on the secondary axis of the figures.

While the concentration methods NMBs, CFCD, and PGM-MBs yielded several orders of magnitude higher concentrations of SARS-CoV-2, compared to the direct extraction (DEW and DES), in the PCR reaction for both WWTPs, the direct extractions from wastewater (DEW) or supernatant (DES) showed similar viral concentration trends. For WWTP B, the DEW and DES methods had negative results for the third sample on Dec 8th, while the concentration methods showed a low viral concentration. The SARS-CoV-2 concentration trends yielded by NMBs, DEW, and DES matched well for WWTP B. For WWTP C, the viral load was high at the beginning of the study period and after a sharp decrease, it increased in the final week. However, the DEW method did not show the increase at the last point.

While direct extraction can be used to track the concentration of SARS-CoV-2 in wastewater, it starts with a small volume of sample (in our case 250 μL), which can result in big variation due to the heterogeneity of wastewater. Moreover, for samples that contain low concentrations of the virus of interest, direct extraction can result in false negatives because of reaching the quantification method limit of detection. For WWTP B, the NMBs method resulted in 100 % positivity in all replicates (24/24), while the DEW method resulted in 67 % positivity in all replicates (16/24). For WWTP C, the NMBs method resulted in 79 % positivity in all replicates (19/24), while the DEW method resulted in 54 % positivity in all replicates (13/24). This result suggested that the assay sensitivity increased when incorporating the concentration step compared to direct extraction from wastewater.

3.6. Time and cost analysis

While the performance characteristics of concentration methods play a crucial role in selecting the appropriate method for WBE, it is equally important to consider the feasibility of this technology in low resource settings. WBE is a more cost-effective surveillance tool compared to individual testing, but it remains relatively expensive for low- to middle-income countries (LMICs). A recent report suggested that the estimated cost per individual for WBE in rural areas might be 20 times higher compared to WBE in big cities (Weidhaas et al., 2021). Moreover, a recent study on SARS-CoV-2 WBE in Malawi and Nepal reported that consumable costs were a large share of the costs (ranging from 39 % to 72 %) (Ngwira et al., 2022). Hence, cost becomes a significant determinant in method selection. Additionally, the accessibility of advanced technologies in low resource settings is another important factor to consider. Utilizing existing or easily transportable equipment, reagents, and consumables can facilitate the implementation of WBE in these regions. Additionally, a short processing time can enhance the feasibility of adopting these technologies. Given the absence of expert personnel in rural/remote/low-resource areas, a lengthy process may be too complex to execute. The processing time is particularly vital for the scalability of WBE, especially in regions where routine testing of environmental samples from multiple locations at multiple timepoints is required. To assist in selecting the most suitable method for each specific setting, we

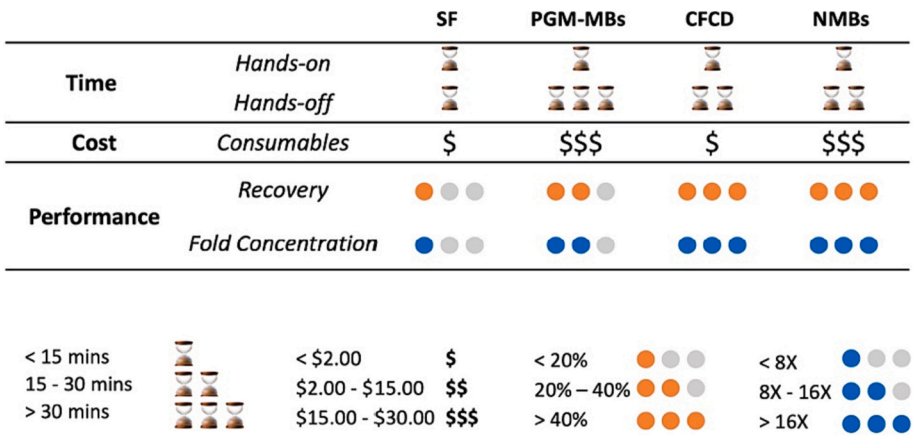

Fig. 6. Time, cost, and performance comparison of different concentration methods.

conducted a time and cost analysis (Fig. 6).

The time analysis is divided into two subcategories. The first category, hands-on time, encompasses all the manual tasks performed by an operator, such as pipetting, labeling, and transferring tubes. The hands-on time for all methods (to process four replicates) was <15 min. The second category, hands-off time, refers to the required incubation times. Among the methods evaluated, the shortest hands-off time was observed for SF (7 min), while PGM-MBs had the longest hands-off time (32 min). Overall, all the concentration methods examined in this study required <45 min to execute, making them considerably faster than conventional ultrafiltration and PEG precipitation methods (1.5 to 6 h) (LaTurner et al., 2021). It is important to note that the reported processing times here apply uniformly to all types of samples, unlike certain methods that require filtration and exhibit varying processing times due to differences in sample turbidity levels (Farkas et al., 2022; Juel et al., 2021). Additionally, it should be emphasized that when running multiple samples simultaneously, the hands-off time remains constant, while the hands-on time may increase. For more detailed information on the time analysis, please refer to Tables S5, S6, and S7.

The cost analysis in this research focused on startup and consumable costs. Startup costs encompass the expenses associated with acquiring equipment, while consumables include materials such as tubes, pipettes, buffers, and reagents. All the methods examined have a startup cost of less than $3500 (except for the DES which has a startup cost of ~$5700), as they only require basic wet lab equipment such as tube rotators, shakers, low-speed centrifuges, and magnets. In contrast, other methods like filtration and PEG precipitation necessitate costly filtration or centrifuge units. Furthermore, the basic equipment is easily transportable, making it particularly advantageous in low resource settings. The SF and CFCD methods require minimal tubes and/or a few chemicals, making them highly cost-effective (less than $2.00 for four replicates). The chemicals used in the CFCD method are available from multiple suppliers at relatively low cost. Additionally, the NMBs method employs commercially available beads and reagents, amounting to approximately $25.00 for four replicates. Notably, the reagents and beads in the NMBs method are shelf-stable and can be shipped and stored without refrigeration. The PGM-MBs method cost $22.00 for four replicates, primarily due to the requirement of carboxyl-derivatized beads, which require refrigeration. For more comprehensive details on the cost analysis, please see Tables S5, S8, S9, S10, S11, S12, and S13.

3.7. Further simplification of wastewater analysis

One of the key parameters in designing wastewater assays in low-resource settings is to minimize electricity consumption and equipment cost. As discussed earlier, removing the solid particles from the wastewater samples adds a centrifugation or filtration step into the process. We used low speed centrifugation as a sample preparation step before some of the concentration methods. We investigated whether we could eliminate the initial centrifugation step without compromising method performance. It is of particular interest in the case of PGM-MBs and NMBs methods, where all other steps can be performed without the need for electricity.

For wastewater samples #18–20, we vigorously mixed the raw wastewater bottles (approximately 500 mL), and then placed them on a flat surface for 10 min, for the solid particles to settle. Next, we collected the top 40 mL of the wastewater sample to continue with the concentration processes. As shown in Fig. 7, the NMBs and PGM-MBs methods yielded lower or equal SARS-CoV-2 copies when performed without centrifugation compared to when performed with the initial centrifugation. As a result, replacing the initial centrifugation with short settling does not damage the sensitivity. However, it might decrease the precision by increasing the variation between replicates. The standard deviations for the replicates without centrifugation were larger, which may be due to heterogeneity caused by the presence of some solid particles in the initial sample.

4. Conclusion

We demonstrated impressive performance of several simple concentration methods to improve the sensitivity and efficiency of WBE. In total, six methods were compared in terms of SARS-CoV-2 recovery, including two direct extraction methods (DEW and DES) and four concentration methods (SF, PGM-MBs, CFCD, NMBs). There was not a significant difference between the SARS-CoV-2 content using DEW and DES methods. However, the targets that are mostly associated with the solid particles (e.g., CrAssphage) were recovered at higher concentrations using the DEW method. While having a high recovery rate, these direct extraction methods start with a small volume of wastewater, resulting in a low number of target nucleic acids in elution that is close to the PCR limit of detection. Moreover, for some endpoints such as sequencing, higher concentrations are required. Therefore, we combined four simple concentration methods with ESP extraction. The addition of concentration methods increased the sensitivity of the assay compared to direct extraction, however, they come at a price of more processing time, complexity, cost, and labor. The four concentration methods (SF, PGM-MBs, CFCD, and NMBs) were able to quantify SARS-CoV-2 in all samples and increased the SARS-CoV-2 copy numbers in the final elution. The CFCD and NMBs methods had the highest concentration performance and recovery rate. The CFCD method, however, requires a low-speed centrifuge to pellet the flocculants, whereas the NMBs method can be performed with no dependence on electricity. Overall, the NMBs and CFCD methods are good options for concentrating mentioned pathogens (i.e., SARS-CoV-2, CrAssphage, IAV, RSV A, and C. Diff) from

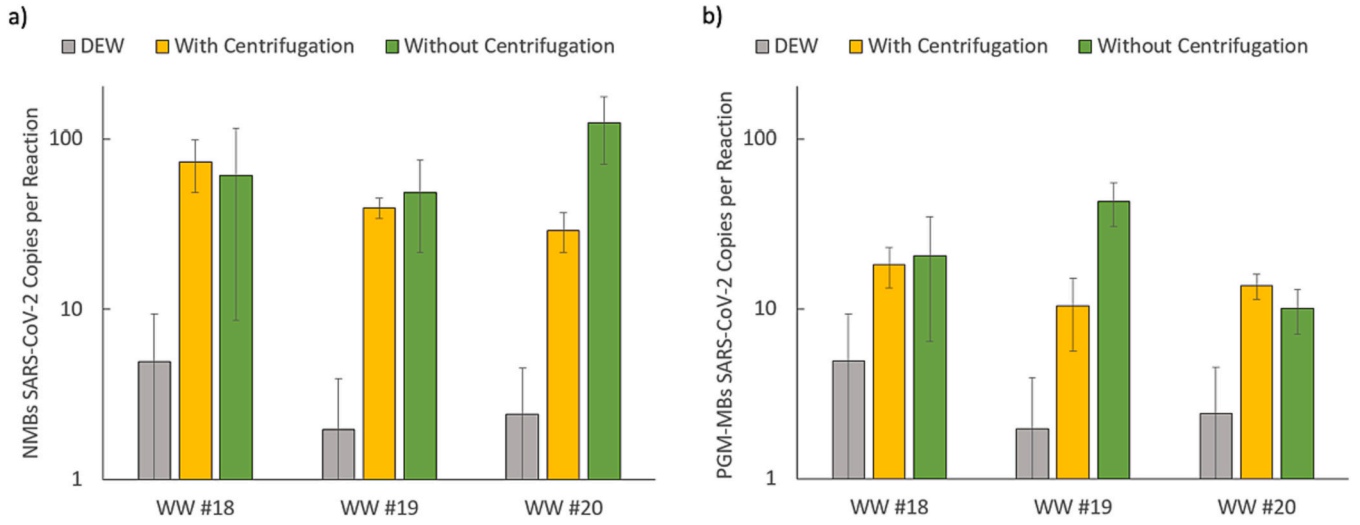

Fig. 7. SARS-CoV-2 copies per reaction for a) NMBs and b) PGM-MBs concentration methods with and without initial centrifugation.

wastewater in low-resource settings.

In low-resource settings where the infrastructure for comprehensive clinical testing of infectious diseases is not available, WBE can be an attractive tool to surveil the prevalence of a pathogen. However, conventional methods associated with WBE require expensive lab equipment and consumables, which might not be available in many parts of the world. To expand access to WBE, fast and cost-effective methods need to be developed and optimized. The WBE workflow includes four main steps: sample collection, target concentration, nucleic acid extraction, and quantification. Recent studies have shown that Moore Swab sampling (putting a piece of gauze in the stream) is a simple, yet efficient sampling technique, which can replace expensive and bulky autosamplers (Bivins et al., 2022a). Our previous work has established a simplified nucleic acid extraction method called ESP. An automated version of this technology is also available (Dehghan Banadaki et al., 2023). Recent studies were able to simplify nucleic acid quantification by replacing qPCR with isothermal amplification methods such as LAMP or RPA (Bivins et al., 2022b; Tang et al., 2023). While qPCR necessitates a thermocycler, LAMP only demands a consistent temperature (typically 60–70 °C), achievable through a basic hot plate. Additionally, the LAMP reaction, typically completed in <45 min, tends to be faster than qPCR, which takes about 90 min, making it more suitable for resource limited settings. Notably, several studies have reported that compared to qPCR, LAMP is less sensitive to inhibition (Kaneko et al., 2007; Lee et al., 2019; Soroka et al., 2021), which is an important factor in environmental samples such as wastewater. On the other hand, LAMP is usually used for qualitative testing and is reported to have a lower sensitivity compared to qPCR (Akter et al., 2024; Amoah et al., 2021). Using a high efficiency concentration method can help compensate the lower sensitivity of LAMP. In this study, we investigated simplifying the concentration step. We selected, optimized, and analyzed several simple methods, which required minimal training, which is essential in settings with limited resources and personnel.

CRediT authorship contribution statement

Mohammad Dehghan Banadaki: Conceptualization, Investigation, Methodology, Software, Writing – original draft. **Soroosh Torabi:** Conceptualization, Investigation, Methodology, Software, Writing – original draft. **Alexus Rockward:** Investigation, Methodology. **William D. Strike:** Investigation. **Ann Noble:** Investigation, Resources. **James W. Keck:** Funding acquisition, Writing – review & editing. **Scott M. Berry:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing.

Declaration of competing interest

Scott Berry has an ownership interest in Salus Discovery, LLC, which has licensed the ESP technology described in the text. Dr. Berry has also been granted patents related to the ESP process.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.168782.

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