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Science of the Total Environment

DOI:
10.1016/j.scitotenv.2021.151916

Published: 20/02/2022

Publisher's PDF, also known as Version of record

Science of the Total Environment 808 (2022) 151916

Contents lists available at ScienceDirect

Science of the Total Environment

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

A comparison of precipitation and filtration-based SARS-CoV-2 recovery methods and the influence of temperature, turbidity, and surfactant load in urban wastewater

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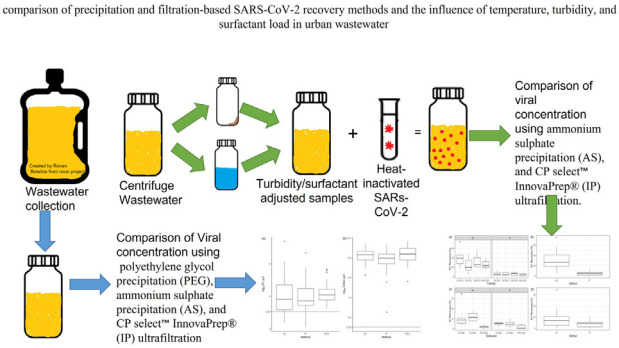
HIGHLIGHTS

- Comparison of wastewater viral recovery using three concentration methods.
- Control experiment looked at effect of turbidity and surfactant on viral recoveries.
- No sig. difference between wastewater viral recovery methods.
- Sequencing result comparable between viral concentration methods.
- Solids and surfactant impact viral recovery dependant on concentration method.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 September 2021
Received in revised form 9 November 2021
Accepted 19 November 2021
Available online 24 November 2021

Editor: Warish Ahmed

Keywords:
COVID-19
Faecal indicator virus
Wastewater concentration

ABSTRACT

Wastewater-based epidemiology (WBE) has become a complimentary surveillance tool during the SARS-CoV-2 pandemic. Viral concentration methods from wastewater are still being optimised and compared, whilst viral recovery under different wastewater characteristics and storage temperatures remains poorly understood. Using urban wastewater samples, we tested three viral concentration methods; polyethylene glycol precipitation (PEG), ammonium sulphate precipitation (AS), and CP select™ InnovaPrep® (IP) ultrafiltration. We found no major difference in SARS-CoV-2 and faecal indicator virus (crAssphage) recovery from wastewater samples (n = 46) using these methods, PEG slightly (albeit non-significantly), outperformed AS and IP for SARS-CoV-2 detection, as a higher genome copies per litre (gc/l) was recorded for a larger proportion of samples. Next generation sequencing of 8 paired samples revealed non-significant differences in the quality of data between AS and IP, though IP data quality was slightly better and less variable. A controlled experiment assessed the impact of wastewater suspended solids (turbidity; 0–400 NTU), surfactant load (0–200 mg/l), and storage temperature (5–20 °C) on viral recovery using the AS and IP methods. SARS-CoV-2

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http://dx.doi.org/10.1016/j.scitotenv.2021.151916

RNA detection
qRT-PCR

recoveries were >20% with AS and <10% with IP in turbid samples, whilst viral recoveries for samples with additional surfactant were between 0–18% for AS and 0–5% for IP. Turbidity and sample storage temperature combined had no significant effect on SARS-CoV-2 recovery ($p > 0.05$), whilst surfactant and storage temperature combined were significant negative correlates ($p < 0.001$ and $p < 0.05$, respectively). In conclusion, our results show that choice of methodology had small effect on viral recovery of SARS-CoV-2 and crAssphage in wastewater samples within this study. In contrast, sample turbidity, storage temperature, and surfactant load did affect viral recovery, highlighting the need for careful consideration of the viral concentration methodology used when working with wastewater samples.

1. Introduction

Coronavirus disease 19 (COVID-19) symptoms were first reported in December 2019, by clinicians treating clusters of patients exhibiting pneumonia-like illness, all originating from the Wuhan district of Hubei province, China (Wang et al., 2020). The pathogen associated with pneumonia was identified as a novel coronavirus in January 2020 (Wang et al., 2020); an enveloped, single-stranded, positive-sense RNA virus in the family *Coronaviridae* (Gorbalenya et al., 2020), later named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Following the sequencing of SARS-CoV-2, a range of laboratory diagnostics for the virus rapidly emerged, predominantly using reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays (Corman et al., 2020; Li et al., 2020). These have been a cornerstone in aiding the understanding of infection and transmission rates, pathology and identifying potential infectious sources such as; infected tissues (Casagrande et al., 2020; Jiang et al, n.d.; Vijayan and Humphreys, 2020), urine, faeces (Cheung et al., 2020; Jeong et al., 2020; Zheng et al., 2020), as well as spill-over events into animals (Koopmans, 2021; McAloose et al., 2020; Sit et al., 2020; World Health Organization, 2003). RT-qPCR studies involving hospital patients show that SARS-CoV-2 can be detected at 10^1 to 10^8 genome copies (gc) g^{-1} in faeces of infected individuals (Cheung et al., 2020; Jeong et al., 2020; Zheng et al., 2020), whilst viral shedding in urine is uncommon (Huang et al., 2020; Lo et al., 2020). The presence of SARS-CoV-2 RNA in faeces has therefore led to the adoption of wastewater-based epidemiology (WBE) to assess levels and lineages of COVID-19 circulating in the community (Ahmed et al., 2020a, 2020b; Gonzalez et al., 2020; Hillary et al., 2021; Kumar et al., 2020; La Rosa et al., 2020; Sherchan et al., 2020; Street et al., 2020; Venugopal et al., 2020; Westhaus et al., 2021). Furthermore, as faecal shedding of SARS-CoV-2 commences several days before clinical symptoms appear, it can potentially provide an early warning system for community-level COVID-19 outbreaks (Zhu et al., 2021).

In addition to viruses, faecal coliform bacteria and human gut-associated bacteriophages (e.g. CrAssphage) can be detected in wastewater and are used as faecal indicators in WBE programs (Doré et al., 2003; Vázquez-Salvador et al., 2020). CrAssphage, a group of bacteriophages infecting *Bacteroides* spp. common in the human gut, are an important human faecal marker as they are regularly detected in wastewater (Farkas et al., 2019; Tandukar et al., 2020), often in parallel with human enteric viruses (Crank et al., 2020; Farkas et al., 2019). When wastewater flow rate is high, due to groundwater infiltration and stormwater runoff, crAssphage concentrations in wastewater are low, allowing these data to be used to normalise viral data and account for dilution (Wilder et al., 2021).

The rapid and widespread utilisation of WBE for the monitoring of SARS-CoV-2 has led to the optimisation and comparison of several methods for concentrating enveloped viruses from wastewater, including: ultracentrifugation (Ahmed et al., 2020c), ultrafiltration (Ahmed et al., 2020c; Forés et al., 2021; Jafferali et al., 2021; LaTurner et al., 2021; McMinn et al., 2021; Philo et al., 2021; Rusiñol et al., 2020), polyethylene glycol (PEG) and salt-based precipitation (Ahmed et al., 2020c; LaTurner et al., 2021; Pérez-Cataluña et al., 2021; Philo et al., 2021), skimmed milk flocculation (Philo et al., 2021; Rusiñol et al., 2020), sludge extraction (Philo et al., 2021), bag-mediated filtration (Philo et al., 2021), magnesium- and aluminium-based absorption (Jafferali et al., 2021; Pérez-Cataluña et al., 2021). The quantification of SARS-CoV-2 from wastewater is performed using reverse-transcriptase quantitative PCR (RT-qPCR); therefore, the addition of internal control viruses to samples is required to assess viral recovery and PCR inhibition. Internal controls include the use of enveloped surrogate viruses, such as human coronavirus OC43 (McMinn et al., 2021; Philo et al., 2021), bovine coronavirus (Jafferali et al., 2021; LaTurner et al., 2021), murine hepatitis virus (Ahmed et al., 2020c), porcine epidemic diarrhoea virus (Pérez-Cataluña et al., 2021), as well as non-enveloped MS2 bacteriophage (Forés et al., 2021), mengovirus (Pérez-Cataluña et al., 2021) and pepper mild mottle virus (Jafferali et al., 2021). All these studies report different virus recoveries, and sensitivities among methods. However, there is consensus in the literature that both ultrafiltration (Forés et al., 2021; Jafferali et al., 2021; LaTurner et al., 2021) and absorption-based methods (Ahmed et al., 2020c; Pérez-Cataluña et al., 2021) have high sensitivity for the detection of SARS-CoV-2.

The efficiency of viral recovery from wastewater is likely to be strongly influenced by the composition of the wastewater, whilst wastewater which varies according to its source (e.g., domestic-to-industrial ratio), degree of groundwater ingress into the sewer system, and prevailing weather conditions (e.g. dilution with rainwater). For example, it is known that high concentrations of suspended solids can negatively affect the recovery of faecal indicator bacteria and pathogenic organisms (Gedalanga and Olson, 2009; Medeiros and Daniel, 2015). This is due to the inability to fully desorb organisms either held electrostatically on particle surfaces or embedded in surface biofilms (Gantzer et al., 2008). Similarly, common domestic cleaning products and surfactants may also influence viral recovery from wastewater (Mousavi and Khodadoost, 2019). In the case of SARS-CoV-2, surfactants may facilitate desorption from surfaces, however, it may also promote envelope damage leading to greater exposure and degradation of its RNA (Jahromi et al., 2020; Shim et al., 2017).

This study aimed to evaluate the influence of concentration method, sample turbidity, temperature and surfactant concentration on the recovery of SARS-CoV-2 and the faecal indicator, crAssphage, from wastewater. We compared three concentration methods: PEG, ammonium sulphate (AS) precipitation and the CP select™ InnovaPrep® filtration unit (IP). We hypothesized that high concentrations of suspended solids and surfactants would reduce SARS-CoV-2 recovery and that high temperatures would also induce viral decay and the loss of viral RNA. The overall aim was to determine the influence of virus concentration methodology and wastewater conditions on the results obtained by national and regional WBE-based COVID-19 surveillance programmes for both RT-qPCR and next generation sequencing (NGS) data.

2. Materials and methods

2.1. Sample collection and storage

A total of 46 untreated wastewater samples (crude influent) were collected between October 2020 and February 2021 by United Utilities and Dŵr Cymru/Welsh Water as part of the UK national SARS-CoV-2 wastewater surveillance programme. At each site, 500 ml of crude influent was collected by manual grab sampling between 08.00 and 10.00 h. This reflected peak flow and aimed to capture the highest faecal load (Hillary et al., 2021). The wastewater treatment plants (WWTP) were located in North Wales ($n = 31$), Liverpool ($n = 12$), and Chester ($n = 3$). These samples were grouped to the environmental wastewater group used for method comparison of PEG vs. AS vs. InnovaPrep and were not seeded with SARS-CoV-2. SARS-CoV-2 detected in these samples is derived from human waste naturally in sewer water. In addition, 20 l of wastewater influent was collected from Chester on the 13th January to be used in the controlled laboratory experiments in which

surfactant and suspended solid load (turbidity) were altered. Samples were transported and stored at 4 °C and concentrated within 24 h of collection. All sample processing was conducted in a Biosafety Level 2 (BSL2) laboratory, adhering to WHO and national biosafety guidelines (OMS, 2004).

2.2. Controlled experiment - effect of turbidity, surfactant load and temperature on the recovery of SARS-CoV-2 from wastewater

The 20 l of influent collected from Chester on the 13th January was used in a series of controlled experiments to assess the effect of chemical, physical, and thermal parameters on SARS-CoV-2 recovery. Aliquots of wastewater (200 ml) in 250 ml polypropylene (PPCO) jars, were centrifuged (10,000*g*, 4 °C, 10 min) and the solids retained. To create a range of samples with differing turbidity, the solids were added back to the supernatant in different amounts within replicate sterile 50 ml polypropylene centrifuge tubes ($n = 2$ per treatment). The turbidity of the samples was adjusted to 25, 50, 100, and 400 Nephelometric units (NTU) using an Orion AQUAfast AQ3010 turbidity meter (Thermo Scientific, Waltham, MA, USA). This range was chosen to reflect the typical range observed in wastewater samples within the national surveillance programme (Fig. S1). For the surfactant experiment, samples were dosed with different levels of sodium dodecyl sulphate (SDS; Thermo Scientific, Waltham, MA, USA, Cat. No. 28364) at concentrations of 10, 50 and 200 mg/l in sterile 50 ml polypropylene tubes. SDS was chosen as it is present in many household cleaning and personal hygiene products. The native surfactant load of wastewater samples from the different sites was assessed using the $[Co(III)\text{-}(5\text{-}Cl\text{-}PADAP)_2]^+$ spectrophotometric method (Yokoyama et al., 2011) and found to range from 0.05 to 4.43 mg/l (mean 0.45 mg/l), values typical of urban wastewater (Palmer and Hatley, 2018). In both the surfactant and turbidity experiments, negative and positive controls consisted of 50 ml distilled water (dH_2O). Once samples were prepared, 1×10^8 genome copies (gc) l^{-1} of heat-inactivated SARS-CoV-2 (heat at 56 °C for 30 min) provided by Cardiff University, was added to all experimental treatments, apart from negative controls and environmental wastewater samples. To determine SARS-CoV-2 genome copies/μl prior to spiking samples, a concentration of 1×10^8 gc/μl of heat inactivated SARS-CoV-2 was added to deionised water and extracted as per the nucleic acid extraction method detailed below. The samples were then placed at 5, 15 or 20 °C on shaking racks set at 140 rpm, for 24 h. All treatments were performed in quadruplicate. Heat-inactivated SARS-CoV-2 was used to mimic its non-infectious nature in wastewater and its different physical states (Wurtzer et al., 2021). A sample of the unmodified and non-seeded wastewater was analysed to assess background levels of SARS-CoV-2 using the AS method detailed below. In total there were 96 samples; 48 with differing turbidity, 36 adjusted for surfactant, 8 positive controls and 4 negative controls.

2.3. Process and extraction controls

Wastewater samples were seeded with Murine norovirus (MNV) and *Pseudomonas* virus phi6 (phi6), which were used a process and extraction controls. MNV stock at a concentration of 1×10^6 gc/μl was added to all samples including the negative control after the initial clarification step, with the exception of sampled collected after February and those included in the control experiment. A 1×10^6 gc/μl phi6 was added to samples collected in February 2021 and the controlled experiment samples. Again, all samples including the negative control were seeded after the initial clarification step, which removed suspended solids by centrifugation from the sample (15,000*g*, 4 °C, 10 min). The percent recovery was then calculated from the copy number obtained from RT-qPCR for either MNV or phi6 using the seeded negative control as a baseline for total viral recovery. Full details of MNV and phi6 propagation are provided in the supplementary materials.

2.4. Concentration method 1: polyethylene glycol (PEG) precipitation

PEG precipitation was based on methods described previously (Farkas et al., 2021; Lewis and Metcalf, 1988) with modifications. A 40% PEG solution (PEG8000, Sigma-Aldrich, St. Louis, MO, USA, Cat. No. P5413) with 8% NaCl (Sigma-Aldrich, St. Louis, MO, USA, Cat. No. S7653) was prepared and autoclaved at 121 °C for 30 min. Wastewater (200 ml, $n = 46$) and dH_2O negative controls were poured into sterile 250 ml PPCO centrifuge jars, and centrifuged (15,000*g*, 4 °C, 10 min). The supernatant (150 ml) was recovered, transferred to a new sterile 250 ml PPCO bottle and pH adjusted to 7.0–7.5 using 1 M NaOH or 0.5 M HCl. PEG-NaCl (50 ml) was then added to each sample, the samples mixed, incubated overnight (4 °C) and then centrifuged (15,000*g*, 4 °C, 30 min), with the pellet resuspended in 200 μl of phosphate buffer saline (PBS). Concentrates were stored at 4 °C until nucleic acid extraction.

2.5. Concentration method 2: ammonium sulphate precipitation

The wastewater (200 ml, $n = 46$) and dH2O negative controls were poured into sterile 250 ml PPCO centrifuge jars, and centrifuged (15,000*g*, 4 °C, 10 min). The supernatant (150 ml) was transferred to a new 250 ml PPCO bottle containing 57 g of AS (Sigma-Aldrich, Cat. No. A4915). After the AS had dissolved, the samples were incubated at 4 °C for 1 h before centrifuging (15,000*g*, 4 °C, 30 min) and the supernatant discarded. The pellet was resuspended in 200 μl of PBS. Concentrates were stored at 4 °C until nucleic acid extraction.

2.6. Concentration method 3: InnovaPrep concentration pipette select

The wastewater samples ($n = 46$) wastewater (200 ml, n = 46) and dH2O negative controls were poured into sterile 250 ml PPCO centrifuge jars, 2 ml of 5% Tween® 20 (Sigma-Aldrich) was added to the samples prior to centrifugation (15,000*g*, 4 °C, 10 min). The CP select™ InnovaPrep® (IP) Concentrating Pipette with 0.05 μM PS hollow fibre filter tips (CP Select™, Drexel, MO, USA) was programmed as per the manufacturer's COVID-19 settings (Concentrating Pipette Select Wastewater Application Note, Revision B.). The IP device was then used to concentrate the virus from 3 × 50 ml aliquots of the supernatant. The tips were changed between samples. Samples were eluted at ~200 μl in 25 mM Tris elution fluid (CP select™, Cat. No. HC08001), which contains 0.075% Tween® 20. Concentrates were stored at 4 °C until nucleic acid extraction.

2.7. Nucleic acid extraction method

Nucleic acids were extracted from concentrates using NucliSens lysis buffer (BioMerieux, Marcy-lÉtoile, France, Cat No. 280134 or 200292), NucliSens extraction reagent kit (BioMerieux, Cat. No. 200293) and a Kingfisher 96 Flex system (Thermo Scientific, Waltham, MA, USA). Full extraction details are provided in supplementary materials.

2.8. RT-qPCR analysis

One-step RT-qPCR for the SARS-CoV-2 N1, Phi6 and MNV targets was performed using an RNA Ultrasense One-step RT-qPCR system (Life Technologies, Carlsbad, CA, USA, Cat. No. 11732927), on a Quant Studio Flex 6 (Applied Biosystems Inc., Waltham, MA, USA). Samples collected as part of the national programme ($n = 96$) were run on a MicroAmp optical 96-well reaction plate (Applied Biosystems Inc., Cat. No. 4306737), at a reaction volume of 25 μl. The samples from the turbidity/surfactant experiment were run on a MicroAmp Endura optical 384 well clear plate (Applied Biosystems Inc., Cat. No. 4483273), at a reaction volume of 20 μl. The mastermix for 96 and 384 well plates contained RNA UltraSense™ 5× Reaction Mix with ROX, 10 pmol of the forward, 20 pmol of the reverse primers and 5 pmol probe, RNA UltraSense™ Enzyme Mix, 16 nmol MgSO4, 1 μg bovine serum albumin (BSA), molecular grade water and 5 μl (96 well plate) or 2 μl (384 well plate) sample/

standard/control. Samples were run in duplicate, against a ssRNA (NI, Phi6) or DNA (MNV, CrAssphage) standard curve dilution series of the target sequence in the range of 1–10^5 copies μl^{-1} per reaction (see supplementary materials for standard curve RNA synthesis). PCR no template controls (molecular-grade water) determined the absence of contamination during the PCR set-up. RT-qPCR settings were: Hold step 55 °C 60 min for reverse transcription, 95 °C 5 min for reverse transcriptase inactivation, followed by 45 amplification cycles of 95 °C 15 s, 60 °C 1 min, 65 °C 1 min at increments of 1.6 °C/s.

The N1 target standard slope ranged from 3.095 to 3.518, for the MNV target 3.095 to 3.580, and for Phi6 3.115 to 3.436. The R^2 for N1 ranged between 0.990 and 0.998, for MNV 0.995 to 0.998, and for Phi6 0.982 to 0.994. The efficiency (*E*) for N1 ranged between 91.82% and 106.43%, for MNV 92% to 110% and for Phi6 95.4% to 109%.

The qPCR for crAssphage was performed using a QuantiFast probe PCR with ROX mix (Qiagen, Hilden, Germany. Cat No. 204354). Each 20 μl reaction mix contained 1× QuantiFast no ROX, 1× QuantiFast with ROX with 10 pmol of the forward, 10 pmol of the reverse primers and 5 pmol probe, 16 nmol MgSO4 and 1 μg bovine serum albumin (BSA) and 5 μl sample/standard/control. Samples were run on a MicroAmp optical 96 well reaction plate, using the Quantstudio Flex 6. The qPCR settings were 98 °C 5 min, followed by 40 cycles at 95 °C 15 s and 60 °C 1 min increments of 1.6 °C/s. Plasmid DNA as described in Farkas et al. (2019) and water for non-template controls were used for quantification and quality control. The crAssphage standard slope ranged from 3.349 to 3.55. The R^2 ranged between 0.932 and 0.999. The efficiency ranged between 91.0 and 98.8%.

Primer sequences have been displayed in Table S1 (supplementary materials).

2.9. RT-qPCR data analysis

The RT-qPCR data was converted to gc/l wastewater and gc/μl nucleic acid extract for statistical analysis. The assay limit of detection (LOD) was tested using 10 replicate dilutions of N1 and crAssphage genomic RNA/DNA, and defined as the minimum concentration whereby 10 replicates all return positive results: 1.7 gc/μl of RNA extract of wastewater for N1 (Farkas et al., 2021) and 2 gc/μl crAssphage (Farkas et al., 2019) per assay. As such, quantities can be detected below this limit but are susceptible to false negatives. The viral load of seeded SARS-CoV-2 was confirmed by RT-qPCR and this number was used to calculate the percent recovery for samples in the controlled experiment.

2.10. SARS-CoV-2 RNA amplicon sequencing

A total of 8 paired AS and IP wastewater RNA extracts from the environmental wastewater sample group containing naturally occurring viruses only (non-seeded), along with negative and positive controls, were treated with TURBO DNase (DNA free kit, Invitrogen) and then purified using 1.8× RNA XP beads (Beckman Coulter) to reduce non-specific amplification. 8 μl of purified RNA was then reverse transcribed using NEB LunaScript. cDNA was amplified using the ARTIC v3 primers (IDT), generating approximately 400 bp amplicons tiling the entire SARS-CoV-2 genome (Tyson et al., 2020). Libraries were generated using the NEBNext Ultra II DNA Library Prep Kit following the 1/3rd volume protocol and indexed with unique dual indexes (IDT). Amplicons were pooled and libraries cleaned (0.7× volume, DNA XP Beads, Beckman Coulter), quantified (Qubit HS DNA Kit, ThermoFisher) and fragments analysed (HS DNA Kit Agilent). The final library was quantified using qPCR and then sequenced on an Illumina MiSeq platform generating 2x250bp paired end reads. PEG samples formed part of routine lab monitoring, where nucleic acids were used for other analysis, not enough sample remained for sequencing comparisons. Full details of bioinformatic and statistical analysis are provided in the supplementary materials.

2.11. Statistical analysis

Data analysis and statistical tests were carried out in R (R Core Team, 2020), utilising the "lme4" package for linear mixed-effects models, "dplyr" for data manipulation, and "ggplot2" for visualisations (Bates et al., 2015; Wickham et al., 2021; Wickham and Sievert, 2016). All data

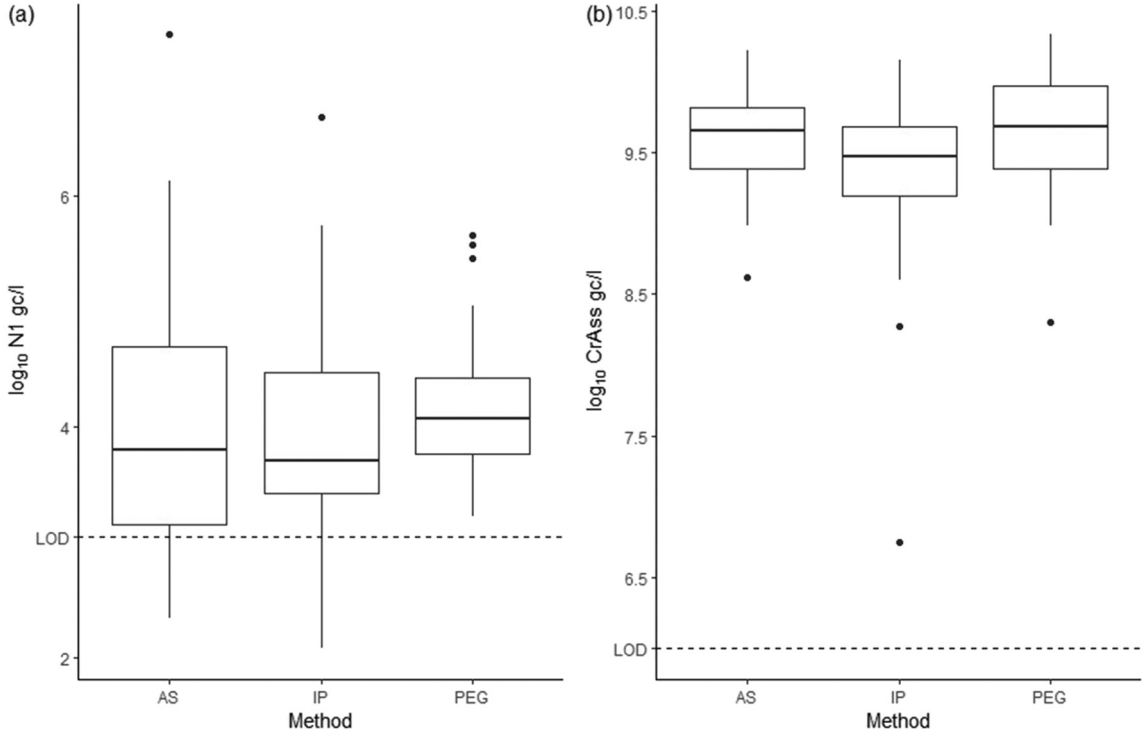

Fig. 1. a) $\log_{10}$ SARS-CoV-2 N1 genome copies per litre (gc/l) among concentration methods ammonium sulphate (AS), polyethylene glycol (PEG) and InnovaPrep (IP). The box depicts percentile ranges from 25, 50, and 75 respectively, the whiskers depict ±1.5× IQR, and the outliers are indicated by points. b) $\log_{10}$ crAssphage genome copies per litre between concentration methods, the dotted line marks the assay LOD for RT-qPCR.

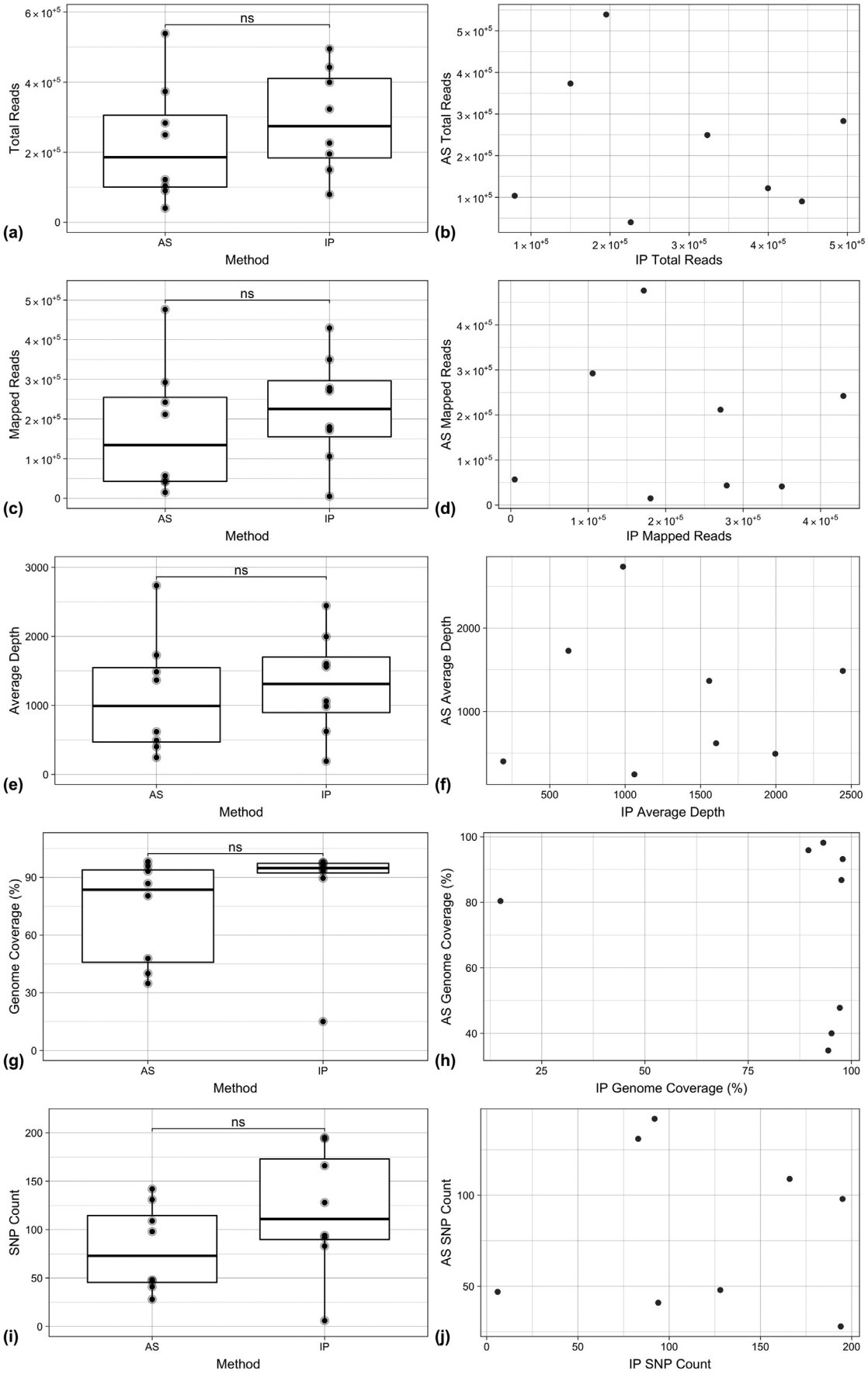

Fig. 2. Summary statistics and sequencing quality indicators for wastewater samples concentrated using IP and AS. a, c, e and i, ANOVA p-value > 0.05. g, Kruskal-Wallis test p-value 0.05 (Table 1). ns = nonsignificant (p-value > 0.05).

Table 1
Summary statistics and sequencing quality indicators for wastewater samples prepared using AS and IP.

Statistic	Method	Mean	SD	SW P-value	BT P-value	ANOVA P-value	CV	Asymptotic test statistic	P-value	Pearson correlation coefficient	P-value
Total reads	AS	225,143.20	169,966.10				0.755				
	IP	288,838.20	149,035.50	0.422	0.737	0.439	0.516	0.548	0.459	−0.182	0.666
Mapped reads	AS	172,444.50	162,617.10				0.943				
	IP	223,966.00	136,171.70	0.394	0.651	0.503	0.608	0.593	0.441	−0.034	0.936
Average coverage	AS	1134.25	162,617.10				0.753				
	IP	1308.01	136,171.70	0.463	0.705	0.670	0.563	0.313	0.576	−0.005	0.991
Genome coverage	AS	72.14	26.70				0.370				
	IP	85.01	28.42	0.000	0.873	0.227[a]	0.334	0.058	0.810	−0.146	0.730
SNP count	AS	80.50	64.48				0.554				
	IP	119.75	44.64	0.722	0.352	0.179	0.538	0.004	0.951	−0.013	0.975

[a] Kruskal Wallis Test as non-normal, SD = standard deviation, SW = Shapiro Wilk Test, BT = Bartlett Test, CV = coefficient of variation.

was checked for normality using the Shapiro-Wilk Test and QQ-plots (provided in the supplementary materials).

For the environmental wastewater samples ($n = 46$), a Kruskal-Wallis test was selected to compare N1 SARS-CoV-2 and crAssphage gene copy (gc) recovery for each method. Wilcoxon signed rank test with a holm adjustment method was used for post hoc pairwise comparisons. Additionally, one-sample Wilcoxon signed rank exact tests were used to compare each method individually with the assay LOD.

The temperature and turbidity datasets for both concentration methods were visualised using boxplots. In addition, a Welch two sample *t*-test was selected to compare SARS-CoV-2 N1 gc between concentration methods. Again, data for the temperature and surfactant dataset were plotted using boxplots. In addition, a Wilcoxon rank sum test with continuity corrections was selected to compare N1 gc recovery between methods. A linear mixed-effects model (LMM) was fitted by maximum likelihood, with turbidity and temperature or surfactant and temperature, as fixed effects, assessing correlations with N1 gc recovery, both controlling for variation between methods with random effects. Variables were normalised to a mean of 0 and a standard deviation of 1 to allow comparison of coefficients. The models and variables were assessed through *p*-values determined by one sample *t*-tests: residual plots of homoscedasticity, prediction accuracy and normality of residuals and by comparing the Akaike information criterion (AIC) with alternate LMM without fixed effects.

3. Results

3.1. Environmental wastewater experiment; comparison of methods

The method comparisons for environmental wastewater samples was non-normally distributed (p-value < 0.05; Supplementary materials Fig. S2). The median SARS-CoV-2 recovery was not significantly different between virus concentration methods (Kruskal-Wallis rank sum test: chi-squared 3.23, df 2, p-value 0.20, Fig. 1a), post hoc pairwise Wilcoxon signed rank comparisons supported this conclusion finding no significant differences between pairs (p-value > 0.60). Furthermore, sample medians were compared to the assay LOD for each method, which revealed all methods could be considered different to the LOD for SARS-CoV-2 (Wilcoxon signed rank exact tests: p-value < 0.001; Fig. 1a). However, a Bartlett test indicated variances were not homogeneous between groups (K-squared 10.89, df 2, p-value < 0.01), suggesting that the PEG method has significantly reduced

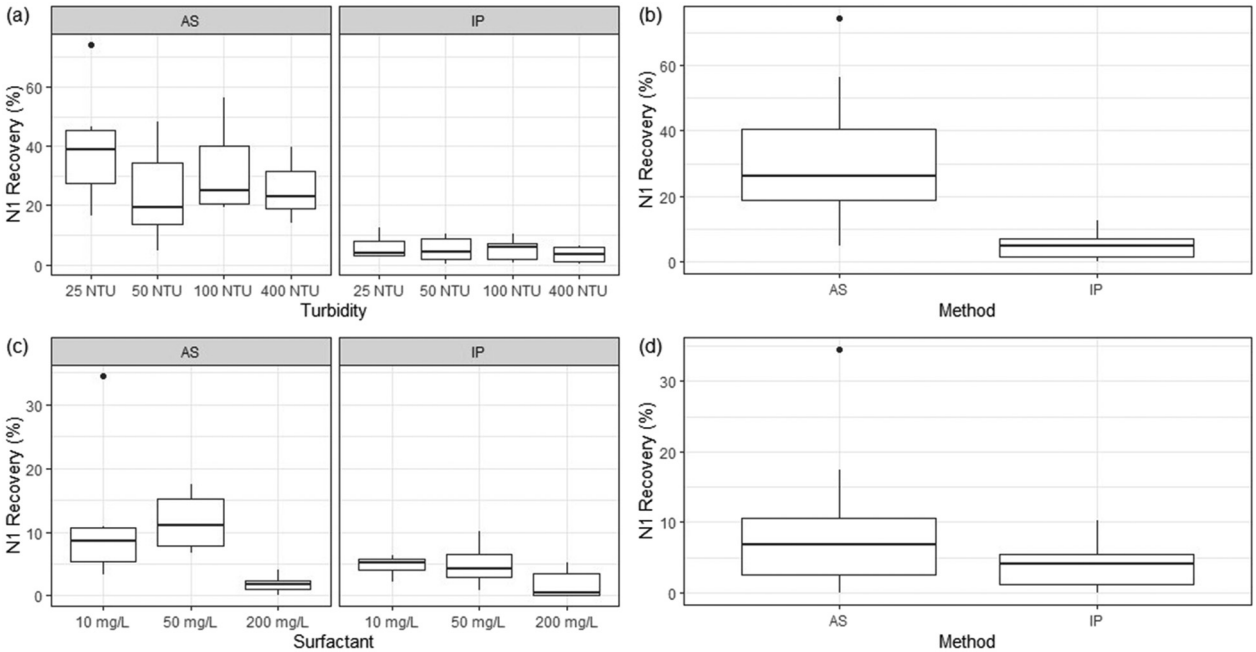

Fig. 3. a) SARS-CoV-2 N1 gene recovery (%) dependent on turbidity (NTU) using the AS and IP method. b) N1 recovery (%) for all samples with adjusted NTU, concentrated using the AS and IP method. c) N1 recovery (%) dependent on surfactant load using the AS and IP method. d) N1 recovery (%) for all samples with adjusted surfactant load, concentrated by the AS or IP method.

variance in viral recovery. A comparison of the crAssphage data further indicated no significant differences between methods (Kruskal-Wallis rank sum test: chi-squared 2.53, df 2, *p*-value 0.28; Fig. 1b).

A comparison of the NGS data for AS and IP methods revealed that for all summary statistics and sequencing quality indicators IP showed an increase in performance and a reduction in variance when compared to AS (Fig. 2, Table 1); however, none of the NGS summary statistics had significantly different mean values (ANOVA and Kruskal.test; *p*-value > 0.05, Table 1) or coefficients of variation (Asymptotic Test p-value > 0.05, Table 1). Interestingly there was no linear relationship between IP and AS summary statistics and sequencing quality indicators (Fig. 2, Table 1).

3.2. Laboratory controlled experiments on viral recoveries

3.2.1. Viral recovery (%) of SARS-CoV-2 in turbid and surfactant-adjusted samples

The viral recoveries of SARS-CoV-2 from seeded samples revealed that AS precipitation is significantly more efficient than IP, in both treatments containing large amounts of suspended solids and surfactant (turbid samples Wilcoxon rank sum exact test: *p*-value < 0.001; surfactant samples Wilcoxon rank sum test with continuity correction: p-value < 0.05; Fig. 3b & d). Concentration of SARS-CoV-2 N1 in the turbid samples with AS had median recoveries of 20–40%, whilst IP had median recoveries of

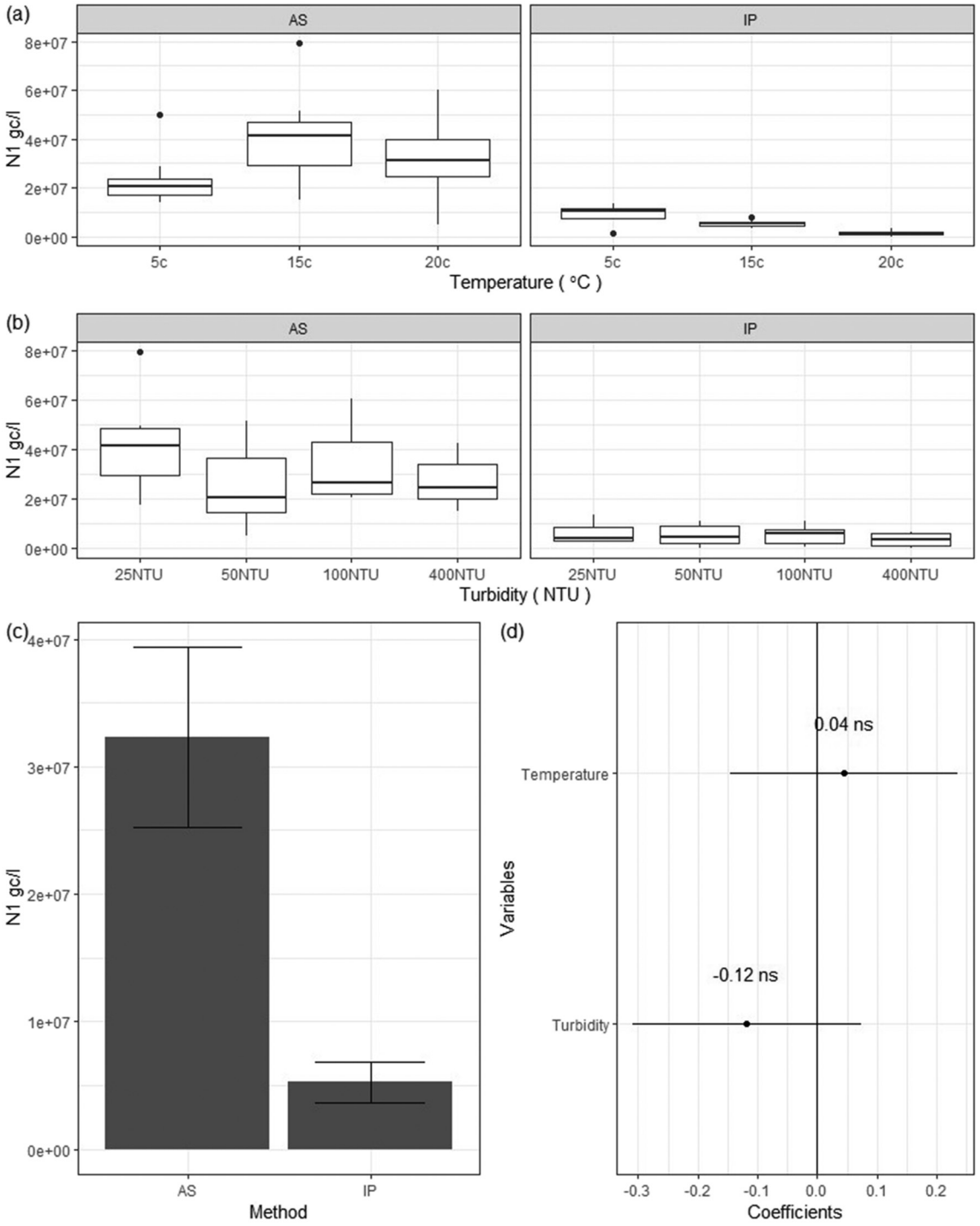

Fig. 4. Median N1 gc/l and percentile ranges between storage temperatures (a) and sample turbidity (b). Comparison of mean N1 gc/l and 95% confidence intervals between concentration methods (c). Linear mixed model with standardised variables (d).

0–10%, suggesting IP efficiency reduced considerably for samples with high turbidites (Fig. 3a & c). Surfactant load had a considerable effect upon SARS-CoV-2 N1 recovery, with samples in both methods recovering <20%: median recoveries being 2–13% and 0–6% for AS precipitation and IP respectively (Fig. 3a & c).

3.2.2. Assessment of turbidity and temperature upon the recovery of SARs-CoV-2

The AS concentration method yielded higher gc/l when compared to that of the IP method, regardless of sample storage temperature and suspended solid load (turbidity). The mean gc/l for samples stored at 5, 15, and 20 °C for AS were 2.36×10^7, 4.10×10^7, and 3.22×10^7, whilst for IP they were 9.01×10^6, 5.33×10^6 and 1.15×10^6, respectively (Fig. 4a). The mean gc/l for samples with an NTU of 25, 50, 100 and 400 for AS were 4.28×10^7, 2.33×10^7, 2.91×10^7 and 2.71×10^7, whilst for the IP method they were 6.36×10^6, 5.47×10^6, 5.66×10^6, and 3.65×10^6, respectively (Fig. 4b). Prior to statistical analysis a Shapiro-Wilk test revealed the data followed a normal distribution (p-value > 0.05). A comparison of the N1 mean gc/l for each concentration method revealed that the means differ significantly between the AS and IP methods (Welch two sample t-test: $t = 7.49$, df = 25, p-value < 0.001, Fig. 4c). The results of the linear mixed model for the effect of temperature and turbidity on SARS-CoV-2 N1 recovery show that both temperature and turbidity levels

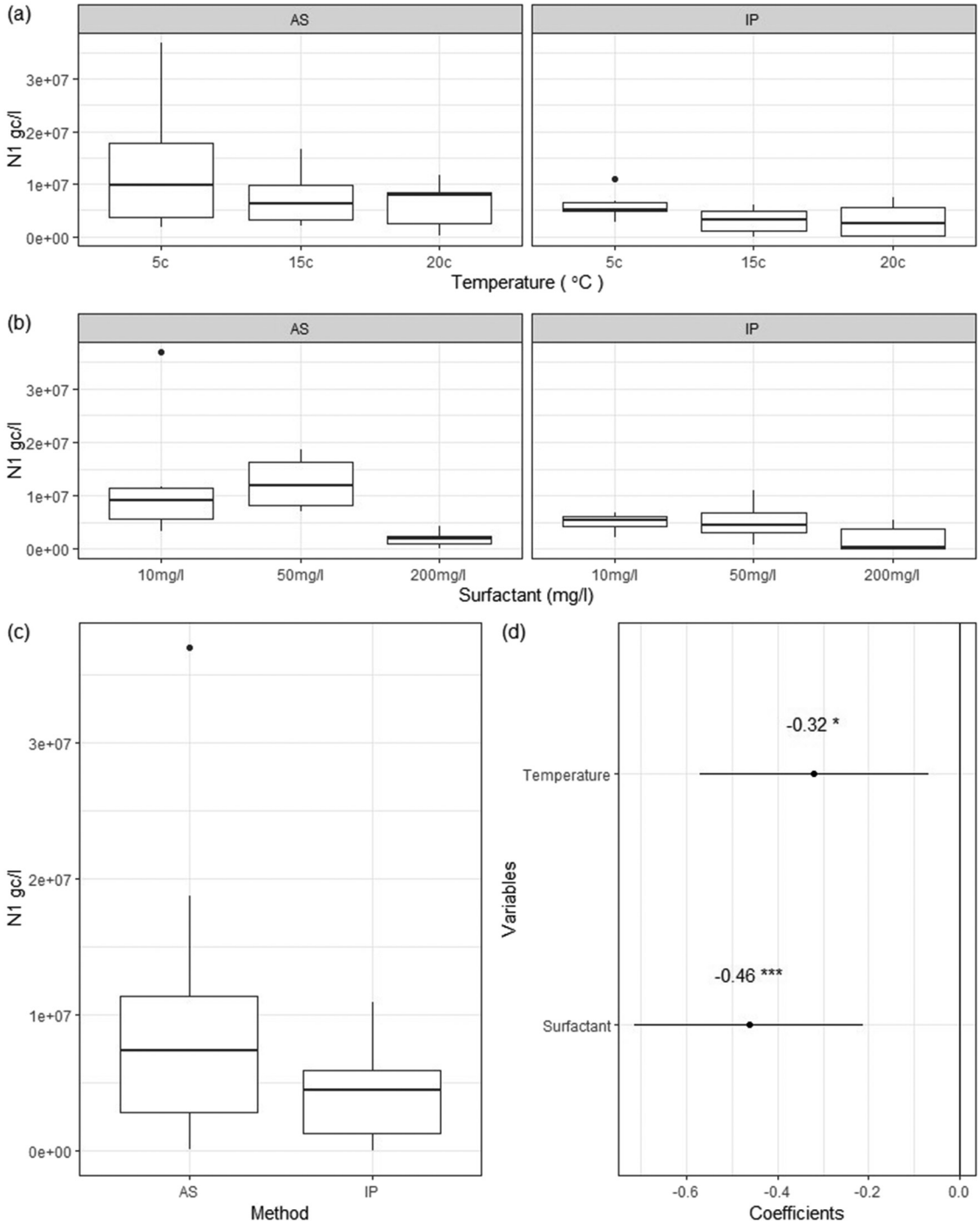

Fig. 5. Median N1 gc/land percentile ranges between storage temperatures (a) and surfactant levels (b). Comparison of median N1 gc/l and percentile ranges between concentration methods (c). Wilcoxon rank sum test with continuity correction suggest medians are significantly different (W = 225, p-value < 0.05). Linear mixed model with standardised variables (d).

were not significant predictors of gene recovery (ns = p-value > 0.05, Fig. 4d). Residual plots of homoscedasticity, prediction accuracy and normality of residuals are provided in the supplementary materials (Fig. S3).

3.2.3. Assessment of surfactant and temperature upon the recovery of SARS-CoV-2

The AS concentration method yielded similar gc/l when compared to that of the IP method, regardless of sample storage temperature and surfactant load, except for 5 °C which had higher viral recovery. The mean gc/l for samples stored at 5 °C, 15 °C, and 20 °C for AS were 1.34×10^7, 7.37×10^6, and 6.14×10^6, whilst for the IP method they were 5.95×10^6, 3.04×10^6 and 3.08×10^6, respectively (Fig. 5a). The mean gc/l for samples with surfactant loads of 10, 50, and 200 mg/l for AS were 1.26×10^7, 1.24×10^7, and 1.95×10^6, whilst for the IP method they were 5.03×10^6, 5.20×10^6, and 1.86×10^6, respectively (Fig. 5b). Prior to statistical analysis a Shapiro-Wilk test revealed data was not normally distributed (p-value < 0.05). A significant difference between AS and IP recovery methods occurred between the median SARS-CoV-2 N1 gc (Wilcoxon rank sum test with continuity correction: W = 225, p-value < 0.05, Fig. 5c). Furthermore, the mixed effects model revealed that both temperature and surfactant levels were significant negative correlates with gene recovery (* = p-value < 0.05; *** = p-value < 0.001, Fig. 5d). Residual plots of homoscedasticity, prediction accuracy and normality of residuals are provided in the supplementary materials (Fig. S4).

4. Discussion

As the COVID-19 pandemic continues and WBE surveillance intensifies, the need for high-throughput sampling and analysis are required. The comparison of different methodologies for concentrating viruses from wastewater shows that there are many considerations when deciding upon a protocol and trade-offs regarding cost, time, and viral recovery may need to be made dependant on the overall and downstream application of the data and samples. Our results show that for the environmental wastewater samples SARS-CoV-2 and crAssphage recovery was consistent regardless of concentration method. Nonetheless, PEG slightly (albeit non-significantly), outperformed AS and IP for the detection of SARS-CoV-2, as less variance in viral load was observed for the SARS-CoV-2 N1 data. This is likely due to the overnight incubation required for PEG (Farkas et al., 2021), which enhances recovery without degrading the viruses or their genome (Farkas et al., 2017). The AS and IP methods are significantly less time consuming than PEG precipitation, due to shorter incubation for AS (1 h) and lack of incubation for IP (Table 2). By examining the literature, we found one pre-print study that precipitated SARS-CoV-2 using AS (Vatansever et al., 2020), and despite this method being used to precipitate a range of proteins and viruses (Park et al., 2008; Safferman et al., 1988; Shields and Farrah, 1986), it is the first time that a comparison for the precipitation of SARS-CoV-2 has been made available. Due to the simplicity of the method, the short processing time involved, and availability of AS to be supplied in large volumes, we favoured this method over PEG or IP (Table 2). Furthermore, the sequencing data shows that AS and IP environmental wastewater samples, had non-significant differences for all quality indicators, meaning that samples concentrated with AS can be used for further downstream analysis. However, it is worth noting that the sequencing quality was slightly better for the IP concentrated samples, a reduction in variance was also observed (this is due to a reduction in sample handling when using the IP method, maintaining the integrity of RNA/DNA).

The amount of SARS-CoV-2 recovered from the seeded samples in the controlled experiment was significantly higher for AS than IP. Overall, AS SAR-CoV-2 viral recoveries were between 5%–58%, and 0%–17%, whereas for IP they were between 0%–12% and 0%–10% for samples with adjusted turbidity and surfactant load, respectively. The amount of SARS-CoV-2 recovered from seeded samples with adjusted turbidity differed significantly with AS viral recoveries being ranging between 5%–58% and IP between 0%–10%. The mean viral load of SARS-CoV-2 seeded samples with increased turbidity also significantly differed between AS and IP concentration methods, as the AS concentration method yielded higher mean viral loads at $\sim 1 \times 10^7$ than that of the IP method which were ten-fold less at $\sim 1 \times 10^6$. SARS-CoV-2 recovery from seeded samples containing high concentrations of SDS were significantly lower for AS and IP compared to what was observed in samples with less SDS. Samples containing SDS at 10 and 50 mg/l concentrated using AS and IP had viral recoveries ranging between 7%–15% and 3%–10%, respectively. Whilst samples with SDS at 200 mg/l had recoveries of <5% for both methods. Showing that increased surfactant load, severely reduced viral recovery within the study. One limitation of the study is that heat-inactivated SARS-CoV-2 may not exactly mimic endogenous SARS-CoV-2 present in wastewater, therefore in the control SARS-CoV-2 seeded experiments we cannot account for what effect this may have on viral recovery. However, the viral recoveries reported within this study are in line with those reported by other comparative studies with recoveries of no greater than 50% for the majority of samples (Jafferali et al., 2021; McMinn et al., 2021; Philo et al., 2021). A study comparing IP elution methods upon the recovery of betacoronavirus strain OC43 from large volumes of autoclaved wastewater (McMinn et al., 2021), showed that viral recovery could be increased from <30% with one elution step, to >40% by including a second elution step. In our study, only one elution step occurred, therefore, it is possible that viral recovery may have been enhanced for the IP method by an including an additional elution step. Consequently, our IP protocol maybe refined in the future. In addition, we recommend that a comparison of sample matrix (i.e. surfactant loads, turbidity) and extraction method (i.e. bead based, column based) is required for future research to determine the inhibitory effects of the sample upon extraction method, as all SARS-CoV-2 seeded samples had a 10-fold or greater loss of virus.

This study was conducted within a temperature range that mimics sample storage at 5 °C and UK sewer temperatures, which span a range from 10 °C to 25 °C, with a yearly average of 17 °C (Ali, 2019). The typical transit time of wastewater in the UK sewer system is approximately 6–12 h. We

Table 2
Details the time, cost, equipment needed, suability for wastewater samples with high turbidity and surfactant loads and false negative rate.

	PEG	AS	IP
Time/sample required for concentration	18 h	3 h	1 h
Bench time/sample required for concentration	30 min	30 min	30 min
Equipment required for concentration	Temperature controlled centrifuge with rotor for 10,000 ×*g* speed	Temperature controlled centrifuge with rotor for 10,000 ×*g* speed	Temperature controlled centrifuge with rotor for 10,000 ×*g* speed CP select™ InnovaPrep® (IP) Concentrating Pipette
Consumables costs/sample	<£1	<£1	>£10
Suitable for high turbidity samples	NA	Yes	No
Suitable for samples with high levels of surfactants	NA	Yes if <200 mg/l	No
False negative rate	6%	11%	10%

showed that temperature over a 24-hour period influences viral recovery in the presence of high surfactant loads, therefore, it is recommended to obtain surfactant load when working with wastewater, particularly urban wastewaters that can have high surfactant loads (Camacho-Muñoz et al., 2014). The turbidity of samples only affected the IP concentration method, due to the increased concentration of inhibitory factors, and possibly the addition Tween® 20 surfactant as per the suggested wastewater protocol. This paper shows that precipitation by flocculation using AS and PEG methods are robust when paired with bead-based extraction methods.

5. Conclusions

Cost, time, and sample chemistry need to be carefully considered when choosing a concentration method for viral recovery from wastewater. We show all concentration methods we tested are suitable for extracting and quantifying SARS-CoV-2 RNA and crAssphage DNA from wastewater samples. In environments where wastewater turbidity and surfactant load are extremely high it is recommended that a precipitation method rather than an ultrafiltration method is employed. Further work on method refinement should focus on other human viruses that can be detected in wastewater, such as respiratory syncytial virus (RSV), noroviruses, and influenza viruses. In addition, research efforts should be conducted to improve viral recovery from wastewater samples, as the controlled experiment had recoveries of less than 50% for most of the samples. Determining where viruses are lost during the process would be of great benefit, especially in samples with low virus abundance.

Funding

Funding was provided by the UK Natural Environment Research Council National under the COVID-19 Wastewater Epidemiology Surveillance Programme (NE/V010441/1) and the Centre for Environmental Biotechnology Project funded though the European Regional Development Fund (ERDF) by Welsh Government. We particularly thank Tony Harrington at Dŵr Cymru-Welsh Water and Jo Harrison and Charmian Abbott at United Utilities PLC alongside staff at the wastewater treatment facilities for their support in this project. We thank Prof Ian Goodfellow (University of Cambridge, UK) for providing MNV and BV2 stocks.

CRediT authorship contribution statement

Conceptualisation; JLK, KF, SM, & DJ. Laboratory work; JK, & KF. Data analysis and curation; JK, KF, CP. Sequencing data and analysis; SP, MRB, IB & HD. Authorship; JK, DJ & CP. Final suggested edits; KF, CP, JM, SM, DJ, JW, NE & JP. Funding acquisition; DJ & AS.

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

We thank Prof Andrew Weightman (Cardiff University, UK) for providing the inactivated SARS-CoV-2, and Ian Goodfellow (University of Cambridge, UK) for providing the original MNV stock.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.151916.

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