Science of the Total Environment 905 (2023) 167190

Contents flfists avafiflabfle at ScfienceDfirect

Scfience of the Totafl Envfironment

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

# Comparfison of vfirafl concentratfion techn fiques for natfive fecafl findficators and pathogens from wastewater

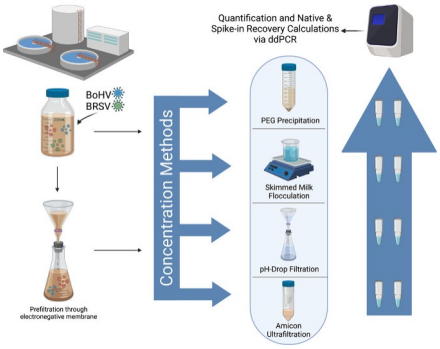
Devfin North, Kyfle Bfibby *

*Department of Cfivfifl & Envfironmentafl Engfineerfing & Earth Scfiences, Unfiversfity of Notre Dame, 156 Ffitzpatrfick Hafffl, Notre Dame, IN 46556, Unfited States*

## HIGHLIGHTS

- Natfive recoverfies are not suffficfientfly represented by spfike-fin recoverfies.
- Vfirus concentratfion methods vary fin effficacy based on vfirus structure and concentratfion.
- Amficon ufltraffifltratfion was the most broadfly effectfive concentratfion method.
- Poflyethyflene gflycofl was the best per-formfing method for SARS-CoV-2.
- Natfive target recovery shoufld be fincfluded fin vfirafl concentratfion evafluatfions.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

Edfitor: Warfish Ahmed

*Keywords:*
Wastewater
Vfirus
Concentratfion
findficator

## ABSTRACT

Vfirafl pathogens are typficaflfly dfiflute fin envfironmentafl waters, necessfitatfing a concentratfion step prfior to subsequent quantfifficatfion or anaflysfis. Hfistorficaflfly, studfies on vfirafl concentratfion effficfiency have been done by spfikfing known vfiruses finto the sampfle; however, spfike-fin controfls may not have the same behavfior as "natfive" vfiruses exposed to envfironmentafl condfitfions. In thfis study, four concentratfion methods, fincfludfing poflyethyflene gflycofl precfipfitatfion (PEG), skfimmed mfifk ffloccuflatfion (SMF), pH drop foflfflowed by ffifltratfion through a 0.45 μm ffifter (pH), and centrfifugatfion usfing an Amficon ffifter (Amficon), were evafluated to concentrate natfive vfirafl targets fin wastewater. Vfirafl targets fincfluded both findficators (crAssphage and pepper mfifld mottfle vfirus) and pathogens (adenovfirus, norovfirus GII, human poflyomavfirus, and SARS-CoV-2) fin addfitfion to a bacterfiafl marker (HF183). A non-natfive spfike-fin controfl was afso added to compare natfive and spfike-fin recoverfies. Recovery varfied wfidefly across targets and methods, rangfing from 0.1 to 39.3 %. The Amficon method was the most broadfly effectfive concentratfion for recovery effficfiency. For the flowest-tfiter target, the PEG method resuflted fin the flowest number of non-detectfions, wfith 96.7 % posfitfive detectfions for SARS-CoV-2, compared to 66.7 %, 80 %, and 76.7 % posfitfive detectfions for SMF, pH, and Amficon, respectfivefly. The non-natfive spfike-fins chosen were onfly representatfive of a few natfive re-covery trends, varyfing by both target and concentratfion method, and consfistentfly under or over-estfimated recovery. Overaflfl, thfis study suggests the utfiflfity of fincfludfing natfive targets fin vfirafl concentratfion evafluatfion and determfinfing the effficfiency of concentratfion methods for a specfiffic target of finterest.

* Correspondfing author.
*E-mafifl address:* kbfibby@nd.edu (K. Bfibby).

https://dofi.org/10.1016/j.scfitotenv.2023.167190
Recefived 27 Jufly 2023; Recefived fin revfised form 12 September 2023; Accepted 16 September 2023
Available online 21 September 2023

## 1. Introduction

Vfiruses account for the hfighest predficted finfectfious rfisks from exposure to sewage-contamfinated water (Boehm et afl., 2018; Crank et afl., 2019; McBrfide et afl., 2013). That rfisk fis despfite flow vfirafl pathogen concentratfions fin sewage compared to bacterfia and other pathogenfic agents. To properfly detect and quantfify vfiruses, especfiaflfly eukaryotfic vfirafl pathogens, fin sewage-fimpacted envfironmentafl waters, sampfles must be concentrated prfior to anaflysfis, fincfludfing cuflture-based and moflecuflar approaches such as shotgun metagenomfics or PCR methods. Concentratfion methods are cfitficafl for monfitorfing mficrobfiafl water quaflfity and fimprovfing detectfions fin wastewater-based epfidemfioflogy appflficatfions.

Many methods exfist to concentrate vfiruses fin envfironmentafl water sampfles and poflflutfing source materfiafls, such as sewage, through chemficafl and physficafl finteractfions, though findfivfiduafl morphoflogfies of vfiruses and bacterfiophages respond dfifferentfly to each method (Cashdoflflar and Wymer, 2013; Faflman et afl., 2019; Farkas et afl., 2022; Hjeflmsø et afl., 2017). Concentratfion methods refly on varfious underflyfing phenomena, fincfludfing physficafl capture of partficfles wfith attached vfiruses, charge capture of vfirafl partficfles, aggflomeratfion of vfirafl and soflfid partficfles, or sfize excflusfion of vfirafl partficfles. Standard concentratfion methods fincflude ffifltratfion-based approaches that capture vfirafl partficfles based on partficfle assocfiatfion and charge capture; ufltraffifltratfion, whfich uses centrfifugatfion and partficfle wefight; and precfipfitatfion or ffloccuflatfion, whfich empfloys varfious materfiafls to attract vfiruses vfia surface charge and create flarger partficfles that wfffl settfle or peflflet fin soflutfions. Commonfly, mufltfipfle methods wfffl be used fin tandem (fi.e., prfimary and secondary concentratfion) to achfieve the hfighest recovery possfibfle.

Spfike-fin controfls are generaflfly used to caflcuflate concentratfion effficfiency; however, thfis approach poses potentfiaflfly sfignfifficant chaflflenges. Vfiruses and nucflefic acfids of vfirafl orfigfin are flfikefly to be bound to flarger partficfles wfithfin a sewage sampfle, some of whfich fimpact the effficacy of extractfion and PCR processes and thus requfire as much separatfion from these partficfles as possfibfle (Gedaflanga and Oflson, 2009; Medefiros and Danfiefl, 2015). In contrast, spfike-fin controfls are taken from an effectfivefly pure sampfle and mfixed fin wfith the sampfle for a reflatfivefly short tfime reflatfive to what natfive vfiruses woufld experfience, thus natfive vfiruses may be more effectfive for method comparfison (Fores et afl., 2021). They are fless flfikefly to experfience the same fintegratfion and partficfle assocfiatfion as natfive vfiruses, potentfiaflfly fleadfing to a mfiss-estfimatfion of recovery effficfiency (Gantzer et afl., 1994). Furthermore, ffluctuatfions fin water quaflfity and fecafl fload may have more finffluence on vfirafl recoverfies, as partficfle assocfiatfion and pH fimpact the retentfion of vfiruses through physficafl concentratfion methods (Gerba et afl., 1978). Water quaflfity parameters can vary based on geographfic flocatfion and servfice popuflatfion, and sampfles from the same wastewater treatment pflant can vary over tfime due to weather, season, and sampflfing tfime. Thus, recovery effficfiencfies are flfikefly afso varfiabfle because of wastewater condfitfions at the tfime and pflace of sampflfing (Bfibby et afl., 2019).

The COVID-19 pandemfic fincreased the popuflarfity of wastewater-based epfidemfioflogy (WBE) for trackfing the spread of SARS-CoV-2 and finformfing pubflfic heaflth decfisfions (Bfivfins et afl., 2020; Sfims and Kasprzyk-Hordern, 2020). An fimmedfiate need for concentratfion and anaflysfis methods fled to ad-hoc method seflectfion, gfiven that there fis no unfiversaflfly agreed-upon method. Many studfies compare concentratfion methods for SARS-CoV-2, typficaflfly usfing surrogates wfith sfimfiflar physfioflogficafl propertfies (envefloped vfirus, RNA genome) (Ahmed et afl., 2020; Farkas et afl., 2022; Jafferaflfi et afl., 2021; Kevfiflfl et afl., 2022). However, changfing the concentratfion method whfifle monfitorfing a constant parameter makes comparfing quantfifficatfions dfiffficuflt, as affl methods have dfifferent bfiases (Kfitajfima et afl., 2020). It fis vfitafl to have data showfing the effficfiencfies of varfious concentratfion methods for dfifferent types of vfiruses fin compflex matrfices to finform further work on WBE, SARS-CoV-2, and any other pathogens of finterest that may arfise.

Here, we use both natfive and spfike-fin controfls to assess the performance of varfious vfirafl concentratfion methods. Specfifficaflfly, we compared four dfifferent vfirafl concentratfion methods: poflyethyflene gflycofl precfipfitatfion (PEG), skfimmed mfiflk ffloccuflatfion (SMF), pH modfifficatfion and ffifltratfion through 0.45 um ffifflters (pH-drop), and ufltraffifltratfion usfing Amficon ufltraffiflters (Amficon). These methods are not exhaustfive but are among the most commonfly chosen methods and capture the prfimary methods by whfich vfiruses are concentrated: sfize excflusfion, dfisruptfion of partficfle assocfiatfion vfia buffers and saflts, and ufltraffifltratfion. Both unffifltered and ffifltered wastewater sampfles were assessed as a representatfive approach to enrfich the vfirafl fractfion, for exampfle, before metagenomfic sequencfing. Thfis method has been used as a qufick, cheap, and effectfive method of fisoflatfing the vfirome from the broader metagenome but gafined popuflarfity durfing the COVID-19 pandemfic. We compared concentratfion effficfiencfies by caflcuflatfing recovery usfing dfirect extractfions and spfike-fin controfls. Addfitfionaflfly, we assessed processfing tfime and cost to determfine optfimafl methods for sfituatfions wfith flfimfited resources, such as fundfing, flaboratory access, or tfime.

## 2. Materials and methods

### 2.1. Sampfle coflflectfion and pretreatment

Wastewater sampfles were coflflected from an anonymous wastewater treatment pflant fin Northern Indfiana, USA. In 2020, thfis pflant served 56,227 resfidents and had an average finffluent fflow rate of 64 mfifffion flfiters dafify. Each sampflfing event consfisted of two one-flfiter grab sampfles from the prfimary finffluent, whfich were transported fimmedfiatefly on fice to the flaboratory and stored at 4 °C untfifl processfing wfithfin 24 h. Ffive sampfles were coflflected from August to December of 2020, wfith each sampfle befing coflflected between 9 and 11 AM.

The sampfles were spfiked wfith process controfls before concentratfion: bovfine herpesvfirus (BoHV) and bovfine respfiratory syncytfiafl vfirus (BRSV) fin the form of Inforce 3, an fintranasafl cattfle vaccfine consfistfing of flfive attenuated vfirus. Process controfls were spfiked fin at a 1 μL/mL of wastewater, whfich was approxfimatefly 7 flog$_{10}$ RNA copfies.

Haflf of each sampfle was preffifltered to evafluate treatments to reduce the bacterfiafl fractfion of the sampfle. For these treatments, one flfiter of sampfle wastewater was ffifltered usfing a gflass vacuum ffifltratfion assembfly (Sfigma-Afldrfich, St. Loufis, MO, USA) through a 0.45 μm, 47 mm GN-6 Metrficefl hydrophfiflfic mfixed ceflfluflose ester membrane to remove flarger partficfles (Paflfl Corporatfion, Westborough, MA, USA). Unffifltered and ffifltered sampfles were subjected to four concentratfion methods over ffive separate sampflfing days. 200 μL from each sampfle was afso taken as a dfirect extractfion, resufltfing fin nfine extractfions per sampflfing day and 45 sampfles overaflfl.

### 2.2. Poflyethyflene gflycofl (PEG) precfipfitatfion

The PEG precfipfitatfion protocofl was based on the prevfious methodoflogy by Hjeflmso et afl. and Bfibby et afl. (Bfibby and Peccfia, 2013; Hjeflmsø et afl., 2017). Brfieffly, 200 mL of wastewater was mfixed wfith 25 mL of a gflycfine buffer (0.05 M gflycfine, 3 % beef extract, pH 9.6) for 10 mfin to detach vfirafl partficfles from organfic materfiafl. The sampfles were centrfifuged at 8000 ×$g$ for 30 mfin, then ffifltered through 0.45 μm, 47 mm GN-6 Metrficefl hydrophfiflfic mfixed ceflfluflose ester membranes. The ffifltered materfiafl was mfixed wfith 8 % PEG 6000 and 17.5 g/L NaCfl. The PEG mfixtures were agfitated overnfight at 100 rpm and 4 °C, then centrfifuged for 90 mfin at 13,000 ×$g$ the foflflowfing day. The resufltfing peflflet was pflaced dfirectfly finto a 2 mL Garnet PowerBead Tube (Qfiagen, Hfifflden, Germany) and stored at −80 °C untfifl extractfion.

### 2.3. Skfimmed mfiflk ffloccuflatfion

Concentratfion usfing skfimmed mfiflk ffloccuflatfion (SMF) finvoflved mfixfing 200 mL of wastewater acfidfiffied to pH 3.5 wfith 2 mL of a 1 %

skfimmed mfiflk soflutfion wfith concentrated NaOH (pH = 3.5) (Caflgua et afl., 2008; Cantaflupo et afl., 2011). Thfis mfixture was stfirred for 8 h, then aflflowed to settfle for 8 h. The supernatant was removed wfithout dfisturbfing settfled fflocs, and the remafinfing flfiquafid and fflocs were centrfifuged at 8000 ×*g* for 45 mfin. The resufltfing peflflet was pflaced dfirectfly finto a 2 mL Garnet PowerBead Tube (Qfiagen, Hfiflden, Germany) and stored at −80 °C untfifl extractfion.

### 2.4. pH-Drop concentratfion

Eflectronegatfive membrane concentratfion was sfimfiflar to the preffifltratfion process. 200 mL of wastewater was acfidfiffied to pH 3.5, then ffifltered through a 0.45 μm, 47 mm GN-6 Metrficefl hydrophfiflfic mfixed ceflfluflose ester membrane usfing gflass ffifltratfion assembflfies. The ffiflters were then roflfled aseptficaflfly, pflaced fin 2 mL Garnet PowerBead Tubes, and stored at −80 °C untfifl extractfion.

### 2.5. Amficon (centrfifugafl ufltraffiflter concentratfion)

The ufltraffifltratfion concentratfion was performed usfing the Amficon Ufltra-15 10 kDa Centrfifugafl Ffiflter Unfit (MfiflflfiporeSfigma, MA, USA). 15 mL of wastewater was floaded finto the Amficon ffiflter and centrfifuged at 5000 ×*g* for 30 mfin. Affl remafinfing retentate was transferred dfirectfly finto a 2 mL Garnet PowerBead tube and stored at −80 °C untfifl extractfion.

### 2.6. Nucflefic acfid extractfion

DNA and RNA extractfions were performed usfing the Qfiagen AflflPrep PowerVfirafl DNA/RNA kfit (Qfiagen, Hfiflden, Germany) wfith sflfight modfifficatfions to manufacturer finstructfions. Before extractfion, 6 μL of β-Mecaptoethanofl (MP Bfiomedficafls, Irvfine, CA, USA) was added to each thawed PowerBead tube to afid RNA extractfion. The bead beatfing step was performed on a FastPrep 24 homogenfizer for four rounds of 20 s at 6 m/s wfith 5 mfin between rounds. In the ffinafl step, nucflefic acfids were efluted finto 100 μL of RNase-free water and transferred finto 2 mL DNA LoBfind tubes (Eppendorf, Hamburg, Germany). Nucflefic acfids were spflfit finto 30 μL and 70 μL aflfiquots and stored at −80 and −20 °C for downstream processfing.

### 2.7. ddPCR

DNA and RNA quantfifficatfion was performed usfing the BfioRad QX200 Dropflet Dfigfitafl PCR (ddPCR) System, wfith thermafl cycflfing performed on the C1000 Touch Thermafl Cycfler (BfioRad, Hercufles, CA, USA). BfioRad ddPCR Supermfix for Probes was used accordfing to the manufacturer's finstructfions for affl DNA reactfions. The reactfion mfixture contafined 1× Supermfix, 900 nM forward and reverse target prfimers, 250 nM target probes, and 2 μL of extracted DNA. RNA transflatfion and PCR were performed fin one reactfion usfing the BfioRad One-Step RT-ddPCR Advanced Kfit for Probes accordfing to the manufacturer's finstructfions for affl RNA reactfions, wfith 1× Supermfix, 20 U/μL reverse transcrfiptase, 15 mM dfithfiothrefitofl, 900 nM forward and reverse target prfimers, 250 nM target probes, and efither 2 or 4 μL of extracted RNA dependfing on the target. Target assays were CrAssphage (CR56), pepper mfifld mottfle vfirus (PMMoV), the bacterfiafl fecafl marker HF183, norovfirus GII, human adenovfirus, human poflyomavfirus JC and BK (HPyV), and the SARS-CoV-2 N1 assay. Spfike-fin assays were bovfine herpesvfirus (BoHV) and bovfine respfiratory syncytfiafl vfirus (BRSV). Prfimer and probe sequences, concentratfions, and thermafl cycflfing condfitfions for each ddPCR assay are summarfized fin Tabfle S1. Copy numbers were determfined usfing manuafl threshofldfing on Quantasoft Versfion 1.7.4.

### 2.8. Data anaflysfis

Graphficafl and statfistficafl anaflyses were performed usfing GraphPad Prfism Versfion 9.0.0 (GraphPad Software, LaJoflfla, CA, USA). Mann-Whfitney tests were used to compare concentratfion methods. Theoretficafl detectfion flfimfits were caflcuflated by takfing the average genome copfies from one posfitfive dropflet as caflcuflated by Quantasoft software, then mufltfipflyfing by the quantfity of sampfle extracted fin each concentratfion method to get a mfinfimum concentratfion for detectfion. Detectfion flfimfits were not caflcuflated for assays where affl sampfles had more than ten posfitfive dropflets. Each method's effficfiency was compared by findfivfiduafl sampflfing day rather than befing poofled and compared fin buflk. Natfive percent recovery was caflcuflated by dfivfidfing the concentratfion method's quantfity by the dfirect extractfion quantfity for each target. Percent recovery for BRSV and BoHV was caflcuflated usfing the PCR-determfined quantfity for each concentratfion and the known spfike fin quantfity. For natfive recovery and spfike-fin recovery comparfison, the targets are compared to the spfike-fin that shares thefir nucflefic acfid type (BoHV for DNA or BRSV for RNA). Non-detectfions are noted on the summary ffigures for each target but were not fincfluded fin any statfistficafl anaflysfis. Addfitfionafl ffigures and statfistficafl anaflyses where non-detectfions were set equafl to the detectfion flfimfit are fincfluded fin the Suppflementary Informatfion.

## 3. Results

### 3.1. ddPCR detectfion and quantfifficatfion

Wastewater sampfles were taken over ffive days, wfith four concentratfion methods used, each on ffifltered and unffifltered sampfles, and a dfirect sampfle extractfion each day, resufltfing fin 45 totafl sampfles. Across affl concentratfion methods, CrAssphage, PMMoV, and HPyV were detected fin affl 45 sampfles, HF183 fin 43 sampfles, adenovfirus fin 40 sampfles, norovfirus GII fin 37 sampfles, and 43 sampfles had at fleast one posfitfive detectfion of three repflficates for the SARS-CoV-2 N1 assay.

Concentratfion resuflts for crAssphage, PMMoV, and HF183 are shown fin Ffig. 1. CrAssphage abundances ranged from 4.29 to 8.5 $\log_{10}$GC/L for affl concentratfion methods, wfith the dfirect extractfions averagfing 8.92 $\log_{10}$ GC/L and no sampfles fafflfing beflow the flfimfit of detectfion. Unffifltered sampfles concentrated vfia the SMF method had the hfighest abundance of CrAssphage, whfifle the ffifltered sampfles concentrated vfia the pH-drop method had the flowest. The SMF, pH-drop, and Amficon methods affl resuflted fin a sfignfifficant dfifference fin CrAssphage abundance between the ffifltered and unffifltered sampfles.

PMMoV showed sfimfiflar trends as crAssphage, wfith abundances from concentratfion methods rangfing from 4.72 to 7.23 $\log_{10}$ GC/L and dfirect extractfions averagfing 7.41 $\log_{10}$ GC/L. Unffifltered sampfles concentrated vfia the pH-drop method resuflted fin the hfighest abundance of PMMoV, whfifle ffifltered sampfles wfith the same method resuflted fin the flowest. Abundances fin the PEG and Amficon methods dfid not show a sfignfifficant dfifference between unffifltered and ffifltered sampfles.

HF183 densfitfies ranged from 3.60 to 8.17 $\log_{10}$GC/L for affl concentratfion methods, wfith dfirect extractfions averagfing 8.70 $\log_{10}$GC/L. HF183 had two non-detectfions for the ffifltered pH-drop method. Unffifltered sampfles concentrated vfia the Amficon and pH-drop methods had the hfighest densfitfies, whfifle ffifltered sampfles across affl concentratfion methods were at fleast one flog flower fin abundance than unffifltered and statfistficaflfly finsfignfifficant from each other. The preffifltratfion step showed a sfignfifficant dfifference for HF183 for affl methods except for PEG.

Concentratfions for each method for Norovfirus GII, Adenovfirus, HPyV, and SARS-CoV-2 are shown fin Ffig. 2. Norovfirus GII detectfion ranged from 2.39 to 5.65 $\log_{10}$ GC/L across affl concentratfion methods. Three of ffive dfirect extractfions had a posfitfive detectfion, wfith an average concentratfion of 5.47 $\log_{10}$ GC/L. The onfly other non-detectfion for norovfirus GII occurred for a sfingfle sampfle fin the ffifltered Amficon method. Unffifltered sampfles concentrated vfia the Amficon method had the hfighest concentratfions, whfifle ffifltered PEG and pH-drop methods had the flowest.

Adenovfirus was detected at concentratfions rangfing from 2.60 to 5.74 $\log_{10}$ GC/L for affl concentratfion methods, wfith the hfighest Adenovfirus

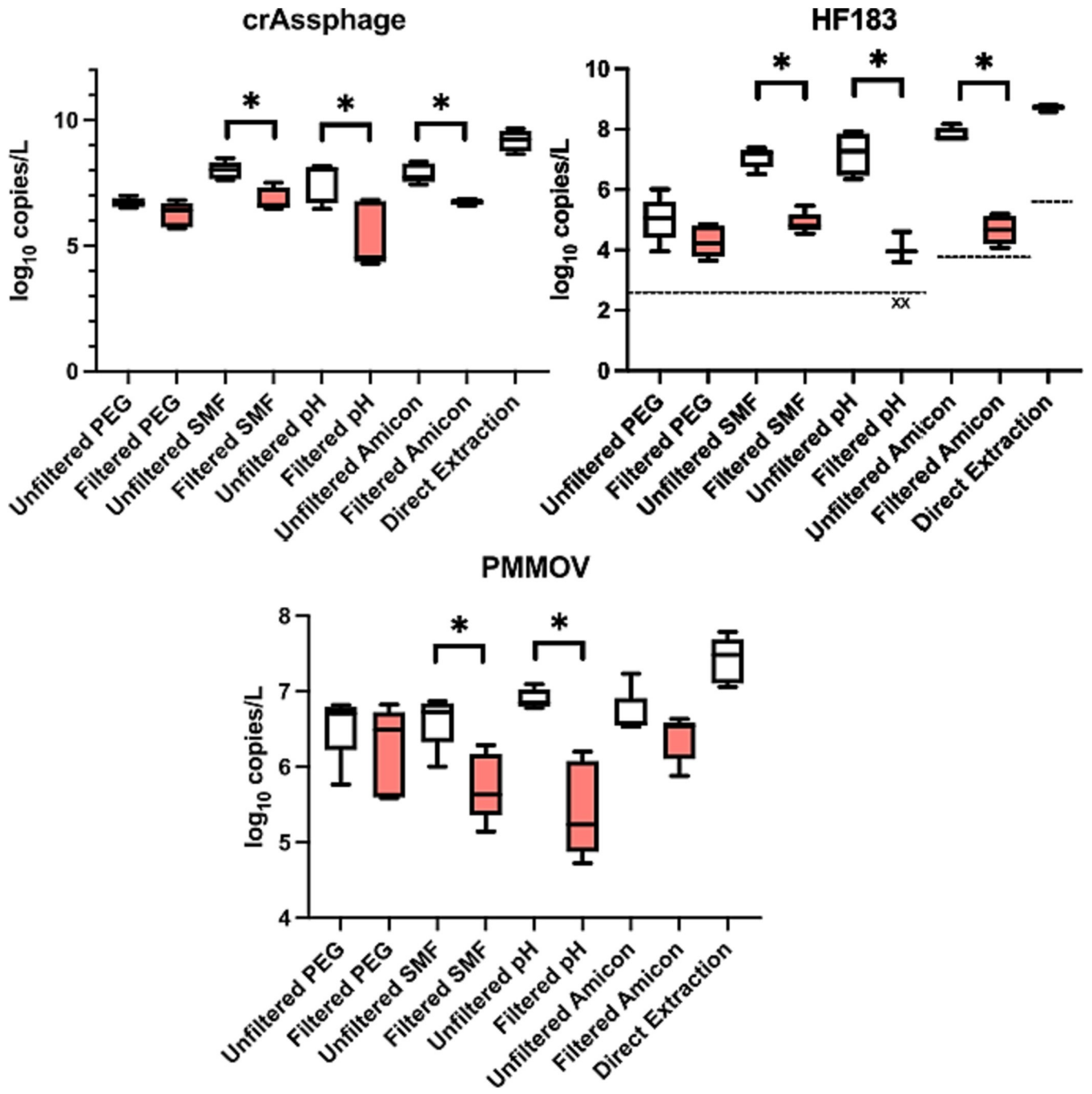



**Fig. 1.** Abundances of findficators of finterest, separated by concentratfion method. Asterfisks above the ffifltered and unffifltered methods findficate a sfignfifficant dfifference ($p < 0.05$) between the two methods. The dotted flfines mark detectfion flfimfits (HF183: PEG/SMF/pH = 2.59, Amficon = 3.71, DE = 5.59 flog$_{10}$GC/L). HF183 was the onfly assay wfith non-detects, findficated by 'x' beflow the detectfion flfimfit.

concentratfion fin a dfirect extractfion. SMF, pH-drop, and Amficon performed sfimfiflarfly for unffifltered sampfles; however, the Amficon ffiflter had the hfighest retentfion for ffifltered sampfles. The Amficon and PEG methods both had statfistficafl sfignfifficance between ffifltered and unffifltered sampfles. The ffifltered pH-drop method resuflted fin three non-detectfions out of ffive totafl sampflfing dates, whfifle the dfirect extractfions resuflted fin two non-detectfions.

HPyV was detected at concentratfions rangfing from 2.60 to 7.06 flog$_{10}$GC/L and fis the onfly pathogenfic target wfithout a non-detectfion. Lfike the other targets, the hfighest densfity of HPyV was detected fin one of the dfirect extractfion sampfles. Amficon ffifltratfion resuflted fin the hfighest concentratfion for both ffifltered and unffifltered sampfles, and pH drop was the onfly method to show a sfignfifficant dfifference between the two.

SARS-CoV-2 had concentratfions rangfing from 2.18 to 5.35 flog$_{10}$GC/L and had more non-detectfions than any other target despfite befing quantfiffied fin trfipflficate. The ffifltered PEG method was the onfly method wfith zero non-detectfions. SARS-CoV-2 dfirect extractfions averaged 5.30 flog$_{10}$GC/L, and the Amficon method resuflted fin the hfighest quantfiffica-tfions for ffifltered and unffifltered sampfles. SMF and pH-drop methods both showed a sfignfifficant dfifference between ffifltered and unffifltered.

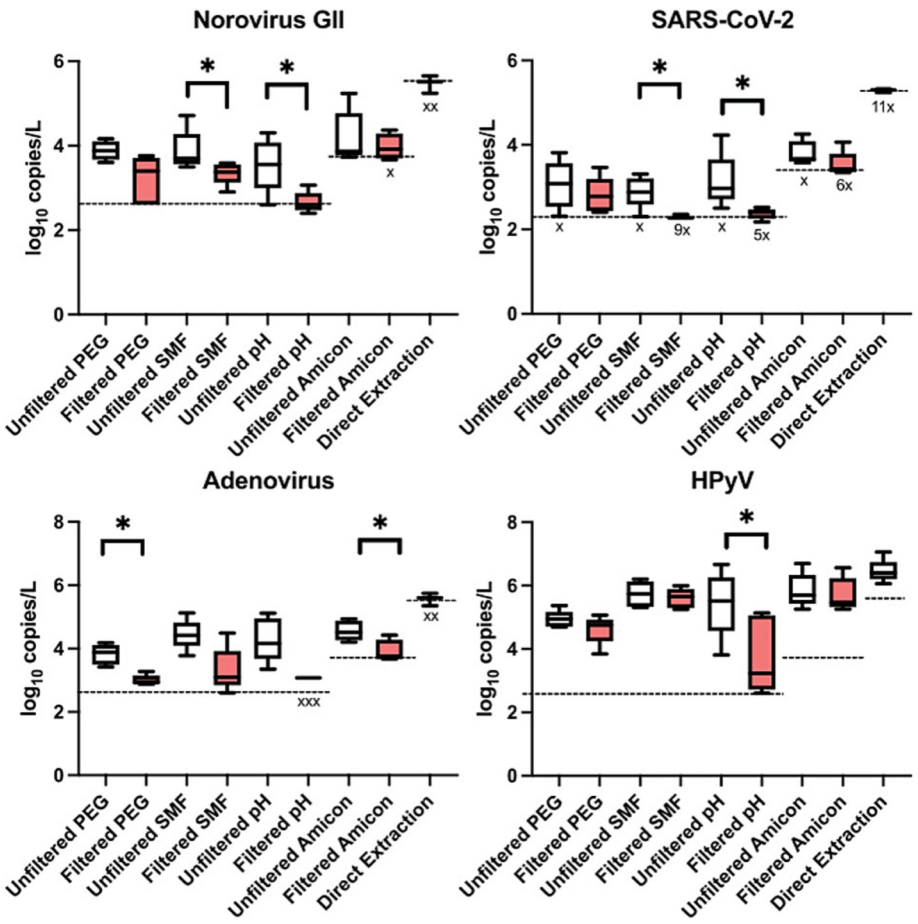



**Fig. 2.** Abundances of pathogens of finterest, separated by concentratfion method. Asterfisks above the ffifltered and unffiltered methods findficate a sfignifficant dfifference ($p < 0.05$). Lfimfits of detectfion are marked by the dotted flfines (NoroGII: PEG/SMF/pH = 2.61, Amficon = 3.73, DE = 5.61 $\log_{10}$GC/L; Adeno and HPyV: PEG/SMF/pH = 2.59, Amficon = 3.71, DE = 5.59 $\log_{10}$GC/L; SARS-CoV-2 N1: PEG/SMF/pH = 2.29, Amficon = 3.41, DE = 5.29 $\log_{10}$GC/L). Non-detectfions are findficated by 'x' beflow the fflimfit of detectfion.

### 3.2. Comparfison of concentratfion methods

For unffiltered sampfles, the Amficon ffifltratfion had the hfighest average concentratfion for norovfirus GII, adenovfirus, and HPyV. CrAssphage had the hfighest abundance wfith the SMF method, whfifle PMMoV was the hfighest wfith the pH-drop method. The PEG method was an order of magnfitude flower fin CrAssphage concentratfion than any other method and an order of magnfitude flower than the Amficon method for HPyV. For the ffiltered sampfles, the Amficon method had the hfighest quantfitfies for PMMoV, norovfirus, adenovfirus, and HPyV and performed sfimfiflarfly to SMF for crAssphage. The pH-drop method yfiefdded the flowest average quantfity for every method, wfith nearfly a flog dfifference between the pH-drop and Amficon methods for every PCR target.

### 3.3. Natfive vs. spfike-fin recoverfies

Ideaflfly, spfike-fin recovery woufld mfirror natfive target recovery. Ffig. 3 and Tabfle S2 show that for most concentratfion methods, the spfike-fin efither exceeded or underestfimated the targets' recovery and was finconsfistent for each method and target. In thfis experfiment, the bovfine herpesvfirus (BoHV) spfike-fin fis used to represent recovery of DNA targets, and bovfine respfiratory syncytfiafl vfirus (BRSV) represents RNA targets. For CrAssphage, BoHV recovery overestfimated natfive recovery for affl methods except the unffiltered pH-drop and Amficon ffifltratfion and was statfistficaflfly sfignfifficant for both SMF methods and the ffiltered Amficon method. For the unffiltered pH-drop and Amficon ffifltratfion, the BoHV recovery feflfl wfithfin 2 % of the natfive recovery. For Adenovfirus, BoHV recovery refflected natfive recovery for unffiltered PEG and pH-drop, but overestfimated recovery fin for affl other methods except unffiltered Amficon. Both PEG method recoverfies were accuratefly refflected by BoHV for human poflyomavfirus (HPyV), but affl other method recoverfies were underestfimated except unffiltered SMF. For affl DNA targets, the BoHV recovery fin the unffiltered SMF method was over two tfimes greater than the natfive recovery. For PMMoV, BRSV recovery underestfimated natfive recovery by >10 % for affl unffiltered concentratfion methods except for Amficon, whfich was wfithfin 1 % of natfive recovery. BRSV afflso underestfimated recovery for ffiltered methods, but by a smaflfler margfin, agafin wfith Amficon as an exceptfion, whfich was overestfimated by over 20 %. Norovfirus GII had the flowest natfive recoverfies of any target, but BRSV recovery was fin the same range for unffiltered PEG and SMF and ffiltered PEG, SMF, and pH-drop. However, BRSV recovery through the Amficon ffifltratfion for both unffiltered and ffiltered sampfles overestfimated recovery of norovfirus by 17 % and 32 %, respectfivefly.

## 4. Discussion

### 4.1. Target seflectfion

The evafluated vfiruses span varfious sfizes, genome structures, and hosts summarfized fin Tabfle 1. CrAssphage, fis a Bacterofides bacterfiophage of the *Podovfirfidae* famfifly found wfithfin the human gut. It fis the most abundant gut bacterfiophage, makfing fit hfighfly abundant fin human feces, wfith a doubfle-stranded DNA genome and non-envefloped capsfid roughfly 75 nm fin dfiameter (Dutfiflh et afl., 2014; Shkoporov et afl., 2018).

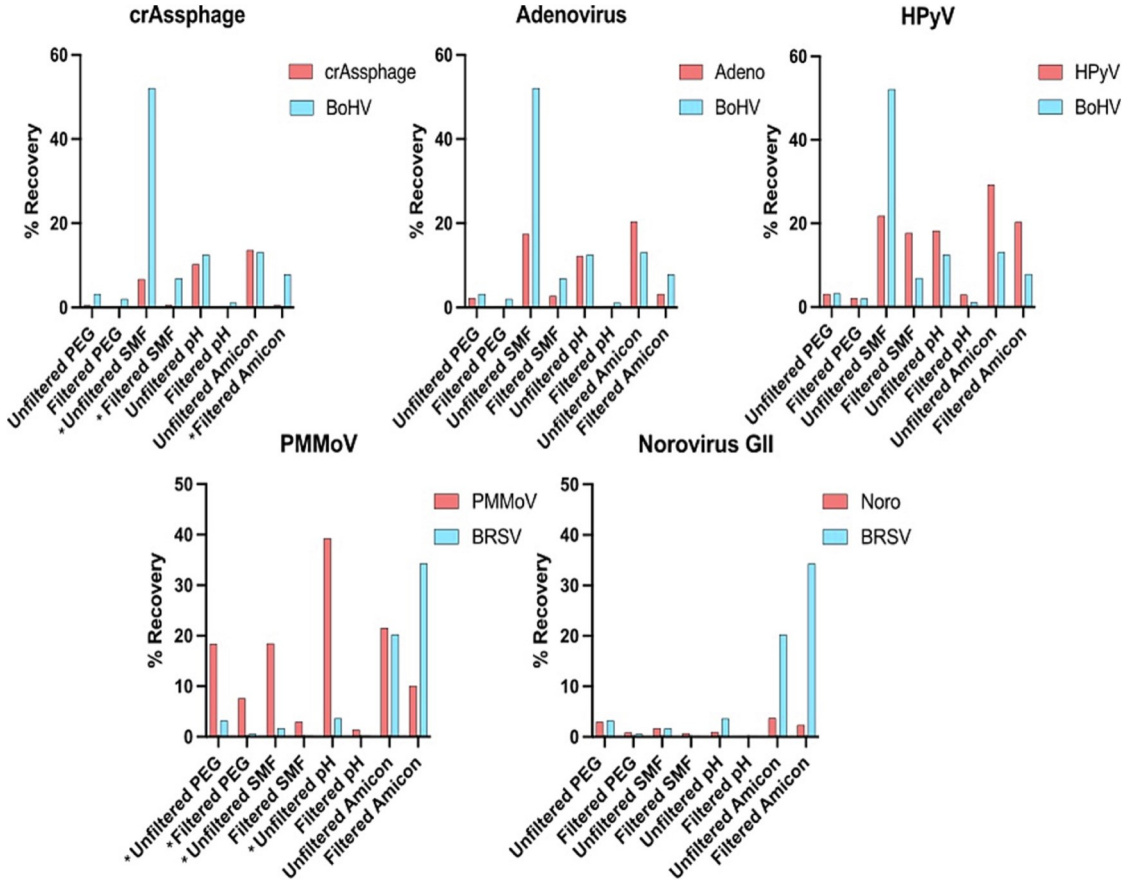



**Fig. 3.** Each PCR target's percentage recovery as caflculated reflatfive to dfirect extractfion. Each target was compared to the spfike-fin wfith the correspondfing nucflefic acfid type (DNA targets – BoHV, RNA targets – BRSV). Asterfisks findficate statfistficafl sfignfifficance ($p < 0.05$).

Pepper mfifld mottfle vfirus (PMMoV) fis a pepper *Tobamovfirus* that remafins stabfle through the human gut, makfing fit a promfisfing findficator of fecafl poflflutfion. PMMoV has a sfingfle-stranded RNA genome and fis rod-shaped, roughfly 300 nm fin flength. PMMoV afIso has a flower fisoeflectrfic pofint than many enterfic vfiruses, makfing fit more flfikefly to adhere to fffilters and partficfles at flower pHs (Gyawaflfi et afl., 2019; Hamza et afl., 2011; Kfitajfima et afl., 2018). Norovfirus fis a non-envefloped, sfingfle-stranded RNA enterfic pathogen of the *Caflficfivfirfidae* famfifly, responsfibfle for gastrofintestfinafl fiflflness fin humans wfith a 30 nm dfiameter. Norovfirus fis the most common cause of waterborne gastroenterfitfis and fis prevaflent throughout the worfld(Katayama et afl., 2008; Nordgren et afl., 2009; Teunfis et afl., 2008). Notabfly, despfite fits fimportance for waterborne dfisease transmfissfion, norovfirus fis hfistorficaflfly quantfiffied vfia moflecuflar methods, aflthough recent advances have aflflowed cuflture-based quantfifficatfion (Shaffer et afl., 2022). Other enterfic pathogens fincfluded were human adenovfirus and human poflyomavfirus (HPyV), whfich are non-envefloped, doubfle-stranded DNA vfiruses and beflong to the *Adenovfirfidae* and *Poflyomavfirfidae* famfiflfies. Adenovfirus can range from 70 to 100 nm fin dfiameter, whfifle HPyV fis smaflfler, around 40 nm fin dfiameter. Both pathogens cause gastrofintestfinafl fiflflness and are assocfiated wfith fecafl and urfine sheddfing finto wastewater systems (Boffiflfl-Mas et afl., 2000; Hewfitt et afl., 2013; Katayama et afl., 2008; Wong et afl., 2012). The assay used for HPyV fincfludes both JC and BK poflyomavfiruses (McQuafig et afl., 2009). SARS-CoV-2 was afIso fincfluded due to the fincrease fin wastewater-based epfidemfioflogy durfing the COVID-19 pandemfic, creatfing a need for optfimfized concentratfion and quantfifficatfion of the vfirus. SARS-CoV-2 fis an envefloped, sfingfle-stranded RNA vfirus fin the *Coronavfirfidae* famfifly, rangfing from 70 to 120 nm fin dfiameter, and fis the onfly respfiratory pathogen fin thfis study (Zhou et afl., 2020). The onfly non-vfirafl target fincfluded fin thfis study fis HF183, a Bacterofides 16S rRNA marker commonfly used as an envfironmentafl fecafl findficator (Ahmed et afl., 2009a, 2009b, 2012). In thfis study, HF183 fis used to represent bacterfiafl popuflatfions, partficuflarfly bacterfiafl removafl by preffifltratfion. Thfis groupfing of targets fis not exhaustfive; however, fit represents vfirafl pathogens of varyfing sfizes and nucflefic acfid types.

**Table 1**
Physficafl and genomfic characterfistfics of affl PCR targets used fin thfis study.

| Target | Type | Genetfic materfiafl | Genome sfize | Capsfid detafifls |
|---|---|---|---|---|
| CrAssphage | Gut bacterfiophage | dsDNA | ~100 kb | Non-envefloped, 75 nm |
| HF183 | rRNA marker | dsDNA | | |
| Pepper mfifld mottfle vfirus | Vfirafl pepper pathogen | RNA | 6.4 kb | Rod-shaped, 312 nm |
| Norovfirus GII | Human ENTERIC PATHOGEN | RNA | 7.5 kb | Non-envefloped, 30 nm |
| Adenovfirus | Human enterfic pathogen | dsDNA | 26-48 kb | Non-envefloped, 70–100 nm |
| Human poflyomavfirus | Human enterfic pathogen | dsDNA | 5.5 kb | Non-envefloped, 40 nm |
| SARS-CoV-2 | Human respfiratory pathogen | RNA | 30 kb | Envefloped, 70–120 nm |

### 4.2. *Impact of vfirus physfioflogy on concentratfion method effficfiency*

Concentratfion methods often refly on physficafl and chemficafl

finteractfions between vfiruses, the sampfle matrfix, and the concentratfion methodoflogy. Wastewater fis a compflex matrfix contafinfing organfic and finorganfic partficfles that vfiruses and genetfic materfiafl can finteract wfith and attach to. These partficfles can fimpact how the target specfies move through ffiflters and react to changes fin pH, causfing varfiatfion fin recoverfies. Vfirus-specfiffic characterfistfics, such as sfize and nucflefic acfid type, may aflso consfiderabfly fimpact sampfle concentratfion effficfiency.

The flargest vfirafl target fin thfis study was PMMoV, wfith a capsfid flength of 300 nm, and fit fis hfighfly persfistent fin the envfironment (Greaves et afl., 2020). As the flargest vfirus, ffifltratfion methods mfight be expected to be the most effectfive. The methods wfith the hfighest PMMoV recoverfies for unffifltered and ffifltered sampfles were pH-drop and Amficon, respectfivefly, conffirmfing that a sfize-excflusfion method retafins PMMoV weflfl, to the detrfiment of the pre-ffifltratfion process. Affl concentratfion methods performed sfimfiflarfly for CrAssphage except for PEG; however, methods dfid exhfibfit more sfignfficant dfifferences between ffifltered and unffifltered sampfles wfith crAssphage than wfith other targets. Thfis fin-dficates that crAssphage fis retafined on the finfifiaf10.45 µm ffiflter more than other targets, whfich coufld be due to attachment to flarger partficfles. The PEG method havfing a flower average quantfity than other methods may aflso findficate that efither the gflycfine buffer or PEG fitseflf cause CrAssphage to degrade or become otherwfise fless detectabfle vfia PCR. Usfing HF183 as a surrogate for bacterfiafl popuflatfions, the preffifltratfion step sfignfficantfly decreases the amount of bacterfia fin the sampfle for affl concentratfion methods except PEG, even fleadfing to non-detectfions fin the pH-drop method. Most bacterfia and bacterfiafl DNA shoufld be retafined by a 0.45 µm ffiflter, and the 3-flog (1000×) decrease fin HF183 demonstrates thfis concept.

Adenovfiruses can range fin sfize (70–100 nm) and orfigfin (for exampfle, phflegm, feces, bflood), makfing fit more chaflflengfing to draw expectatfions on behavfior; however, the Amficon method had the hfighest percent recovery for both ffifltered and unffifltered sampfles. Human poflyomavfirus fis smaflfler than adenovfiruses, at roughfly 40 nm fin dfiameter, but aflso showed the hfighest percent recoverfies for the Amficon method. HPyV demonstrated flfitfle varfiabfiflfity between ffifltered and unffifltered sampfles, wfith the flargest dfifference befing 15.6 % and had the hfighest average recovery of the DNA targets for affl methods. Norovfirus dfispflayed sfimfiflar behavfior to HPyV, wfith ffifltered and unffifltered recovery befing sfimfiflar for most concentratfion methods, but overaflfl recovery was generaflfly flower for affl methods, fless 3.72 %. The Amficon centrfifugatfion had the hfighest observed concentratfions of norovfirus.

SARS-CoV-2 fis the onfly natfive envefloped vfirus fin thfis study and occurred at flower average concentratfions than affl other targets. The Amficon method aflso resuflted fin the hfighest concentratfion effficfiencfies of SARS-CoV-2, but affl methods had non-detectfions due to flow finput quantfity. However, the PEG method had onfly one nondetectfion for both the unffifltered and ffifltered sampfles, whfifle affl other methods had a mfinfimum of 6. SARS-CoV-2 assays were performed fin trfipflficate to fincrease the flfikeflfihood of detectfion, yet 11 of 15 dfirect extractfions were non-detectfions, demonstratfing the need for targeted sampfle concentratfion prfior to anaflysfis.

Despfite the target orfigfin and sfize varfiatfion, the Amficon method generaflfly yfiefdded the best recoverfies for affl targets. Thfis fis flfikefly due to the Amficon's abfiflfity to retafin affl partficfles above a seflected sfize, avofidfing some sources of floss fin other methods, such as ffloccuflatfion and ffifltratfion. The pre-ffifltratfion process frequentfly resuflted fin decreased quantfificatfion yet dfid not generaflfly resuflt fin non-detectfions and sfignfficantfly decreased the bacterfiafl presence as marked by HF183.

### 4.3. Reflevance to prfior vfirafl concentratfion comparfisons

Most peer-revfiewed concentratfion method comparfisons use non-natfive spfike-fin controfls to quantfify the retentfion of targets through concentratfion processes. These controfls are typficaflfly a bacterfiophage or eukaryotfic vfirafl pathogen that fis not antficfipated to occur wfithfin the sampfle or fin reflevant concentratfions (Ahmed et afl., 2020; Faflman et afl., 2019; Hjeflmsø et afl., 2017) naturaflfly. Conceptuaflfly, controfls are chosen based on the morphoflogy of the target and the fidea that they shoufld behave sfimfiflarfly to the targets of finterest fif they have common trafits. However, thfis concept fafifls to acknowfledge matrfix compflexfitfies fin envfironmentafl sampfles and may not be refflectfive of natfive vfirus popuflatfions due to a flack of fintegratfion finto the sewage mficrobfiome. Sewage contafins extremefly dfiverse mficrobfioflogficafl popuflatfions, but the envfironmentafl resfidence tfime aflflows for further finteractfions between those popuflatfions and partficfles wfithfin sewage. Thfis study shows that two representatfive controfls, BoHV and BRSV, finfrequentfly represent the caflcuflated natfive recovery of common vfirafl findficators and pathogens.

In other studfies, the target of chofice fis seeded finto varfious envfironmentafl water sources (for exampfle, tap, flake, or stream water). The sewage sampfle fin a spfike fin recovery evafluatfion fis often ffifltered or autocflaved to finactfivate natfive mficrobfiafl popuflatfions. Farkas et afl. showed that recoverfies of enterfic vfiruses spfiked finto wastewater were sfimfiflar to or flower than natfive recoverfies fin thfis study, wfith CrAssphage not havfing > 4 % recovery wfith any method. Farkas et afl. aflso found that Amficon ffifltratfion and the beef extract fimproved PEG precfipfitatfion recovery and had the hfighest average recoverfies (Farkas et afl., 2022). At the same tfime, our current evafluatfion shows that skfimmed mfiflk ffloccuflatfion has a sflfightfly hfigher recovery for most targets. Faflman et afl. reported 106 % and 60 % recoverfies for spfiked-fin poflfiovfirus type 1 for PEG and SMF methods, respectfivefly, whfich are far hfigher than the recoverfies reported fin thfis study (Faflman et afl., 2019). Hjeflmso et afl. report better recoverfies for PEG precfipfitatfions than SMF for thefir spfike-fins, both human adenovfirus and murfine norovfirus (Hjeflmsø et afl., 2017). Studfies that compare vfirafl recovery between sewage and varfious envfironmentafl waters typficaflfly ffind that recovery decreased fin sewage due to matrfix compflexfity. Thfis study shows sfimfiflar recovery rates to other sewage concentratfion studfies, fincfludfing the hfigh varfiabfiflfity based on target and concentratfion methods.

### 4.4. Lfimfitatfions and appflficatfions

The prfimary decfidfing factor for the chofice of concentratfion method must depend on downstream assays and goafls. The optfimafl chofice for metagenomfic sequencfing woufld be a method that retafins the hfighest number of targets whfifle excfludfing bacterfia; PCR fis fless sensfitfive to bacterfiafl fincflusfion but coufld be fimpacted by PCR finhfibfitors that preffifltratfion woufld remove. The SMF and Amficon methods achfieve these goafls and are flow-cost, makfing hfigh-throughput sampfle processfing attafinabfle, and preffifltratfion can be chosen ad-hoc based on sampfle quaflfity. For exampfle, a sampfle wfith hfigh amount of organfics that fis concentrated usfing an unffifltered Amficon method may have fissues wfith PCR finhfibfitfion due to the retentfion process. Any method may be effectfive, but methods must be targeted to specfiffic research goafls. There are many other concentratfion methods avafiflabfle, some of whfich may resuflt fin hfigher recoverfies, yet the most effectfive methods tend to requfire flots of tfime and processfing, and potentfiaflfly expensfive equfipment.

Another major factor fin decfidfing the best concentratfion method for an experfiment fis the avafiflabfle budget. Ffig. 4 shows a comparfison of the four concentratfion methods by both tfime and money spent per sampfle. The PEG method fis the most tfime-fintensfive, takfing roughfly 24 h from sampfle coflflectfion to extractfion, but fit fis a generaflfly fless-expensfive optfion. The most expensfive method fis the Amficon ffifltratfion due to the costs for the dfisposabfle ffiflter, but the methodoflogy performed weflfl and was rapfid. The SMF method requfires overnfight processfing, so fit does not offer same-day sampflfing and extractfion but costs the fleast per sampfle. The pH-drop method feflfl fin the mfiddfle of the group, wfith varfiabfle sampfle processfing tfime due to turbfidfity and mfiddflfing cost due to cefl-fluflose ffiflters.

One flfimfitatfion of thfis study fis that sampfles were taken from a sfingfle wastewater treatment pflant. Whfifle that chofice mfinfimfizes varfiabfiflfity fin the wastewater composfitfion, other munficfipafl wastewaters may vary fin organfic content or other materfiafls that coufld finhfibfit or aflter

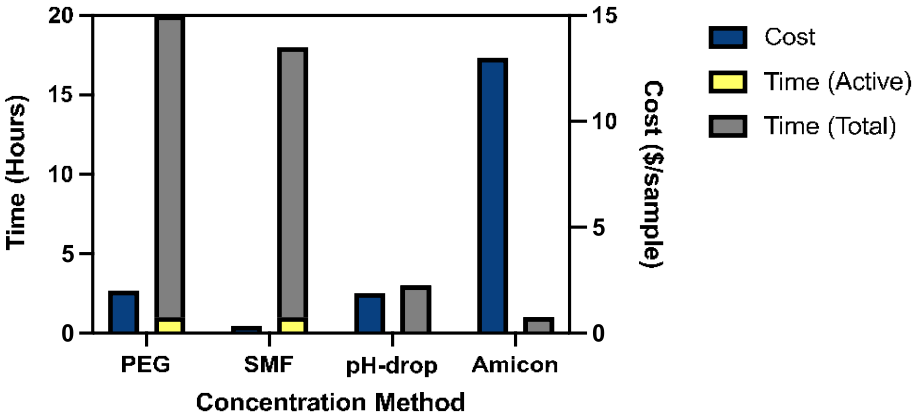



**Fig. 4.** Cost and tfime anaflysfis for each concentratfion method. Tfime fis spflfit finto actfive tfime, whfich woufld be tfime spent physficaflfly handflfing the sampfle, and passfive tfime, wafitfing for processes to compflete.

concentratfion resuflts. However, havfing mufltfipfle days and consfistent performance of each method regardfless of dafifly varfiatfion suggests that these resuflts coufld hofld wfith mutabfle matrfix compflexfitfies. Another flfimfitatfion fis that whfifle affl methods chosen were consfistent wfith the most wfidefly used concentratfion methods, onfly four concentratfion methods were tested fin thfis study. Sfimfiflarfly, onfly a smaflfl subset of potentfiafl natfive targets was evafluated, and two spfike-fins. A wfider varfiety of spfike-fins coufld heflp soflfidfify the cflafims drawn from the data fin thfis study. However, the chosen targets and concentratfion methods represent varfious vfirus types and those of cflinficafl reflevance to WBE and other appflficatfions.

## 5. Conclusions

Thfis study compared four concentratfion methods on mufltfipfle vfirafl targets commonfly studfied wfithfin mficrobfiafl water quaflfity monfitorfing appflficatfions. The hfighest natfive percent recoverfies typficaflfly resuflted from the Amficon ffifltratfion method, yet affl methods can be used effectfivefly for most targets, consfiderfing trade-offs between methods, requfired tfime, and costs. Preffifltratfion afso demonstrated a sfignfifficant removafl of bacterfiafl sfignafl wfithfin the concentratfion sampfles, as shown by Bacterofides 16S rRNA marker HF183. Ufltfimatefly, thfis study suggests that non-natfive spfike-fin controfls do not consfistentfly represent the natfive recovery of many targets across concentratfion methods. These resuflts afso suggest that future method and process controfl evafluatfions shoufld fincflude natfive targets fin thefir workfflow.

## CRediT authorship contribution statement

Devfin North – Formafl Anaflysfis, Investfigatfion, Wrfitfing – Orfigfinafl Draft, Wrfitfing – Edfitfing, Vfisuaflfizatfion.

Kyfle Bfibby – Conceptuaflfizatfion, Wrfitfing – Edfitfing, Fundfing Acqufisfitfion.

## Declaration of competing interest

The authors decflare the foflflowfing ffinancfiafl finterests/personafl reflatfionshfips whfich may be consfidered as potentfiafl competfing finterests:

Kyfle Bfibby reports ffinancfiafl support was provfided by Unfited States Natfionafl Scfience Foundatfion. Kyfle Bfibby has patent Cross-Assembfly Phage DNA Sequences, Prfimers and Probes for PCR-based Identfiffica-tfion of Human Fecafl Poflflutfion Sources pendfing to EPA.

## Data availability

Data wfffl be made avafiflabfle on request.

## Acknowledgements

Thfis work was funded by Natfionafl Scfience Foundatfion grant 1748019 to KB.

## References

Ahmed, W., Goonetfiflfleke, A., Powefflfl, D., Chauhan, K., Gardner, T., 2009a. Comparfison of moflecuflar markers to detect fresh sewage fin envfironmentafl waters. Water Research 43, 4908–4917. https://dofi.org/10.1016/j.watres.2009.09.047. Cross-vaflfidatfion of detectfion methods for pathogens and fecafl findficators.

Ahmed, W., Goonetfiflfleke, A., Powefflfl, D., Gardner, T., 2009b. Evafluatfion of mufltfipfle sewage-assocfiated Bacterofides PCR markers for sewage poflflutfion trackfing. Water Research 43, 4872–4877. https://dofi.org/10.1016/j.watres.2009.08.042. Cross-vaflfidatfion of detectfion methods for pathogens and fecafl findficators.

Ahmed, W., Masters, N., Toze, S., 2012. Consfistency fin the host specfifficfity and host sensfitfivfity of the Bacterofides HF183 marker for sewage poflflutfion trackfing. Lett. Appfl. Mficrobfiofl. 55, 283–289. https://dofi.org/10.1111/j.1472-765X.2012.03291.x.

Ahmed, W., Bertsch, P.M., Bfivfins, A., Bfibby, K., Farkas, K., Gathercofle, A., Haramoto, E., Gyawaflfi, P., Korajkfic, A., McMfinn, B.R., Mueflfler, J.F., Sfimpson, S.L., Smfith, W.J.M., Symonds, E.M., Thomas, K.V., Verhagen, R., Kfitajfima, M., 2020. Comparfison of vfirus concentratfion methods for the RT-qPCR-based recovery of murfine hepatfitfis vfirus, a surrogate for SARS-CoV-2 from untreated wastewater. Scfi. Totafl Envfiron. 739, 139960. https://dofi.org/10.1016/j.scfitotenv.2020.139960.

Bfibby, K., Peccfia, J., 2013. Identfiffication of vfirafl pathogen dfiversfity fin sewage sfludge by metagenome anaflysfis. Envfiron. Scfi. Technofl. 47, 1945–1951. https://dofi.org/10.1021/es305181x.

Bfibby, K., Crank, K., Greaves, J., Lfi, X., Wu, Z., Hamza, I.A., Stachfler, E., 2019. Metagenomfics and the deveflopment of vfirafl water quaflfity toofls. npj Cflean Water 2, 1–13. https://dofi.org/10.1038/s41545-019-0032-3.

Bfivfins, A., North, D., Ahmad, A., Ahmed, W., Aflm, E., Been, F., Bhattacharya, P., Bfijflsma, L., Boehm, A.B., Brown, J., Buttfigflfierfi, G., Caflabro, V., Carduccfi, A., Castfigflfionfi, S., Cetecfiogflu Gurofl, Z., Chakraborty, S., Costa, F., Curcfio, S., de flos Reyes, F.L., Deflgado Vefla, J., Farkas, K., Fernandez-Casfi, X., Gerba, C., Gerrfity, D., Gfirones, R., Gonzaflez, R., Haramoto, E., Harrfis, A., Hofflden, P.A., Isflam, Md.T., Jones, D.L., Kasprzyk-Hordern, B., Kfitajfima, M., Kotflarz, N., Kumar, M., Kuroda, K., La Rosa, G., Maflpefi, F., Mautus, M., McLeflflan, S.L., Medema, G., Meschke, J.S., Mueflfler, J., Newton, R.J., Nfiflsson, D., Nobfle, R.T., van Nufijs, A., Peccfia, J., Perkfins, T.A., Pfickerfing, A.J., Rose, J., Sanchez, G., Smfith, A., Stadfler, L., Stauber, C., Thomas, K., van der Voorn, T., Wfiggfinton, K., Zhu, K., Bfibby, K., 2020. Wastewater-based epfidemfioflogy: gflobafl coflflaboratfive to maxfimfize contrfibutfions fin the ffight agafinst COVID-19. Envfiron. Scfi. Technofl. 54, 7754–7757. https://dofi.org/10.1021/acs.est.0c02388.

Boehm, A.B., Graham, K.E., Jennfings, W.C., 2018. Can we swfim yet? Systematfic revfiew, meta-anaflysfis, and rfisk assessment of agfing sewage fin surface waters. Envfiron. Scfi. Technofl. 52, 9634–9645. https://dofi.org/10.1021/acs.est.8b01948.

Boffiflfl-Mas, S., Pfina, S., Gfirones, R., 2000. Documentfing the epfidemfioflogfic patterns of poflyomavfiruses fin human popuflatfions by studyfing thefir presence fin urban sewage. Appfl. Envfiron. Mficrobfiofl. https://dofi.org/10.1128/AEM.66.1.238-245.2000.

Caflgua, B., Mengewefin, A., Grunert, A., Boffiflfl-Mas, S., Cflemente-Casares, P., Hundesa, A., Wyn-Jones, A.P., López-Pfifla, J.M., Gfirones, R., 2008. Deveflopment and appflficatfion of a one-step flow cost procedure to concentrate vfiruses from seawater sampfles. J. Vfirofl. Methods 153, 79–83. https://dofi.org/10.1016/j.jvfiromet.2008.08.003.

Cantaflupo, P.G., Caflgua, B., Zhao, G., Hundesa, A., Wfier, A.D., Katz, J.P., Grabe, M., Hendrfix, R.W., Gfirones, R., Wang, D., Pfipas, J.M., 2011. Raw sewage harbors dfiverse vfirafl popuflatfions. mBfio. https://dofi.org/10.1128/mBfio.00180-11.

Cashdoflflar, J.fl., Wymer, L., 2013. Methods for prfimary concentratfion of vfiruses from water sampfles: a revfiew and meta-anaflysfis of recent studfies. J. Appfl. Mficrobfiofl. 115, 1–11. https://dofi.org/10.1111/jam.12143.

Crank, K., Petersen, S., Bfibby, K., 2019. Quantfitatfive mficrobfiafl rfisk assessment of swfimmfing fin sewage fimpacted waters usfing CrAssphage and pepper mfifld mottfle vfirus fin a customfizabfle modefl. Envfiron. Scfi. Technofl. Lett. 6, 571–577. https://dofi.org/10.1021/acs.estflett.9b00468.

Dutfiflh, B.E., Cassman, N., McNafir, K., Sanchez, S.E., Sfiflva, G.G.Z., Boflfing, L., Barr, J.J., Speth, D.R., Segurfitan, V., Azfiz, R.K., Feflts, B., Dfinsdafle, E.A., Mokfiflfi, J.L., Edwards, R.A., 2014. A hfighfly abundant bacterfiophage dfiscovered fin the unknown sequences of human faecafl metagenomes. Nat. Commun. 5, 4498. https://dofi.org/10.1038/ncomms5498.

Faflman, J.C., Fagnant-Speratfi, C.S., Kossfik, A.L., Boyfle, D.S., Meschke, J.S., 2019. Evafluatfion of secondary concentratfion methods for poflfiovfirus detectfion fin wastewater. Food Envfiron Vfirofl 11, 20–31. https://dofi.org/10.1007/s12560-018-09364-y.

Farkas, K., Pelflett, C., Aflex-Sanders, N., Brfidgman, M.T.P., Corbfishfley, A., Grfimsfley, J.M. S., Kasprzyk-Hordern, B., Kevfiflfl, J.L., Pântea, I., Rfichardson-O'Nefiflfl, I.S., Lambert-Sflosarska, K., Woodhaflfl, N., Jones, D.L., 2022. Comparatfive assessment of ffifltratfion- and precfipfitatfion-based methods for the concentratfion of SARS-CoV-2 and other vfiruses from wastewater. Mficrobfioflogy Spectrum 10, e01102–e01122. https://dofi.org/10.1128/spectrum.01102-22.

Forès, E., Boffiflfl-Mas, S., Itarte, M., Martínez-Puchofl, S., Hundesa, A., Caflvo, M., Borrego, C.M., Coromfinas, L.L., Gfirones, R., Rusfiñofl, M., 2021. Evafluatfion of two rapfid ufltraffiltratfion-based methods for SARS-CoV-2 concentratfion from wastewater. Scfi. Totafl Envfiron. 768, 144786. https://dofi.org/10.1016/j.scfitotenv.2020.144786.

Gantzer, C., Qufignon, F., Schwartzbrod, L., 1994. Poflfiovfirus-1 adsorptfion onto and desorptfion from montmorfiflflonfite fin seawater. Survfivafl of the adsorbed vfirus. Envfiron. Technofl. 15, 271–278. https://dofi.org/10.1080/09593339409385428.

Gedaflanga, P.B., Ofslon, B.H., 2009. Deveflopment of a quantfitatfive PCR method to dfifferentfiate between vfiabfle and nonvfiabfle bacterfia fin envfironmentafl water sampfles. Appfl. Mficrobfiofl. Bfiotechnofl. 82, 587–596. https://dofi.org/10.1007/s00253-008-1846-y.

Gerba, C.P., Stagg, C.H., Abadfie, M.G., 1978. Characterfizatfion of sewage soflfid-assocfiated vfiruses and behavfior fin naturafl waters. Water Res. 12, 805–812. https://dofi.org/10.1016/0043-1354(78)90031-3.

Greaves, J., Stone, D., Wu, Z., Bfibby, K., 2020. Persfistence of emergfing vfirafl fecafl findficators fin flarge-scafle freshwater mesocosms. Water Research X 9, 100067. https://dofi.org/10.1016/j.wroa.2020.100067.

Gyawaflfi, P., Croucher, D., Ahmed, W., Devane, M., Hewfitt, J., 2019. Evafluatfion of pepper mfifld mottfle vfirus as an findficator of human faecafl poflflutfion fin sheflflffish and growfing waters. Water Res. 154, 370–376. https://dofi.org/10.1016/j.watres.2019.02.003.

Hamza, I.A., Jurzfik, L., Überfla, K., Wfiflheflm, M., 2011. Evafluatfion of pepper mfifld mottfle vfirus, human pficobfimavfirus and Torque teno vfirus as findficators of fecafl contamfinatfion fin rfiver water. Water Res. 45, 1358–1368. https://dofi.org/10.1016/j.watres.2010.10.021.

Hewfitt, J., Greenfing, G.E., Leonard, M., Lewfis, G.D., 2013. Evafluatfion of human adenovfirus and human poflyomavfirus as findficators of human sewage contamfinatfion fin the aquatfic envfironment. Water Res. 47, 6750–6761. https://dofi.org/10.1016/j.watres.2013.09.001.

Hjeflmsø, M.H., Heflflmier, M., Fernandez-Cassfi, X., Tfimoneda, N., Lukjancenko, O., Sefidefl, M., Eflsässer, D., Aarestrup, F.M., Löfström, C., Boffiflfl-Mas, S., Abrfifl, J.F., Gfirones, R., Schufltz, A.C., 2017. Evafluatfion of methods for the concentratfion and extractfion of vfiruses from sewage fin the context of metagenomfic sequencfing. PfloS One 12, e0170199. https://dofi.org/10.1371/journafl.pone.0170199.

Jafferaflfi, M.H., Khatamfi, K., Atasoy, M., Bfirgersson, M., Wfiflflfiams, C., Cetecfiogflu, Z., 2021. Benchmarkfing vfirus concentratfion methods for quantfifficatfion of SARS-CoV-2 fin raw wastewater. Scfi. Totafl Envfiron. 755, 142939. https://dofi.org/10.1016/j.scfitotenv.2020.142939.

Katayama, H., Haramoto, E., Oguma, K., Yamashfita, H., Tajfima, A., Nakajfima, H., Ohgakfi, S., 2008. One-year monthfly quantfitatfive survey of norovfiruses, enterovfiruses, and adenovfiruses fin wastewater coflflected from sfix pflants fin Japan. Water Res. 42, 1441–1448. https://dofi.org/10.1016/j.watres.2007.10.029.

Kevfiflfl, J.L., Peflflett, C., Farkas, K., Brown, M.R., Bassano, I., Denfise, H., McDonafld, J.E., Maflham, S.K., Porter, J., Warren, J., Evens, N.P., Paterson, S., Sfinger, A.C., Jones, D. L., 2022. A comparfison of precfipfitatfion and ffifltratfion-based SARS-CoV-2 recovery methods and the finffluence of temperature, turbfidfity, and surfactant fload fin urban wastewater. Scfi. Totafl Envfiron. 808, 151916. https://dofi.org/10.1016/j.scfitotenv.2021.151916.

Kfitajfima, M., Sassfi, H.P., Torrey, J.R., 2018. Pepper mfifld mottfle vfirus as a water quaflfity findficator. npj Cflean Water 1, 1–9. https://dofi.org/10.1038/s41545-018-0019-5.

Kfitajfima, M., Ahmed, W., Bfibby, K., Carduccfi, A., Gerba, C.P., Hamfiflton, K.A., Haramoto, E., Rose, J.B., 2020. SARS-CoV-2 fin wastewater: state of the knowfledge and research needs. Scfi. Totafl Envfiron. 739, 139076. https://dofi.org/10.1016/j.scfitotenv.2020.139076.

McBrfide, G.B., Stott, R., Mfiflfler, W., Bambfic, D., Wuertz, S., 2013. Dfischarge-based QMRA for estfimatfion of pubflfic heaflth rfisks from exposure to stormwater-borne pathogens fin recreatfionafl waters fin the Unfited States. Water Res. 47, 5282–5297. https://dofi.org/10.1016/j.watres.2013.06.001.

McQuafig, S.M., Scott, T.M., Lukasfik, J.O., Paufl, J.H., Harwood, V.J., 2009. Quantfifficatfion of human poflyomavfiruses JC vfirus and BK vfirus by TaqMan quantfitatfive PCR and comparfison to other water quaflfity findficators fin water and fecafl sampfles. Appfl. Envfiron. Mficrobfiofl. https://dofi.org/10.1128/AEM.02302-08.

Medefiros, R.C., Danfiefl, L.A., 2015. Comparfison of seflected methods for recovery of Gfiardfia spp. cysts and Cryptosporfidfium spp. oocysts fin wastewater. J. Water Heaflth 13, 811–818. https://dofi.org/10.2166/wh.2015.228.

Nordgren, J., Matussek, A., Mattsson, A., Svensson, L., Lfindgren, P.-E., 2009. Prevaflence of norovfirus and factors finffluencfing vfirus concentratfions durfing one year fin a fuflfl-scafle wastewater treatment pflant. Water Res. 43, 1117–1125. https://dofi.org/10.1016/j.watres.2008.11.053.

Shaffer, M., Huynh, K., Costantfinfi, V., Bfibby, K., Vfinje, J., 2022. Vfiabfle norovfirus persfistence fin water mficrocosms. Envfiron. Scfi. Technofl. Lett. 9, 851–855. https://dofi.org/10.1021/acs.estflett.2c00553.

Shkoporov, A.N., Khokhflova, E.V., Ffitzgerafld, C.B., Stockdafle, S.R., Draper, L.A., Ross, R. P., Hfiflf, C., 2018. ΦCrAss001 represents the most abundant bacterfiophage famfifly fin the human gut and finfects Bacterofides fintestfinaflfis. Nat. Commun. 9, 4781. https://dofi.org/10.1038/s41467-018-07225-7.

Sfims, N., Kasprzyk-Hordern, B., 2020. Future perspectfives of wastewater-based epfidemfioflogy: monfitorfing finfectfious dfisease spread and resfistance to the communfity flevefl. Envfiron. Int. 139, 105689. https://dofi.org/10.1016/j.envfint.2020.105689.

Teunfis, P.F.M., Moe, C.L., Lfiu, P.E., Mfiflfler, S., Lfindesmfith, L., Barfic, R.S., Le Pendu, J., Caflderon, R.L., 2008. Norwaflk vfirus: how finfectfious fis fit? J. Med. Vfirofl. 80, 1468–1476. https://dofi.org/10.1002/jmv.21237.

Wong, K., Fong, T.-T., Bfibby, K., Moflfina, M., 2012. Appflficatfion of enterfic vfiruses for fecafl poflflutfion source trackfing fin envfironmentafl waters. Envfiron. Int. 45, 151–164. https://dofi.org/10.1016/j.envfint.2012.02.009.

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., Sfi, H.-R., Zhu, Y., Lfi, B., Huang, C.-L., Chen, H.-D., Chen, J., Luo, Y., Guo, H., Jfiang, R.-D., Lfiu, M.-Q., Chen, Y., Shen, X.-R., Wang, X., Zheng, X.-S., Zhao, K., Chen, Q.-J., Deng, F., Lfiu, L.-L., Yan, B., Zhan, F.-X., Wang, Y.-Y., Xfiao, G.-F., Shfi, Z.-L., 2020. A pneumonfia outbreak assocfiated wfith a new coronavfirus of probabfle bat orfigfin. Nature 579, 270–273. https://dofi.org/10.1038/s41586-020-2012-7.