Science of the Total Environment 829 (2022) 154431

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Evaluation of a virus concentration method based on ultrafiltration and wet foam elution for studying viruses from large-volume water samples

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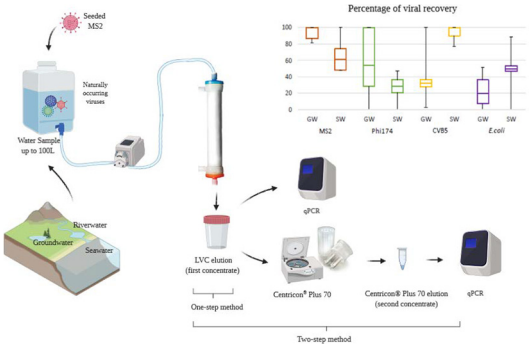
HIGHLIGHTS

- LVC kit is a rapid method for viral detection in water samples up to 100 L.
- The novel wet foam elution technology allows viral elution in the field.
- Method sensitivity can be increased by coupling a secondary concentration step.
- HAdV, NoV GI and NoV GII have been detected in freshwater and sea water.
- Cell culture and viral NGS studies can be conducted with the LVC eluates.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 December 2021
Received in revised form 4 March 2022
Accepted 5 March 2022
Available online 9 March 2022

Editor: Warish Ahmed

Keywords:
Viral detection
Viral metagenomics
Large volume concentration
Freshwater
Sea water
Ultrafiltration

ABSTRACT

Assessing the presence of viruses in large-volume samples involves cumbersome methods that require specialized training and laboratory equipment. In this study, a large volume concentration (LVC) method, based on dead-end ultrafiltration (DEUF) and Wet Foam Elution™ technology, was evaluated in different type of waters and different microorganisms. Its recovery efficiency was evaluated through different techniques (infectivity assays and molecular detection) by spiking different viral surrogates (bacteriophages PhiX174 and MS2 and Coxsackie virus B5 (CVB5) and *Escherichia coli* (*E. coli*). Furthermore, the application of a secondary concentration step was evaluated and compared with skimmed milk flocculation. Viruses present in river water, seawater and groundwater samples were concentrated by applying LVC method and a centrifugal ultrafiltration device (CeUF), as a secondary concentration step and quantified with specific qPCR Human adenoviruses (HAdV) and noroviruses (NoVs). MS2 was used as process control, obtaining a mean viral recovery of 22.0 ± 12.47%. The presence of other viruses was also characterized by applying two different next-generation sequencing approaches. LVC coupled to a secondary concentration step based on CeUF allowed to detect naturally occurring viruses such as HAdV and NoVs in different water matrices. Using HAdV as a human fecal indicator, the highest viral pollution was found in river water samples (100% of positive samples), followed by seawater (83.33%) and groundwater samples (66.67%). The LVC method has also proven to be useful as a virus concentration method in the filed since HAdV and NoVs were detected in the river water and groundwater samples concentrated in the field. All in all, LVC method presents high concentration factor and a low limit of detection and provides viral concentrates useful for subsequent molecular analysis such as PCR and massive sequencing.

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http://dx.doi.org/10.1016/j.scitotenv.2022.154431

1. Introduction

Humans and animals excrete viruses in feces and urine. Among these, members of the *Picornaviridae*, *Caliciviridae*, *Reoviridae*, *Hepeviridae* and *Adenoviridae* families, cause gastroenteritis (adenovirus, norovirus, rotavirus, and astrovirus) or hepatitis (hepatitis A and E viruses) as well as other pathologies such as meningitis (enterovirus and others). Their most important routes of transmission are the fecal-oral route and/or the direct contact with infected individuals (Cook, 2013). Therefore, many viral infections occur through consumption or contact with sewage-contaminated water arising from the discharge of untreated or inadequately treated sewage into the aquatic environment (Farkas et al., 2020). Whereas diffuse source pollution occurs when fecal pollution leach into surface waters and groundwater as a result of rainfall, soil infiltration or surface runoff, point source pollution comes from specific discharges. Hence, untreated wastewater can contaminate water sources through different routes. Large volumes of untreated wastewater can be discharged *via* sewer overflows during heavy rainfall events, tidal infiltration, snowmelt or system failures and blockages. Considering the different uses of water, such as drinking, household needs, recreational uses, as well as agricultural uses including irrigation and farming animals, its use or consumption may pose a risk to humans if the water sources (*e.g.*, groundwater, seawater or surface water) are contaminated by sewage (Fong et al., 2010; Lowther et al., 2012; Sinclair et al., 2009).

Apart from the aforementioned enteric viruses, other viruses have been identified in wastewater such us polyomaviruses (Bofill-Mas et al., 2000), papillomaviruses (La Rosa et al., 2013), small circular viruses (Phan et al., 2015) and coronaviruses including SARS-CoV-2 (Martínez-Puchol et al., 2021; Medema et al., 2020) among others.

The presence of viruses in aquatic environments has been widely studied in water bodies around the world (Farkas et al., 2020). Apart from the commonly analyzed viral indicators (*e.g.*, HAdV, JC polyomavirus (JCPyV), or pepper mild mottled virus (PMMV)), several reports describe the presence of pathogenic viruses such as NoVs, enterovirus and rotavirus in surface waters (Hata et al., 2014; Jurzik et al., 2010; Rusiñol et al., 2015; Sassi et al., 2018).

HAdV, hepatitis A virus, NoVs and sapoviruses have been describe to occur in coastal and brackish water (Dias et al., 2018a, 2018b; Farkas et al., 2018; Fongaro et al., 2015; Kaas et al., 2019; Moresco et al., 2012; Rusiñol et al., 2015; Symonds et al., 2018). Moreover, NoV as the etiological agent responsible for several groundwater-related outbreaks (Carrique-Mas et al., 2003; Giammanco et al., 2014; Moreira and Bondelind, 2017; Papadopoulos et al., 2006; Riera-Montes et al., 2011; Vantarakis et al., 2011).

The infective dose of these pathogens is known to be low (Kirby et al., 2015; Thebault et al., 2013), and has reported to be of between 0.5 and 6 $TCID_{50}$ (Yezli and Otter, 2011). Given that they are present in water matrices at low concentrations, in some cases below of 10^2 *GC/L* (Farkas et al., 2020, 2018; Hata et al., 2014), the detection of waterborne viruses is somehow cumbersome, because large volumes of water are needed to concentrate viruses in smaller volumes suitable for molecular detection, requiring two step processes, skilled staff, long periods of time, resulting most of the time in low viral recovery efficiencies or presence of viruses in the concentrate below the limit of detection of the detection method.

Several methodologies are available to concentrate viruses from water samples as reviewed by Bofill-Mas and Rusiñol, 2020. Most virus concentration methods consist of two steps where a few to hundreds of liters of water are concentrated into smaller volumes by a primary concentration method and then into a few milliliters by a secondary concentration method. Viruses are concentrated from water using different strategies based on the structural properties of viruses, mainly relying on their surface charge (commonly negative) or size (20 to 300 nm). The main methods based on charge are flocculation-precipitation with organic/inorganic flocculant such as skimmed milk flocculation (SMF), beef extract, polyethyleneglycol dextran PEG/Dex, PEG/NaCl, Al(OH)3 (Calgua et al., 2013a, 2013b; Deboosere et al., 2012, 2011) and virus adsorption-elution (VIRADEL methods) using electronegative (nitrocellulose membranes) or electropositive filters (ViroCap/Nanoceram®, glasswool) (Blanco et al., 2019; Ikner et al., 2012). Of all the methods based on size exclusion, those involving filtration (dead end ultrafiltration (DEUF), tangential flow ultrafiltration (TFUF), centrifugal ultrafiltration (CeUF)) are widely used and have been extensively reviewed in the literature (Bofill-Mas and Rusiñol, 2020; Cashdollar and Wymer, 2013; Ikner et al., 2012). A combination of both approaches is commonly used and there are other virus concentration methods that do not rely exclusively on size exclusion or surface charge, such as ultracentrifugation and lyophilization (Calgua et al., 2013a, 2013b; Hjelmsø et al., 2017; Ye et al., 2016). Every single method has its own limitations. In some cases, they are difficult to perform and require specialized staff and laboratory facilities, making their field deployment unfeasible.

Among many concentration methods used for viral recovery, ultrafiltration is based on the retention of low size particles to concentrate viruses in dead-end, tangential, or axial flow configuration (Gallardo et al., 2019; Ikner et al., 2012).

Ultrafiltration does not require sample preacidification, can concentrate multiple pathogens simultaneously and achieve good recoveries (Forés et al., 2021; Ye et al., 2016) allowing water volumes of up to 1000 L to be concentrated (Gunnarsdottir et al., 2020; Pascual-Benito et al., 2020; Smith and Hill, 2009). Most ultrafiltration methods require a second concentration step since the elution of viruses from filters requires the use of 200–500 mL of eluent.

The aim of this study was to validate and characterize a large volume concentration (LVC) protocol for the detection of viruses in large-volume samples as a fast and user-friendly procedure based on dead-end ultrafiltration (DEUF) followed by the Wet Foam Elution™ technology. This protocol can be used either as a one-step method or as a primary method in a two-step procedure. To accomplish this aim, groundwater and seawater samples spiked with several different viruses and *E. coli* were tested and the recovery of the viruses was estimated. The method was also applied to the molecular analysis of the presence of naturally occurring viruses in different types of water. Finally, the possibility of adapting the procedure to *in situ* concentration of large-volume water samples was explored.

2. Materials and methods

2.1. Microorganisms and cell lines

Escherichia coli (EC) (CECT 515) was cultured in trypticasein soy broth (TSB) EP/USP (Pronadisa) and quantified after filtration and dilution in ringer ¼ solution (Scharlau) by plating on trypticasein soy agar (TSA) EP/USP/ISO (Condalab).

Human adenovirus type 35 (HAdV-35) (ATCC® VR-718™) and Coxsackie virus B5 (CVB5) (ATCC® VR-185™) were produced by infecting A549 (ATCC® CCL-185™) and BGM (ECACC 90092601) cells, respectively. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies) containing 2× (maintenance medium) or 10× (growth medium) of heat-inactivated fetal bovine serum (FBS). DMEM was supplemented with 2% glutamine and 1% penicillin-streptomycin (Gibco, Life Technologies). All viruses were released from cells by freezing and thawing the cultures three times. A centrifugation step at 3000 ×*g* for 10 min was then applied to eliminate cell debris. The supernatant obtained was ultracentrifuged for 1 h at 34,500 ×*g* and resuspended in PBS, before being quantified using specific qPCR assays (see Section 2.5) and stored in 10-mL aliquots at −80 °C until use.

PhiX174 (ATCC13706-B1) and MS2 bacteriophages (ATCC 23631) were cultured in *E. coli* WG5 (ATCC 13706), following ISO 10705-2:2000, and in *Salmonella typhimurium* WG49 (NCTC 12484), following ISO 10705-1:1995, respectively.

2.2. Sample collection

2.2.1. Naturally water samples used for method validation

Water samples were collected in sterile plastic carboys and transported to the laboratory under cold conditions (4 °C). The turbidity was measured

using the HI98703 turbidimeter (Hanna Instruments Inc.) just after seeding 10^7 GC/mL of MS2 (1:100, v/v) as a process control. Groundwater with turbidities of 0.35 ± 0.25 NTU (nephelometric turbidity units) and seawater with turbidities of 6.94 ± 3.20 NTU were used to characterize the LVC method.

Groundwater, seawater and river water samples were collected from Barcelona urban area, three different coastal sites close to Barcelona and four different Catalan rivers. Samples were collected in sterile plastic carboys and processed *in situ* or transported to the laboratory under cold conditions. The processing of all samples was done immediately upon arrival at the laboratory.

2.3. E. coli determination

E. coli determination, was carried out with the 96-well microplate systems (MUG/EC 355-3782, BioRad®), according to ISO 9308-2:2012 (International Organization for Standardization, 2012).

2.4. Nucleic acid extraction and RT-qPCR quantification

Viral nucleic acids (NA) were extracted using the QIAmp Viral RNA Mini kit (Qiagen, Inc., Valencia, CA) in an automated QIAcube platform (Qiagen, Inc., Valencia, CA), according to the manufacturer's protocol. The volume of the viral concentrates used for the extraction was 140 μL, which was finally eluted with 60 μL of the kit elution buffer. A negative control of the viral nucleic acid extraction was added per each batch of 11 samples.

Based on previous research, specific real-time qPCRs were used to quantify HAdV (Hernroth et al., 2002) and PhiX174 (Verreault et al., 2010), while RT-qPCR was used to quantify CVB5 (Rusiñol et al., 2020), MS2 (Pecson et al., 2009), NoV-GI (Da Silva et al., 2007; Hoehne and Schreier, 2006; Svraka et al., 2007) and NoV-GII (Kageyama et al., 2003; Loisy et al., 2005). Viral nucleic acids were amplified using hydrolysis probes TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher Scientific) or the RNA UltraSense™ One-Step RT-qPCR System (Invitrogen) for DNA and RNA viruses, respectively. Quantification was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Undiluted and 10-fold dilutions of the nucleic acid extracts were analyzed in duplicate. All the qPCR and RT-qPCR assays included non-template controls to demonstrate that the mix did not produce fluorescence. The standards for viral quantification were prepared using synthetic gBlocks® Gene Fragments (IDT) and quantified with a Qubit® fluorometer (Thermo Fisher Scientific). For all the standards, 10-fold dilutions were prepared from 10^0 to 10^7 copies per reaction. All the qPCR assays performed were considered acceptable under the following parameters: mean Slope, between −3.1 and −3.5; r2, 0.999; and mean Efficiency, between 85 and 110%.

2.5. Infectivity assays

Infectious CVB5 viral particles present in viral concentrates were determined by plaque assays after inoculating cell monolayers with 1 mL of 10-fold dilutions of the concentrates in 90-mm^2 Falcon™ standard tissue culture dishes (Thermo Fisher Scientific). After 1 h of adsorption at 37 °C and 5% CO_2, the infected cells were overlaid with 12 mL of DMEM 2× containing 2% of Oxoid™ Purified Agar (Thermo Fisher Scientific). Following a 48-h incubation at 37 °C and 5% CO_2, the agar was carefully removed and the attached cells were fixed and stained for 45 min with 6 mL of 1% crystal violet (Merck) in 11.4% formaldehyde (Panreac) and 5% 2-propanol (Panreac).

PhiX174 and MS2 bacteriophages were quantified in *E. coli* WG5 and *S. typhimurium* WG49, following ISO 10705-2 and 10705-1, respectively.

2.6. Large volume concentration (LVC) method

Water samples were filtered using the Large Volume Concentration (LVC) kit from InnovaPrep® (CC01116-T), which couples a Rexeed-25A polysulfone hollow-fiver ultrafilter with a Wet Foam Elution™ canister containing the elution fluid. The kit also contains tubing, clamps, and can fitting. The ultrafiltration setup is shown in Fig. 1a. Water samples were forced through the membranes of the filter cell in a dead-end configuration (DEUF), using a Solinst® peristaltic pump (Model 410) set at 350 rpm. Filters were eluted using the patented Wet Foam Elution™ technology by pressing the elution canister into the supplied fitting at the top of the filter cell. The elution fluid contains water, a low concentration of a surfactant (less than 0.1%), a pH buffer, all infused with carbon dioxide. During the extraction process, the dissolved carbon dioxide expands and comes out of the solution to form microbubbles. These microbubbles increase the volume of the fluid by sevenfold or more as it recovers the biological organisms from the filter consumable. Upon elution, the foam immediately offgasses and collapses into a liquid and the concentrated sample is ready for analysis or further concentration.

The elution volume used varied from approximately 30 to 75 mL depending on the type of sample (Fig. 1b).

2.6.1. Selection of elution buffer

Elution fluid canisters (HC08000) containing a mixture of PBS and Tween-20 (0.075%) (n = 3) or a mixture of 25 mM Tris and Tween-20 (0.075%) (n = 3) were compared for the recovery of HAdV, PhiX174, MS2 and CVB5 present in an artificially spiked groundwater sample which was divided into six 10-L replicates, ultrafiltered and eluted with both types of canisters.

2.6.2. Characterization of the LVC method for fresh and seawater samples

To investigate the performance of LVC (25 mM Tris and Tween-20 (0.075%) elution) as a one-step concentration method for large-volume samples, *E. coli*, PhiX174, MS2 and CVB5 were used to spike 100-L groundwater and seawater samples, which were divided into ten 10-L replicates each. The microorganisms were quantified after ultrafiltration by infectivity assays (*E. coli*, PhiX174, MS2 and CVB5). The recovery value was calculated as follows:

$$\text{Microbial recovery}(\%) = \frac{\text{Concentrate titer}\left(\text{GC, PFU or CFU}/_{\text{mL}}\right)\text{x Sample Vol.(mL)}}{\text{Inoculum titer}\left(\text{GC, PFU or CFU}/_{\text{mL}}\right)\text{x Sample Vol.(mL)}}\text{x } 100$$

2.6.3. Optimization of the LVC method by adding a secondary concentration step

Three 10-L river water samples were primarily concentrated by the LVC method, as described above, followed by a secondary concentration step involving ultrafiltration with Centricon® Plus-70 devices with a cut-off of 30 kDa (Merck Millipore), following the manufacturer's instructions.

The theoretical limit of detection (LoD) of the whole method (including primary and secondary concentration steps and RT-qPCR detection) was calculated by running six replicates of 10-fold dilutions of target DNA/RNA suspensions around the detection end point (2.5, 5, 25 and 50 GC/reaction) for each virus analyzed. The concentration that produced at least 95% of the positive replicates was assumed to be the LoD of the qPCR assay, which was transformed into the LoD of the entire method using the sample volume tested and assuming 100% efficiency.

2.6.4. Comparison of the LVC method and the secondary concentration step with the skimmed milk flocculation method

The three 10-L river water samples described in Section 2.6.3 were also concentrated using the skimmed milk flocculation (SMF) procedure. This method, previously described to concentrate viruses from river water samples (Calgua et al., 2013a, 2013b), relies on the adsorption of viral particles to flocculated skimmed milk. It was used here as a reference method to validate the LVC method for environmental samples. The LoD of this concentration method was also estimated as described above.

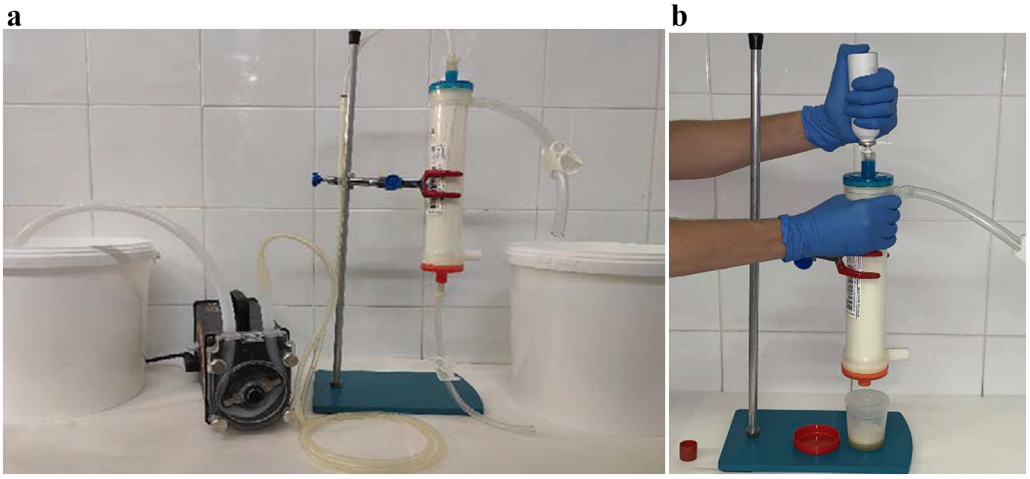

Fig. 1. a. Ultrafiltration setup. b. Elution setup.

2.7. Application of the LVC method to viruses present in diverse environmental samples

Environmental surveillance of HAdV, NoV-GI and NoV-GII was performed in 7 river water (10 L), 6 seawater (50 L) and 12 groundwater (100 L) samples collected from different locations in Catalonia (Northeast Spain) and presenting different turbidities. All the samples were collected in sterile carboys, spiked with MS2 as a control and shipped to the laboratory under cold conditions. Viral particles were concentrated by LVC and a secondary concentration step performed with Centricon® Plus-70 centrifugal ultrafiltration units, obtaining a final volume of 200–300 μL.

2.8. Application of the LVC method for the concentration of viruses in the field

To evaluate the efficiency and applicability of the LVC method in the field, two different approaches were applied. Firstly, four 50-L groundwater and two 100-L river water samples collected from different locations in Catalonia (Northeast Spain) and presenting different turbidities were concentrated in the field by adapting the LVC to the field as shown in Fig. S1a (Supplementary Material). Eluates were transported to the laboratory for further analysis. In this case, the Hyundai HY900Si inverter generator was used to feed the pump. Secondly, one filter unit was sent to the Guara Guara refugee camp in Mozambique and several liters of groundwater were filtrated by manual pumping (Fig. S1b). The cartridge was then sent to the laboratory in Barcelona by plane, which took 10 days. Upon arrival, Tris elution with wet foam canisters was performed. In both cases, a secondary concentration step was performed with the Centricon® Plus-70 CeUF units, obtaining a final volume of 200–300 μL.

2.9. Viral metagenomics

Viral nucleic acid extractions obtained from 2 river water and 6 groundwater samples were used to conduct target enrichment next-generation sequencing (TES) and amplicon deep sequencing (ADS) for NoV-GI and NoV-GII, as recently described by Itarte et al. (2021).

Briefly, for the TES, sequencing libraries were constructed with the KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, Roche) and hybridized with probes to select viruses infecting vertebrates using the VirCapSeq-VERT Capture Panel (Roche). This procedure was applied to examine the sample for the presence of vertebrate viral sequences.

For the ADS assay, specific nested PCRs for the selected genogroups were performed using primers incorporating Illumina adapters to study the genetic diversity of the NoV detected in this samples. In both cases, libraries and amplicons were sequenced in an Illumina 2 × 300 bp platform.

2.10. Data visualization and statistical analysis

Statistical analysis was conducted with the R package (R Core Team, 2021). Welch's test was used in order to compare the two eluents, while a paired *t*-test was performed for the comparison of both methods.

To evaluate a potential association between MS2 recoveries and enzymatic inhibition, a Pearson's correlation coefficient test was run. Significance for all the comparisons was indicated by a *p*-value less than 0.05. Data of infectivity obtained from the characterization of the LVC method for fresh and seawater samples (Section 2.6.2), was analyzed considering a full two factorial model with organism, water type and their interaction as sources of variation. We used a generalized least squares approach implemented in the gls method (Pinheiro et al., 2021), in order to correct the heterokedasticity shown by the data. Welch's test was used for all the pairwise comparisons between organisms and water types of interest, adjusting this multiple testing procedure with the p.adjust method of R, using a false discovery rate level of 5%.

3. Results and discussion

This manuscript analyzed the performance of wet foam elution as a procedure to elute viruses from ultrafilter cells. Wet foam elution is supposed to be much more efficient than liquid rinsing since it contains gas that promotes eluent expansion, maximizing the contact with the filter surface. Its viscosity creates a more uniform flow across the filter surface, with the microbubbles in the foam behaving as deformable solids. As they travel across the surface of the filter, they move as a ridged body with a narrow lubricating layer, effectively squeegeeing the particles off of the surface. As the microbubbles in the foam make impact against one other and burst, the turbulence and energy produced help to lift the particles adhering to the membrane (Innovaprep, 2019).

3.1. Selection of the LVC elution buffer

One groundwater sample was divided into six aliquots and spiked with HAdV, PhiX174, MS2 and CVB5 before being concentrated by LVC. Tris elution was performed in three of the aliquots and PBS elution in the other three. Recovery values were determined by qPCR for HAdV and by infectivity assays for CVB5, MS2 and PhiX174. Although, comparison of the elution buffers showed that Tris elution provided higher mean recovery values for HAdV, PhiX174 and CVB5 and similar values for MS2 (Table S1, Supplementary Material) although no significant differences between the buffers used statistically determined (p-values > 0.05).

Infectivity assays provided recovery values higher than 100% in some cases, as has been previously reported in the literature (Cooksey et al.,

Table 1
Mean recovery values, minimum (min), maximum (max), standard desviation (SD) and coefficient of variation (CV) of seeded viruses in Groundwater (N: 10) and Seawater (N: 10).

	Groundwater				Seawater			
	E. coli	PhiX174	MS2	CVB5	*E. coli*	PhiX74	MS2	CVB5
Min	0.96	3.12	6.79	1.14	17.48	7.66	7.47	27.69
Max	51.33	300.00	270.71	144.00	88.89	47.19	402.86	516.92
Mean	22.58	88.60	158.12	45.72	51.50	27.91	91.14	175.64
SD	18.88	100.78	93.18	49.88	17.63	12.33	112.32	143.16
CV	0.84	1.14	0.59	1.09	0.34	0.44	1.23	0.82

2019; Smith and Hill, 2009). This phenomenon may be caused by the under-quantification of viral stocks used for the spiking, which might contain viral aggregates. Viral stocks tend to aggregate when resuspended in solutions with a pH close to the isoelectric point of the virus under the influence of certain salt concentrations in solution, cationic polymers, and suspended organic matter. The conditions under which aggregates form are highly dependent on the type of virus, type of salts in the solution and pH (Gerba and Betancourt, 2017). Previous studies have also indicated a clear effect of the matrix in which the viral stock is suspended on the final recovery value obtained (Forés et al., 2021). In any case, both elution fluid canisters contain surfactant compounds that may disaggregate the viruses present in the cartridge, providing a highly disaggregated concentrate. Viral disaggregation using detergents has been widely reported in the literature (Brakke, 1959; Gerba and Betancourt, 2017; Konz et al., 2005; Mattle et al., 2011; Sobsey et al., 1988). This could have explained the values higher than 100% for CVB5 and the bacteriophages. For HAdV that was quantified by qPCR, recovery values higher than 100% were not observed.

Taking all this into consideration and based on the results obtained, the Tris elution buffer was selected to be used in the rest of the study.

3.2. Characterization of the LVC method for freshwater and seawater samples

LVC and viral elution with pressurized Tris buffer were evaluated by concentrating 10 groundwater and 10 seawater samples spiked with *E. coli*, PhiX174, MS2 and CVB5. Viral recovery values were determined by infectivity assays for *E. coli*, CVB5, MS2 and PhiX174.

Mean recoveries values for the artificially spiked samples were: 22.58 ± 18.88% for *E. coli*, 88.60 ± 100.78% for PhiX174, 158.12 ± 93.18% for MS2 and 45.72 ± 49.88% for CVB5 in groundwater samples; and 51.50 ± 17.63% for *E. coli*, 27.91 ± 12.33% for PhiX174, 91.14 ± 112.32% for MS2 and 175.64 ± 143.16% for CVB5 in seawater samples. In general, DNA viruses and MS2 were recovered more efficiently from groundwater than from seawater, whereas CVB5 recovery was higher in seawater samples (Table 1 and Fig. 2). Values over 100% were considered as 100% for plotting the recoveries. The concentration factor of the method, considering it as a one-step method, allowed the concentration of 10 L into a range of 20 to 75 mL, producing a 133–500-fold concentration factor and a sample volume of 1.17–8.75 mL analyzed per qPCR reaction. These values could have been even higher if a second concentration step had been performed.

Using Rexeed-25S in the same DEUF configuration, the recovery efficiency for a list of microbes was assessed by Smith and Hill (2009). They reported that the average recovery efficiencies of DEUF for MS2 seeded in 100 L of water were 57 ± 7.7%, 82 ± 14% and 73 ± 13% for water samples with a low range (0.29 NTU), mid-range (1.5 NTU) and high range (4.3 NTU) of turbidity, respectively. This agrees with the recoveries obtained in this study and support Rexeed-25A filters as an effective way of recovering different microorganisms from large-volume water samples with moderate to high turbidity. Previous studies using Rexeed-25A ultrafilters in the tangential flow configuration (TFUF) reported mean recovery values of 70% and 60% for the dsDNA bacteriophages T3 and HS2 in river water and seawater, respectively (Langenfeld et al., 2021).

Overall, viruses were recovered more efficiently from groundwater, except for CVB5 and *E. coli*, which were recovered more efficiently from seawater. It should be noted that different viruses may show different behaviors when using the same concentration method (De Keuckelaere et al., 2013; Forés et al., 2021) and the same virus can behave differently for the same method if the matrix it is embedded in is different.

Statistical analysis showed significant differences for the interactions between the microorganism and the type of water sample (two-way ANOVA *p*-values: microorganism, 0.0156; water, 0.0003; and microorganism:water, 0.0205). Regarding the paired comparisons between different microorganisms evaluated in groundwater, none of the microorganisms showed significant differences in their mean recovery values once the *p*-values had been adjusted. Regarding the paired comparisons between the microorganisms for the seawater samples, only PhiX174 and *E. coli* (adjusted *p*-value, 0.045) showed a significant difference in their mean recovery values. Finally, in the paired comparisons between both

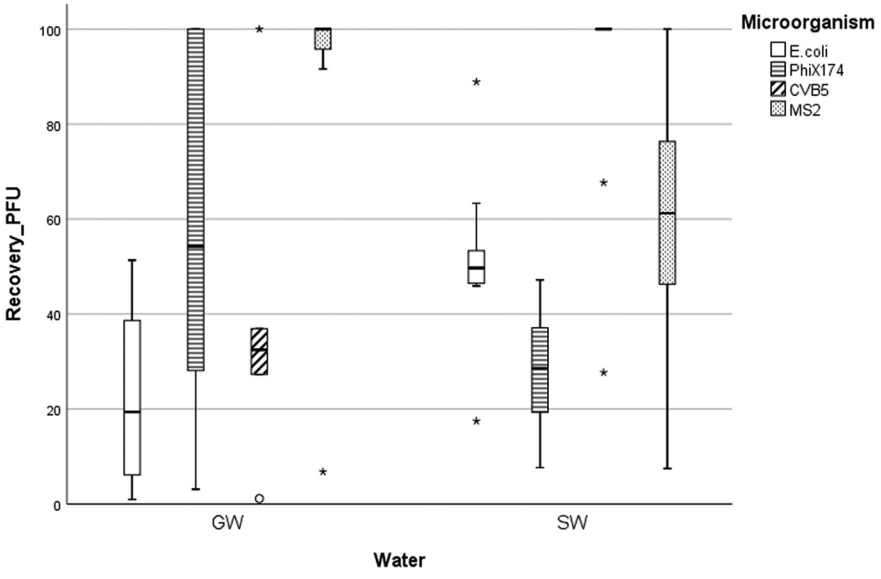

Fig. 2. Barplots showing LVC mean recovery values (%) obtained by infectivity assays (PFU) for different microorganisms used to spike groundwater (GW) and seawater (SW).

Table 2
Characterizacion of LVC compared to SMF using quantification of seeded and naturally ocurring viruses. Limit of detection (LOD), samples volumes equivalent and concentration factor are shown. ND, non detected.

	Seeded MS2		Natural HAdV	
	LVC + CeUF	SMF	LVC	SMF
	Recovery (%)		GC/L of sample	
RIV1	35.27	8.85	3.25E+03	6.96E+03
RIV2	23.13	8.97	6.38E+03	4.23E+03
RIV3	10.34	8.97	2.33E+02	N.D.
LOD (GC/10 L of sample)	2420–3010	6850–8000	907–1130	2571–3000
Sample volume equivalent	100–175 mL	13–15 mL		
Concentration factor	15,000–20,000×	2000–2857×		

water samples for every single microorganism, only *E. coli* (adjusted *p*-value, 0.016) showed significant differences in their recoveries for the two different types of water.

3.3. Optimization of the LVC method by adding a secondary concentration step

When a secondary concentration step was applied to river water samples, mean recovery value of seeded MS2 was 22 ± 12.47% and the quantifications obtained for naturally occurring HAdV were within the range of 2.33×10^2 to 6.38×10^3 GC/L. Rusiñol et al. (2015, 2014), reported similar values from Llobregat River (mean value 10^3 GC/L). The sample volume analyzed per reaction of qPCR increased from 1.17–8.7 mL to 105–175 mL, while the concentration factor increased from 133–550× to 15,000–20,000×, providing greater sensitivity to the method. However, when the concentration factor increases, qPCR inhibitors were observed. Hence, 10-fold dilutions of the samples are recommended for molecular detection.

3.4. Comparison of the LVC method + secondary concentration to SMF method

The LVC method followed by a second concentration step was compared to the SMF method, which has been extensively used for virus concentration from diverse types of environmental samples (Assis et al., 2017; Calgua et al., 2013a, 2013b; Calgua et al., 2008; Gonzales-Gustavson et al., 2017a, 2017b; Melgaço et al., 2018).

Table 2 shows the recovery values of spiked MS2 and naturally occurring HAdV and describes the equivalent sample volume, concentration factor and LoD of each method for the concentration of 10-L water samples. Mean MS2 recovery values for the LVC and SMF were 22.91 ± 12.47% and 8.97 ± 0.07%, respectively. All tested samples (3/3) were positive for HAdV when applying the LVC method, while 67% (2/3) tested positive when using the SMF method, with both methods presenting similar quantifications. This can be explained by the equivalent sample volume analyzed for each method. A higher sample volume in the PCR can be used with the LVC method compared to the SMF method, as commented before. This also means that the LVC method has a smaller LoD than the SMF method. Finally, the observed differences between both methods were statistically significant (paired *t*-test *p*-values were 0.027 for MS2 and 0.02 for HAdV).

3.5. Application of the LVC method for the concentration of viruses from different types of environmental samples

Seawater, groundwater and river water were tested for the presence and concentration of naturally occurring HAdV, NoV-GI and NoV-GII. MS2 was used as a control and used to spike all the tested samples. A second concentration step was performed in these samples using Centricon® Plus-70 CeUF units, which allowed the concentration by up to 250,000× of a 100-L sample and the analysis of 4375–2187 mL of water per qPCR reaction. Enzymatic inhibition was monitored, with seawater followed by river water showing the highest enzymatic inhibition. MS2, which was used as a process control, showed recovery values in accordance with those obtained for the characterization of the method. Nevertheless, a strong influence of the type of water sample was observed. There was an inverse relationship between MS2 recovery values and enzymatic inhibition (Pearson's correlation coefficient, −0.66). River water samples presented the highest level of human fecal viral pollution, as expected.

Table 3 shows the main results obtained. HAdV, an indicator of human fecal contamination, was present in 5/6 seawater samples (83.33%) at concentrations ranging from 2.14 to 2.39×10^2 GC/L, 8/12 (66.67%) urban

Table 3
Viral quantification in environmental samples concentrated by LVC + CeUf for naturally occurring viruses and enzimatic inhibition observed in each sample. Enzimatic levels of inhibition have been considered in the qPCR as follows: (−), no inhibition; (+), direct quantification at the same Ct than diluted sample; (++), Ct of the direct quantification higher than in the diluted sample; and, (+++), No Ct seen in the direct quantification but only in the diluted one.

Water type	Sample	Sample volume (L)	MS2 recovery (%)	HAdV	NoV GI	NoV GII	Enzimatic inhibition	Mean turbidity (NTU)	Location
Seawater GC/50 L	SW1	50	ND	1.43E+02	NT	NT	+++	6.94 ± 3.20	Catalan coast
	SW2		6.1	1.07E+02			+++		
	SW3		4.6	ND			+++		
	SW4		4.5	1.25E+03	9.46E+02	9.72E+02	+		
	SW5		5.6	1.20E+04	4.52E+04	1.21E+04	+++		
	SW6		2.8	6.09E+02	<LOD	2.41E+02	+		
Groundwater GC/100 L	GW1	100	21.42	3.14E+01	ND	ND	−	3.2 ± 2.27	Barcelona Urban area
	GW2		4.58	ND	ND	ND	++		
	GW3		92.24	ND	ND	ND	−		
	GW4		78.07	ND	ND	ND	−		
	GW5		1.62	2.38E+01	ND	ND	++		
	GW6		132.54	2.25E+02	ND	ND	−		
	GW7		68.35	7.85E+01	ND	ND	−		
	GW8		NT	1.18E+01	ND	ND	−		
	GW9		NT	4.67E+02	5.89E+01	ND	++		
	GW10		NT	9.01E+01	ND	ND	−		
	GW11		52.91	ND	ND	ND	−		
	GW12		44.41	4.67E+00	1.69E+02	ND	++		
Riverwater GC/L	RV1	10	23.69	3.47E+04	6.36E+03	1.33E+04	−	6.01 ± 1.00	Besós
	RV2		23.13	6.38E+03	1.33E+03	ND	++		Llobregat
	RV3		10.34	2.33E+02	1.81E+02	<LOD	++		
	RV4		10.32	5.71E+02	<LOD	ND	+++		
	RV5		5.94	1.73E+02	1.19E+02	ND	+++		
	RV6		35.27	3.25E+03	<LOD	<LOD	++		Anoia
	RV7		38.24	9.26E+02	1.50E+03	5.32E+02	++		

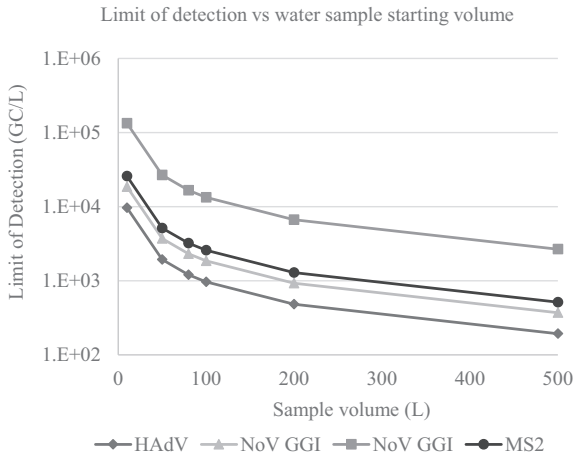

Fig. 3. Estimated LOD according to the starting sample volume. LOD has been calculated assuming 7.5, 7.2, 51.8 and 10 GC as the minimum amount of genomes detected in one qPCR or RT-qPCR reaction for HAdV, NoVGI, NoVGII and MS2 respectively and a mean volume of sample concentrate for each concentration methodology. A 100% recovery has been assumed for the whole concentration, nucleic extraction and detection process.

groundwater samples with concentrations ranging from 4.67 to 4.67 × 10^2 GC/100 L and 7/7 river water samples with values ranging from 2.33 × 10^1 to 3.47 × 10^3 GC/L.

Two groundwater samples positive for HAdV also tested positive for NoV-GI while 6/7 (85%) and 4/7 (57%) river water samples tested positive for NoV-GI and GII, with concentrations ranging from 1.81 × 10^1 to 6.36 × 10^2 GC/L and 5.32 × 10^1 to 1.33 × 10^3 GC/L, respectively.

These results are in accordance with previous ones described in the literature. Rusiñol et al. (2015) found concentrations around 10^3 GC/L for HAdV and up to 10^4 GC/L for NoV-GII in the Llobregat River, while Jurado et al. (2019) describe concentrations of 10^3 GC/L for HAdV and 10^1 and 10^3 GC/L for NoV-GI and NoV-GII respectively in Besós River, whose concentrations correlated with the distance of the sampling point from the source of contamination (wastewater treatment plant). Seawater samples were collected after heavy rainfall events and the quantifications obtained for HAdV and NoV were also in accordance with those of other studies. For example, after rainfall events, Farkas et al. (2018) described HAdV concentrations ranging from 10^2 to 10^4 GC/L at Conwy estuary, Wales, while Rusiñol et al. (2015) reported concentrations ranging from 10^1 to 10^5 GC/L in the Catalan coast and Kaas et al. (2019) found that the concentrations of human fecal indicators and NoV-GII were approximately 10^4 GC/L at the Tahiti coast. As for the groundwater samples, several studies have described the presence of enteric viruses in groundwater at similar concentrations as the ones reported above. Viral pollution in groundwater is mainly due to system failures and blockages (Kauppinen et al., 2018), rainy periods followed by prolonged floodwater injections into groundwater (Fongaro et al., 2015; Masciopinto et al., 2019), or the proximity of a polluted river (Jurado et al., 2019), with viruses present for long periods of time after episodes of pollution. Groundwater pollution is influenced by many factors, such as aquifer typology and the hydraulics and flow dynamics of groundwater, pathogen structure, episodes of extreme climate, proximity of the source of pollution and system failures (Fongaro et al., 2015; Masciopinto et al., 2019). However, a natural decrease in pathogens has also been described when polluted water infiltrates an aquifer (Jurado et al., 2019). Therefore, the HAdV quantifications reported in this study indicate low, but widespread viral pollution in the urban area of Barcelona.

Fig. 3 shows theoretical estimates of the LoD of the different viruses tested in this study, depending on the volume of groundwater filtered. The concentration factor of the method depended on the sample volume. When 10 L of water were processed, the concentration factor ranged from 15,000× to 20,000×, allowing the analysis of an equivalent sample volume per reaction of qPCR ranging from 105 to 175 mL. For large-volume samples (100 L), the concentration factor was 250,000×, meaning that an equivalent sample volume of 4.37 L of groundwater was being tested in the qPCR. In addition, the LVC kit was a very sensitive method for detecting viruses, presenting a LoD (GC/L) ranging from 1.01 GC/L for HAdV to 13.91 GC/L for NoV-GII. This explains the low HAdV concentrations detected in the groundwater samples. Therefore, it seems significant to concentrate a minimum of 100 L of the water sample, with volumes from 100 to 500 L guaranteeing the detection of viruses.

3.6. Application of the LVC method for the concentration of viruses in situ

Two different experiments were conducted as described in the Materials and methods section. Table 4 shows the main results obtained for the samples concentrated completely *in situ*, one river water sample was positive for NoV-GII by with a concentration of 1.34 E + 01 GC/L. Another groundwater sample was filtered *in situ* and the filter unit transported to the laboratory at room temperature. This sample was positive for HAdV at a concentration of 3.01 E + 03 GC/L in the first concentrate obtained after elution. The LVC system proved to be easy to use in the field. Samples can be ultrafiltrated directly through the cartridge (single-pass method), pumping the water directly from the source, or, if sampling with a pressurized system, the system pressure can be used to force the water through the ultrafilter without the need for pumping. The elution takes place rapidly thanks to the pressurized wet foam canisters in the DEUF configuration, which does not require cartridge back flushing (TFUF). This provides the possibility of testing viruses in water samples from remote areas.

3.7. Viral metagenomics

Viral concentrates obtained by using LVC from two river water and six groundwater samples were used to conduct two different metagenomic approaches, TES and ADS, as described in Materials and methods. Massive sequencing results have been extensively described as part of a larger study (Itarte et al., 2021).

TES provided a list of human viral pathogens belonging to *Astroviridae*, serotypes HAstV-1 and HAstV-5, and *Picornaviridae*, serotype Aichi virus 1 (Table 5).

Table 4
Detection of naturally occurring viruses applying LVC kit in the point-of-use. ND, non detected; NT, non tested.

Method	Location	Type of water	Sample volume (L)	Naturally ocurring viruses	qPCR (GC/L)	Positive samples	Mean turbidity (NTU)
LCV Kit, elution and transport	Mataró	GW	50	HAdV	ND	0/2	0.3 ± 0.1
				NoV GI	ND	0/2	
				NoV GII	ND	0/2	
	Òrrius	GW	50	HAdV	ND	0/2	0.42 ± 0.2
				NoV GI	ND	0/2	
				NoV GII	ND	0/2	
	Cardedeu	RW	50	HAdV	ND	0/2	2.00 ± 0.5
				NoV GI	ND	0/2	
				NoV GII	1.34E + 01	1/2	
LVC Kit, cartridge shippment to UB laboratory	Guara Guara (Mozambique)	GW	10	HAdV	3.01E + 03	1/1	NT

Table 5
Target Enrichment Sequencing (TES) results of human viral pathogens. ND, non detected; GW, groundwater; RW, riverwater.

Type of water	TES			
	Family	Genus	Species	Genogroup/genotype/serotype
GW	*Circoviridae*	*Cyclovirus*	*Human associated cyclovirus 6*	*NG12*
GW	*Parvoviridae*	*Dependoparvovirus*	*Adeno-associated dependoparvovirus A*	*AAV2*
RW	*Astroviridae*	*Mamastrovirus*	*Mamastrovirus 1*	*HAstV-1 and HAstV-5*
	Picornaviridae	*Kobuvirus*	*Aichivirus A*	*Aichi virus 1*

ADS revealed the presence of NoV serotypes GI.4, GI.1, GI.5, GII.4 and GII.13 in river water samples even though the sample was negative by q(*RT*)PCR. These results prove that the LVC method can be successfully applied when performing the metagenomics of large water volumes.

4. Conclusions

- LVC involving an ultrafilter cell and elution with a pressurized buffer was useful for one-step virus concentration for all the water matrices and viruses tested. The volume that the method can process depends on the sample nature and up to 100 L can be easily filtered. The concentration factor of the LVC method is of up to 500×. When a secondary concentration step is coupled to LVC, it can be of 250,000×.
- The use of Innovaprep® wet foam elution, which is applied after the DEUF filter cell concentration, reduces the duration of the process and makes it less cumbersome. This makes it possible to deploy the method in the field (which can be conducted by non-specialized staff).
- The LVC method is efficient and can be coupled with CeUF if higher sensitivity is needed for virus concentration from water samples, allowing the quantification and characterization of viruses by molecular assays such as PCR-based methods and next-generation sequencing techniques.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154431.

CRediT authorship contribution statement

Eva Forés: Investigation, Methodology, Formal analysis, Validation, Visualization, Writing - original draft. **Marta Rusiñol:** Conceptualization, Investigation, Methodology, Validation, Resources, Writing-review & editing, Project administration. **Marta Itarte:** Investigation, Methodology, Formal analysis, Writing-review & editing. **Sandra Martínez-Puchol:** Investigation, Methodology, Formal analysis, Writing-review & editing. **Miquel Calvo:** Data curation, Formal analysis, Software, Writing-review & editing. **Sílvia Bofill-Mas:** Conceptualization, Writing-review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was partly supported by grant PCI2019-103643, RTI2018-097346-B-I00 and AGL2017-86797-C2-1-R AGL funded by MCIN/AEI/10.13039/501100011033/FEDER "A way of making Europe", by the European Union. Sílvia Bofill-Mas is a Serra-Hunter Fellow at the University of Barcelona. Eva Forés is an APIF fellow at the University of Barcelona. Marta Itarte is a fellow of the Catalan Government "AGAUR" (FI) at the University of Barcelona. We thank Rosina Girones for her scientific assessment and Mauricio Córdova for his collaboration in the application of the method *in situ*.

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