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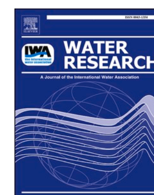

Examining the stability of viral RNA and DNA in wastewater: Effects of storage time, temperature, and freeze-thaw cycles

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ABSTRACT

Wastewater-based epidemiology (WBE) has been demonstrably successful as a relatively unbiased tool for monitoring levels of SARS-CoV-2 virus circulating in communities during the COVID-19 pandemic. Accumulated biobanks of wastewater samples allow retrospective exploration of spatial and temporal trends for public health indicators such as chemicals, viruses, antimicrobial resistance genes, and the possible emergence of novel human or zoonotic pathogens. We investigated virus resilience to time, temperature, and freeze-thaw cycles, plus the optimal storage conditions to maintain the stability of genetic material (RNA/DNA) of viral +ssRNA (Envelope – *E*, Nucleocapsid – *N* and Spike protein – *S* genes of SARS-CoV-2), dsRNA (Phi6 phage) and circular dsDNA (crAssphage) in wastewater. Samples consisted of (i) processed and extracted wastewater samples, (ii) processed and extracted distilled water samples, and (iii) raw, unprocessed wastewater samples. Samples were stored at –80 °C, –20 °C, 4 °C, or 20 °C for 10 days, going through up to 10 freeze-thaw cycles (once per day). Sample stability was measured using reverse transcription quantitative PCR, quantitative PCR, automated electrophoresis, and short-read whole genome sequencing. Exploring different areas of the SARS-CoV-2 genome demonstrated that the *S* gene in processed and extracted samples showed greater sensitivity to freeze-thaw cycles than the *E* or *N* genes. Investigating surrogate and normalisation viruses showed that Phi6 remains a stable comparison for SARS-CoV-2 in a laboratory setting and crAssphage was relatively resilient to temperature variation. Recovery of SARS-CoV-2 in raw unprocessed samples was significantly greater when stored at 4 °C, which was supported by the sequencing data for all viruses – both time and freeze-thaw cycles negatively impacted sequencing metrics. Historical extracts stored at –80 °C that were re-quantified 12, 14 and 16 months after original quantification showed no major changes. This study highlights the importance of the fast processing and extraction of wastewater samples, following which viruses are relatively robust to storage at a range of temperatures.

1. Introduction

Monitoring the amount of SARS-CoV-2 RNA in wastewater has been shown to be effective for the early detection of COVID-19 outbreaks in many regions of the world (Alhama et al., 2022; Hillary et al., 2021; Wurtzer et al., 2021). This approach relies on the shedding of viral RNA in faecal material and other fluids (Kashi et al., 2020), and its subsequent purification from wastewater before quantification by either reverse transcription quantitative or digital PCR (i.e., RT-qPCR or dPCR; Kevill et al., 2022). Viral genetic material can be extracted and purified from wastewater in a wide range of ways (Belouhova et al., 2023; Farkas et al., 2021; Matheri et al., 2023), however, an internationally accredited method has yet to be determined (Michael-Kordatou et al., 2020). Despite this, it is now well established that methodology is

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important in regulating the quantity of RNA recovered from wastewater (Angga et al., 2023; Zheng et al., 2022). The quality of the RNA recovered from wastewater is also important, especially for the downstream sequencing of SARS-CoV-2 lineages which is used for tracking variant dominance, the emergence of novel variants and co-infections (Brunner et al., 2022; Crits-Christoph et al., 2021; Ding et al., 2020).

It has been shown that rapid reporting of SARS-CoV-2 levels and variant identification in wastewater can be useful for effective management of COVID-19 response at both the local and national level (Amman et al., 2022). To ensure this, quantification and sequencing of RNA is normally carried out directly after recovery from the sample, typically within 24 h of collection (Mailepessov et al., 2022). This rapid processing minimises degradation of the viral genetic material and thus the loss of signal (Guo et al., 2023). For example, it is known that failure to store the samples under cool conditions (i.e., 1–4 °C) can lead to substantial degradation of viral RNA (Ahmed et al., 2020; Chandra et al., 2021). Further, while there appears to be little loss of RNA signal in refrigerated samples over short storage times (< 48 h), long-term storage can lead to significant RNA degradation (Hokajärvi et al., 2021; Markt et al., 2021; Thapar et al., 2023). In contrast to refrigeration, freezing wastewater samples for long-term storage (i.e., biobanking) may induce a large loss of signal that hinders the use of archived samples (Simpson et al., 2021). The large volumes of wastewater needed for subsequent recovery of genetic material also makes storage of wastewater at either −20 °C or −80 °C impractical, especially from large regional- or national-scale monitoring programmes where thousands of samples are collected (Wade et al., 2022).

In most cases, the preferred option for long-term biobanking involves the storage of a small volume of extracted genetic material (ca. 100 μl) which has been concentrated and purified from a large volume (50–1000 ml) of raw wastewater. In the case of SARS-CoV-2 in wastewater, the RNA is likely to be highly fragmented (Thapar et al., 2023; Wurtzer et al., 2021), however, the stability of extracted RNA is not well understood. Being able to utilise the extensive biobank of wastewater extracts that have been collected throughout the pandemic would be useful for evaluating the presence of a wide range of human and zoonotic viruses, that may exist in wastewater and for tracing the origin, evolution and spread of future disease outbreaks (Henderson et al., 2020; Simeon-Dubach and Henderson, 2020). The degradation and loss of genetic material in wastewater starts immediately after it enters the sewer network due to the wide diversity of heterotrophic microbes present (Ho et al., 2022; Parra-Arroyo et al., 2023). It is known from clinical samples that tissue storage at −80 °C can preserve DNA and protein for decades but that RNA is much more susceptible to degradation (Huang et al., 2017). Regardless of the initial processing stage at which wastewater samples are frozen for biobanking, they inherently require freezing and repeated thawing over time for subsequent analysis, progressively losing integrity of genetic material.

Understanding how different areas of the SARS-CoV-2 genome react to single and repeated freeze-thaw cycles and to different storage temperatures is important to the success of subsequent data mining of archived samples, and will aid in the design of better standard operating procedures for biobanking of wastewater samples. Here, we assessed the stability of wastewater-derived viral RNA and DNA after storage under a wide range of conditions, manipulating both the number of freeze-thaw cycles and the time spent in storage. We made comparisons with viruses used as surrogates, and viruses that are typically used to normalise data. Further, we used short-read whole genome sequencing to quantify the effects on SARS-CoV-2. We also utilised our own existing historical collection of samples to demonstrate the utility of archived samples already in storage. Our aim was to assess the value of quantifying and sequencing samples that have been tested with time and temperature at various stages of processing.

2. Methods

2.1. Sample collection and processing

Wastewater samples used in all the experiments described below were collected at 09.00 h on 1st December 2021 from the Treborth wastewater treatment plant (WWTP) located in Bangor, North Wales, UK (53°12′34.04′'N, 4°10′58.56′'W). This sampling time was chosen to reflect peak flow and aimed to capture the highest faecal load (Hillary et al., 2021). The WWTP is mainly composed of domestic wastewater, including sewage from clinical settings, with few industrial inputs. Here, 15 litres of crude influent wastewater were taken from behind the primary screen (flow 273 l s^{-1}) in polypropylene bottles and immediately transported to the laboratory at 4 °C (within 5 km of the WWTP). Due to the SARS-CoV-2 concentration in the wastewater at the time of sampling being relatively low (conclusion made from COVID-19 Wales Situational Report, 19 Nov 2021), each 200 ml wastewater sample was spiked with 100 μl of 2×10^5 genome copies (gc)/μl heat-inactivated (56 °C, 30 min) SARS-CoV-2 (Wuhan strain; kindly provided by Prof Richard Stanton, Cardiff University, UK). The samples were also spiked with 100 μl of a 1×10^6 gc/μl Phi6 phage, an enveloped dsRNA virus, which infects *Pseudomonas* spp. Phi6 is often used as a process control for wastewater concentration and is also a surrogate of SARS-CoV-2 in environmental persistence studies (Dey et al., 2022; Kevill et al., 2022). At the time of collection, the pH of the wastewater was 7.37, the electrical conductivity was 2610 μS cm^{-1}, the turbidity was 3.9 NTU and the ammonium and phosphate concentrations were 9.3 mg N l^{-1} and 0.03 mg P l^{-1}, respectively, measured as described by Hillary et al. (2021). The wastewater composition was typical of that collected during the English wastewater monitoring programme for COVID-19 across 47 urban sites (Wade et al., 2020). To account for the effect of the wastewater matrix, distilled water samples were spiked and processed in tandem with the wastewater samples.

Pre-processing consisted of centrifuging the raw wastewater samples (10,000 *g*, 10 min, 4 °C) in 200 ml aliquots. Distilled water samples were distributed throughout the samples and included as processing controls. After discarding the pellet, 150 ml of the supernatant was recovered, and 60 g of ammonium sulfate (Sigma-Aldrich, USA) added to the bottle. After dissolving the salt, the samples were stored at 4 °C for 1 h. The samples were then centrifuged (10,000 *g*, 30 min, 4 °C) and the supernatants discarded, and the remaining pellet resuspended in 800 μl of NucliSens lysis buffer (bioMerieux, France). No template controls (PBS) were included as extraction controls for reference. DNA/RNA extractions were carried out on the Kingfisher 96 Flex system (Thermo Scientific, USA) using NulciSens extraction reagents and eluted into 100 μl of elution buffer (Kevill et al., 2022). Extracts were pooled and divided into 50 μl aliquots of eluted RNA/DNA for subsequent experimentation.

2.2. Experimental design

We investigated the effect of repeated freeze-thaw cycles and storage time on viral +ssRNA (SARS-CoV-2), dsRNA (Phi6) and circular dsDNA (crAssphage) stability. All samples were spiked with known concentrations of heat-inactivated SARS-CoV-2 (provided by Prof. Richard Stanton, Cardiff University), and with known concentrations of Phi6 bacteriophage, cultured in-house (Kevill et al., 2022) and used as a process control. The faecal-marker virus crAssphage is ubiquitous in human faeces (Farkas et al., 2019). All samples were pooled by sample type (processed and extracted spiked wastewater samples, processed and extracted spiked distilled water samples, and raw, unprocessed spiked wastewater samples - based on samples per treatment listed in Experiments) to ensure equal starting concentrations and to reduce/remove the effect of the wastewater matrix within groups. Viruses in the sample pool were quantified prior to any storage time or freeze thaw cycles (on day 0), to establish baseline concentrations.

Experiments were carried out with 142 samples divided into (i)

processed and extracted spiked wastewater samples, (ii) processed and extracted spiked distilled water samples (i.e., RNA/DNA in elution buffer), and (iii) raw, unprocessed spiked wastewater samples (Fig. 1). The number of sample replicates varied due to logistical constraints related to processing and storage space. A treatment refers to a time/temperature combination. Three experiments were undertaken as follows:

Experiment 1 (Fig. 1A): Processed and extracted spiked wastewater samples ($n = 5$ replicates per treatment), processed, and extracted spiked distilled water samples ($n = 2$ per treatment), and raw unprocessed spiked wastewater samples ($n = 1$ per treatment) were stored at both −20 °C and −80 °C for up to 10 days. They were put through 1, 3, 7, or 10 freeze-thaw cycles and then quantified. Processed and extracted samples were quantified immediately after thawing on days 1, 3, 7, 10. Raw unprocessed wastewater samples were processed and extracted before quantification (described above).

Experiment 2 (Fig. 1B): Processed and extracted spiked wastewater samples ($n = 5$ per treatment), processed, and extracted spiked distilled water samples ($n = 2$ per treatment), and raw unprocessed spiked wastewater samples ($n = 1$ per treatment) were stored at both 4 °C for up to 14 days, and 20 °C (room temperature) for up to 10 days. Processed and extracted samples were quantified on days 1, 3, 7, 10. Raw unprocessed wastewater samples were processed and extracted before quantification (quantification took place on the day of extraction).

Experiment 3 (Fig. 1C): Samples kept in storage as a stable control, and not subjected to freeze-thaw cycles; processed and extracted spiked wastewater samples ($n = 4$ per treatment) remained frozen at −20 °C and −80 °C before thawing on days 3 or 10.

In the freeze-thaw treatments, samples were removed from the freezer, allowed to defrost at 4 °C (ca. 4 h) and then returned to the freezer; this was considered one freeze-thaw cycle. Wastewater and distilled water extracts were stored in 50 μl aliquots, and raw unprocessed samples were stored in 200 ml aliquots. Samples were not reused (i.e. not re-frozen or re-refrigerated) between conditions, and so after each freeze-thaw cycle, one aliquot was quantified following quantification protocols, and then removed from the experiment. One day is equal to one freeze-thaw cycle (Fig. 1), making the storage times for the −20 °C, −80 °C, 4 °C and 20 °C treatments comparable. Wastewater samples and distilled water samples were thawed for equal amounts of time.

2.3. Sample quantification

RT-qPCR and qPCR were run to determine starting concentrations (referred to as day 0) immediately following extraction. Quantification then took place after the designated number of freeze thaw cycles, or the number of days in storage.

The concentrations of SARS-CoV-2 and Phi6 were determined using RT-qPCR targeting four genetic markers: three targeting different genes of the SARS-CoV-2 genome: the Envelope (*E*), the Nucleocapsid (*N*1), and the Spike proteins (*S*) genes (CDC, 2020; Corman et al., 2020), and the enveloped *Pseudomonas* virus Phi6 (Phi6; Table 1) as a viral comparison for SARS-CoV-2. *N* and Phi6 targets were run in one duplex assay, and *S* and *E* were run in a second duplex assay. Both assays ran all samples, negatives, and standards in duplicate. RT-qPCR was run using TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, USA) with a reaction volume of 20 μl. PCR negatives were included for reference. Reactions were run according to methods previously described (Kevill et al., 2022) at an annealing temperature of 58 °C and 60 °C for *E*/*S* and *N*/Phi6, respectively. An internal amplification control was not added,

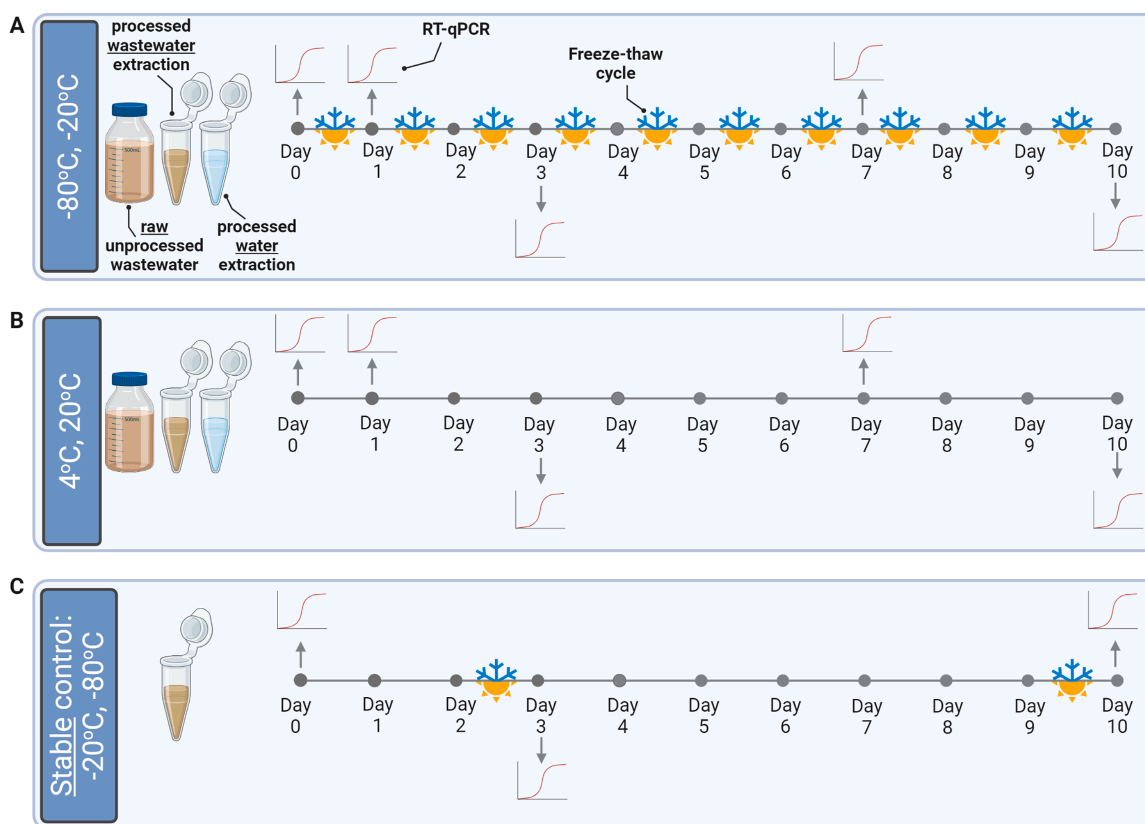

Fig. 1. Experimental overview. Samples were all quantified on day 0, before storage and freeze-thaw cycling to establish baseline starting quantification values. Groupings used in analysis are underlined. (A), Samples were stored at −20 °C and −80 °C, and were freeze-thawed every day for up to 10 days with quantification on days 1, 3, 7, and 10. (B), Samples were stored at 4 °C and 20 °C and quantified on days 1, 3, 7, and 10. (C), Samples remained frozen at −20 °C and −80 °C, before thawing and quantification on days 3 and 10.

Table 1
Primers and probes used for the different viral targets quantified within this study.

Target	Reference	Primer/Probe Name	Sequence (5′–3′)
Phi6	Gendron et al. (2010)	Phi6-F	TGGCGGCGGTCAAGAGC
		Phi6-R	GGATGATTCTCCAGAAGCTGCTG
		Phi6-P	VIC—CGGTCGTCGCAGGTCTGACACTCGC-QSY
SARS-CoV-2 N(1) gene	CDC (2020)	N1-F	GACCCCAAAATCAGCGAAAT
		N1-R	TCTGGTTACTGCCAGTTGAATCTG
		N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-MGB
SARS-CoV-2 E gene	Corman et al. (2020)	E-F	VIC-ACAGGTACGTTAATAGTTAATAGCGT-QSY
		E-R	ATATTGCAGCAGTACGCACACA
		E-P	ACACTAGCCATCCTTACTGCGCTTCG
SARS-CoV-2 S gene	In-house assay	S-F	TGAAGTCAGACAAATCGCTCC
		S-R	CAAGCTATAACGCAGCCTGT
		S-P	FAM-AGGGCAAACTGGAAAGATTGCTG-MGB
CrAssphage	Stachler et al. (2017)	CrAss-F	CAGAAGTACAAACTCCTAAAAAACGTAGAG
		CrAss-R	GATGACCAATAAACAAGCCATTAGC
		CrAss-P	FAM-AATAACGATTTACGTGATGTAAC-TAMRA

however bovine serum albumin (BSA) was included in the reaction (Kevill et al., 2022) to increase amplification efficiency in the presence of inhibitors found in wastewater. In a previous study (Farkas et al., 2022), we also trialled the sample process method, where no inhibition was observed when diluted samples were subject to RT-qPCR.

We used qPCR to quantify the DNA virus crAssphage using primers and probes adopted from Farkas et al. (2019). The CrAssphage qPCR was run using QuantiNova Master Mix (Qiagen, Germany) following manufacturer's recommendations and an annealing temperature of 60 °C.

A six-point standard curve of known quantity was included for each marker on every plate to quantify the targets (RT-qPCR and qPCR assay statistics and standard curve settings in Table S2). PCR non template controls (molecular-grade water) determined the absence of contamination during the PCR set-up. Reactions were performed on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, USA).

2.4. SARS-CoV-2 RNA amplicon sequencing

Wastewater RNA extracts were purified using Mag-Bind® TotalPure NGS beads (Omega Bio-Tek, USA) and reverse transcribed using LunaScript® RT SuperMix Kit (New England Biolabs, USA). Sequencing libraries were prepared using EasySeq™ RC-PCR SARS CoV-2 Whole Genome Sequencing kit v3.0 (Nimagen, Netherlands) and the V1.0 protocol (Jeffries et al., 2021). Amplicons were pooled and libraries cleaned using Mag-Bind® Total Pure NGS beads (Omega Bio-Tek, USA) before sequencing on a NovaSeq™ 6000 platform (Illumina, USA) generating 2 × 150 bp paired-end reads. Basecalling, demultiplexing and trimming of adapter and indexes took place on Illumina's sequencing cloud BaseSpace. Sequencing metrics were generated using the Nextflow execution of the ARTIC pipeline (https://github.com/connor-lab/ncov2019-artic-nf), mapping sequences to the SARS-CoV-2 reference sequence (Genbank accession MN908947.3).

2.5. RNA sample quality

To assess the quality of the RNA after different numbers of freeze-thaw cycles, a subset of both processed and unprocessed wastewater samples (61 total) were run on a 4150 TapeStation (Agilent Technologies Inc., USA). Samples were run using Agilent RNA or High Sensitivity RNA ScreenTape and Agilent RNA or High Sensitivity RNA ScreenTape Buffer. Results were calibrated using RNA or High Sensitivity RNA Ladder (Agilent).

2.6. Historical comparison

Samples obtained from urban wastewater treatment plants across England and analysed and extracted as part of the national COVID-19 surveillance programme (see Morvan et al., 2022 for further details) have been stored in our biobank at −80 °C. A subset of samples that were collected between February and June 2021 were thawed and re-quantified alongside the newly collected samples. These historical extracts were pre-processed upon collection by polyethylene glycol (PEG) precipitation to concentrate viruses as described in Farkas et al. (2021). Briefly, 200 ml of sample was centrifuged, and 150 ml of supernatant combined with PEG/NaCl for a final concentration of 10 % and 2 %, respectively after pH adjustment to 7–7.5. Following a 16 h incubation at 4 °C, samples were centrifuged to preserve viral nucleic acids in the pellet. Samples were extracted using the NucliSens method described, and then frozen at −80 °C.

2.7. Data analysis

The qPCR and RT-qPCR were analysed using the QuantStudio Real-Time PCR Software v1.7.2 (Applied Biosystems, USA). To reduce variation and allow comparison between runs, the threshold of quantification was manually set to 0.04 for each target. All subsequent analysis was carried out in R version 4.2.2 (R Core Team, 2022). Data were tested for normality using a Shapiro-Wilk test. For qPCR markers (SARS-CoV-2 *E, N, S*, Phi6 and CrAssphage), to understand change over experiment days, generalised additive models (GAMs) were implemented due to non-linear relationships. GAMs were constructed using the package *mgcv* (Wood, 2010) with a different model for each marker. Each model contained quantity in gene copies per µl as the response variable and a smooth term for days, stratified by temperature and treatment, and a spline with 4 degrees of freedom.

Sequencing metrics (percentage N bases, percentage mapped reads, percentage covered bases and longest number of Ns) were used to understand change over experiment days, linear models were implemented. Comparison of means between the temperatures and treatment groups, for both qPCR and sequencing, was carried out using linear models with the terms 'temperature', 'treatment', 'days' as well as their interaction terms. Pairwise comparisons were produced using the package *emmeans* (Lenth, 2023).

3. Results

3.1. Change in quantifiable virus over time

Quantifying how viral load changes over time in storage allows us to determine optimal storage conditions; we looked at virus quantity and time, and then compared this relationship across the viruses tested. For SARS-CoV-2 *E* gene, there was a significant relationship with time in the raw 4 °C ($F = 11.89, p < 0.01$), water −80 °C ($F = 2.83, p = 0.03$), water −20 °C ($F = 3.56, p = 0.01$) and water 4 °C ($F = 2.82, p = 0.03$; Fig. 2A). For raw samples in particular, there was an increase of detectable viral load at 14 days. Whereas for the *N* gene, there was a significant

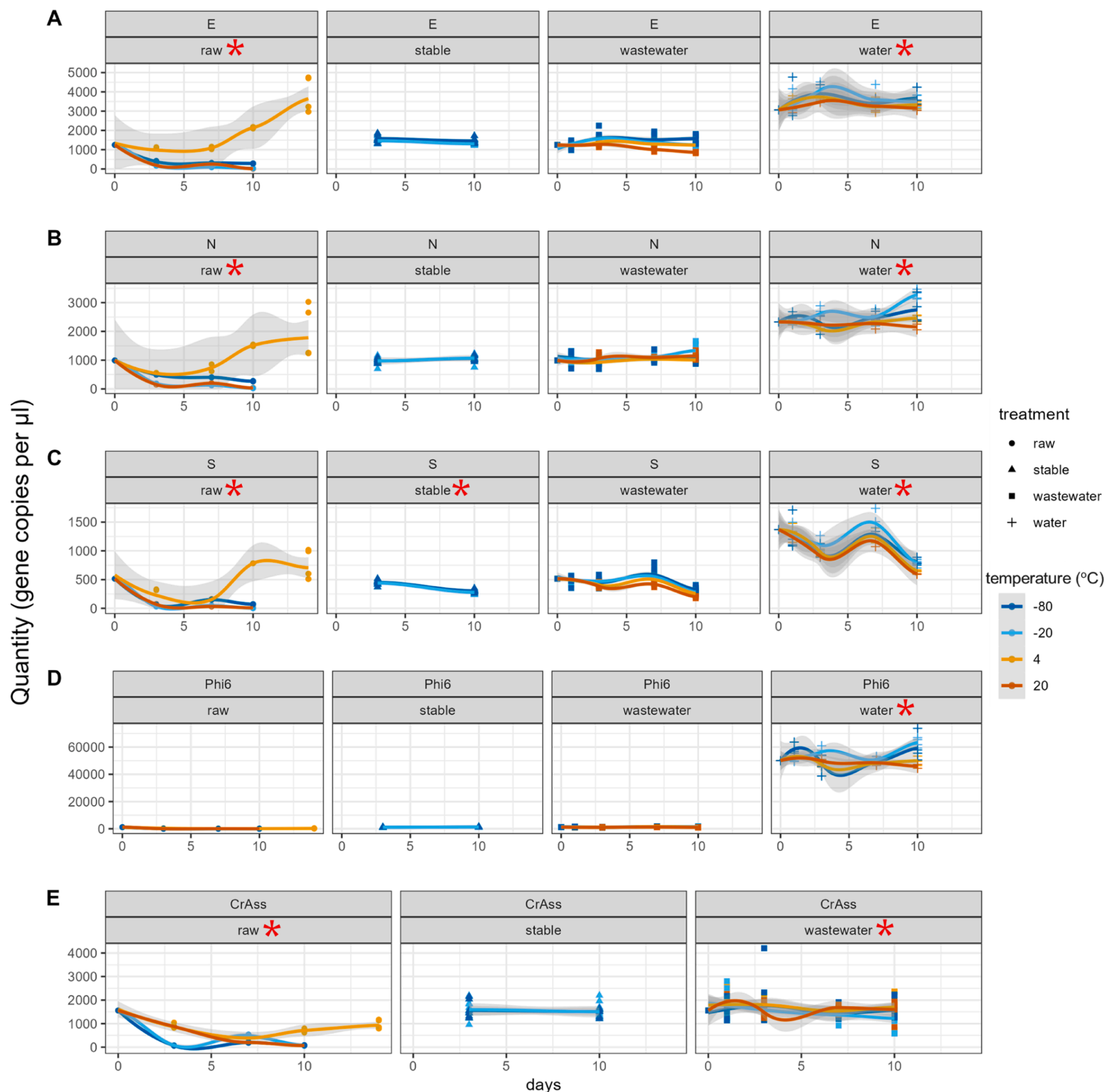

Fig. 2. Gene copies per µl of the viral markers (A) SARS-CoV-2 E gene, (B) SARS-CoV-2 N gene, (C) SARS-CoV-2 S gene, (D) Phi6 and (E) CrAssphage over time under different experimental treatments (see Fig. 1) and storage temperatures (−80 °C, −20 °C, 4 °C, 20 °C), with a LOESS smoother applied over time for each of the experimental groups and temperatures. Red asterisks represent treatments where at least one of the temperatures has a significant smoothing term, assessed using a generalised additive model. Each point represents a sample, technical PCR replicates have been combined. Samples are: raw - unprocessed wastewater; Stable - processed and extracted wastewater not subjected to daily freeze-thaw cycles; wastewater - processed and extracted wastewater; water - processed and extracted distilled water.

relationship with time in the raw 4 °C ($F = 4.10$, $p = 0.04$), raw 20 °C ($F = 4.70$, $p = 0.03$) and water −80 °C ($F = 3.20$, $p = 0.02$; Fig. 2B). The greatest number of significant relationships with time were seen for the *S* gene; there was a significant relationship with time in the raw −80 °C ($F = 4.01$, $p = 0.05$), raw 4 °C ($F = 4.84$, $p = 0.03$), raw 20 °C ($F = 4.01$, $p = 0.02$), stable −20 °C ($F = 2.63$, $p = 0.05$), water −80 °C ($F = 17.75$, $p < 0.01$), water −20 °C ($F = 23.68$, $p < 0.01$), water 4 °C ($F = 19.96$, $p < 0.01$) and water 20 °C ($F = 10.32$, $p < 0.01$; Fig. 2C).

For Phi6, there was a significant relationship with time in water −80 °C ($F = 4.55$, $p < 0.01$), water −20 °C ($F = 3.41$, $p = 0.02$) and water 4 °C ($F = 4.33$, $p < 0.01$; Fig. 2D).

For crAssphage, there was a significant relationship with time in the raw −80 °C ($F = 4.15$, $p < 0.01$), raw 4 °C ($F = 6.55$, $p = 0.01$), raw 20 °C ($F = 6.51$, $p < 0.01$) and wastewater −20 °C ($F = 8.28$, $p < 0.01$; Fig. 2E). CrAssphage was not measured in any of the water treatments due to CrAssphage not being present in water.

3.2. Temperatures within each treatment and its effect on virus concentration

For SARS-CoV-2, the *E* gene in raw sewage showed gene copy numbers at 4 °C that were significantly higher than all other temperatures, including −80 °C ($t = 7.94$, $p < 0.01$), −20 °C ($t = 8.99$, $p < 0.01$) and 20 °C ($t = 9.03$, $p < 0.01$; Fig. 3A and F). In the stable treatment, where processed and extracted wastewater samples were not subjected to daily freeze-thaw cycles (only thawed twice for quantification, hence fewer data points), there was no significant difference in recovery between temperatures (Fig. 3A and F). In freeze-thawed wastewater extracts, there was a significant impact of temperature (−80 °C > −20 °C > 4 °C > 20 °C), where −80 °C gave significantly higher recoveries than 4 °C ($t = 3.42$, $p = 0.05$) and 20 °C ($t = 5.38$, $p < 0.01$), and gene abundance at −20 °C was significantly higher than at 20 °C ($t = 3.55$, $p = 0.04$; Fig. 3A and F). In extracted water samples, there was no significant difference between temperatures (Fig. 3A and F).

For the SARS-CoV-2 *N* gene in raw sewage, 4 °C gave a significantly higher recovery than all other temperatures, including −80 °C ($t = 5.17$, $p < 0.01$), −20 °C ($t = 7.32$, $p < 0.01$) and 20 °C ($t = 7.45$, $p < 0.01$; Fig. 3B and G). In the stable treatment, there was no significant difference in gene abundance between temperatures (Fig. 3B and G). In wastewater extracts, −20 °C was significantly higher recovery than 4 °C ($t = 4.10$, $p = 0.01$; Fig. 3 B and G). In water extracts, there was a significant impact of temperature (−20 °C > −80 °C > 4 °C > 20 °C), where −20 °C gave significantly higher values than 4 °C ($t = 6.00$, $p < 0.01$) and 20 °C ($t = 5.88$, $p < 0.01$) while −80 °C was significantly higher than 20 °C ($t = 3.42$, $p = 0.057$; Fig. 3 B and G).

For the SARS-CoV-2 *S* gene recovery in raw sewage, 4 °C was significantly higher than all other temperatures, including −80 °C ($t = 4.16$, $p < 0.01$), −20 °C ($t = 4.85$, $p < 0.01$) and 20 °C ($t = 4.81$, $p < 0.01$; Fig. 3C and H). In the stable, wastewater and water treatments, there was no significant difference between temperatures (Fig. 3C and H).

For Phi6 in raw sewage, stable treatment and wastewater extracts, there was no significant difference between temperatures (Fig. 3C and H). In contrast, in the water extracts, there was a significant impact of temperature (−20 °C > −80 °C > 4 °C > 20 °C), where −20 °C was significantly higher than 4 °C ($t = 7.78$, $p < 0.01$) and 20 °C ($t = 7.30$, $p < 0.01$) and −80 °C was significantly higher than 4 °C ($t = 5.17$, $p < 0.01$) and 20 °C ($t = 5.15$, $p < 0.01$; Fig. 3C and H).

For the virus CrAssphage in raw sewage, stable treatment and wastewater extracts, there was no significant difference between temperatures (Fig. 3E and J).

3.3. Amplicon sequencing metrics

The 94 samples that underwent sequencing of SARS-CoV-2 showed no significant impact of temperature, treatment, days, or any of their interaction terms when analysed by total reads, aligned reads or average coverage. Only in raw sewage samples was there a significant difference between temperatures within a treatment group in the slope between days and any of the four sequencing metrics (Fig. 4). For percentage N bases, the slope for −80 °C was significantly different from the slope of −20 °C ($t = 4.31$, $p < 0.01$), 4 °C ($t = 7.98$, $p < 0.01$) and 20 °C ($t = 5.03$, $p < 0.01$), due to the −80 °C slope showing a positive correlation, and other slopes showing no correlation (Fig. 4A). Percentage N bases from −80 °C samples were seen to increase over time due to a high value at day 10, which was not seen in temperatures −20 °C or 4 °C but was seen to some extent at 20 °C. For the percentage of mapped reads, the slope for −80 °C was significantly different from the slope of 4 °C ($t = 5.19$, $p < 0.01$), due to the −80 °C slope showing no correlation, and other slopes showing a negative correlation (Fig. 4B). Percentage mapped reads were seen to decrease over time due to a low value at day 10, which was not seen in temperatures −20 °C or 4 °C but was seen to some extent at 20 °C. For percentage covered bases, the slope for −80 °C was significantly different from the slope of −20 °C ($t = 4.33$, $p < 0.01$), 4 °C ($t = 7.98$, $p < 0.01$) and 20 °C ($t = 5.06$, $p < 0.01$), due to the −80 °C slope showing a negative correlation, and other slopes showing no correlation (Fig. 4C). Percentage covered bases was seen to decrease over time due to a low value at day 10, which was not seen in temperature −20 °C or 4 °C but was seen to some extent at 20 °C. Finally, for longest number of Ns, the slope for −80 °C was significantly different from the slope of 4 °C ($t = 5.31$, $p < 0.01$) and the slope for 20 °C was significantly different from the slope of 4 °C ($t = 4.41$, $p < 0.01$), due to the 4 °C slope showing no correlation, and other slopes showing a negative correlation (Fig. 4D). Longest number of Ns was seen to decrease over time due to low values at day 7 and 10, which was seen at all temperatures except 4 °C.

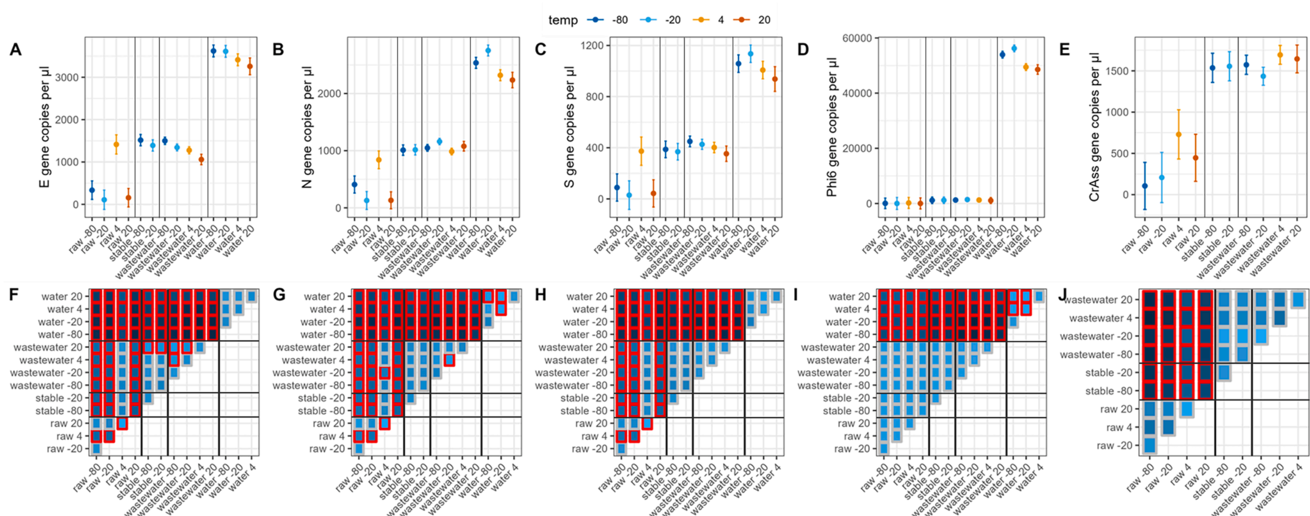

Fig. 3. Mean gene copies per µl nucleic acid extract, with 95 % confidence intervals, of the viral markers (A) SARS-CoV-2 E gene, (B) SARS-CoV-2 N gene, (C) SARS-CoV-2 S gene, (D) Phi6, and (E) CrAssphage under different experimental treatments and temperatures, with all time points merged (apart from time zero). Pairwise comparisons between temperatures and treatment groups are also shown (F) SARS-CoV-2 E gene, (G) SARS-CoV-2 N gene, (H) SARS-CoV-2 S gene, (I) Phi6, and (J) CrAssphage. For the pairwise comparisons, tiles are coloured by t ratio (the smaller the value, the darker the blue), red outlines represent significant comparisons, and bolder lines separate water types. Samples are: raw - unprocessed wastewater; Stable - processed and extracted wastewater not subjected to daily freeze-thaw cycles; wastewater - processed and extracted wastewater; water - processed and extracted distilled water.

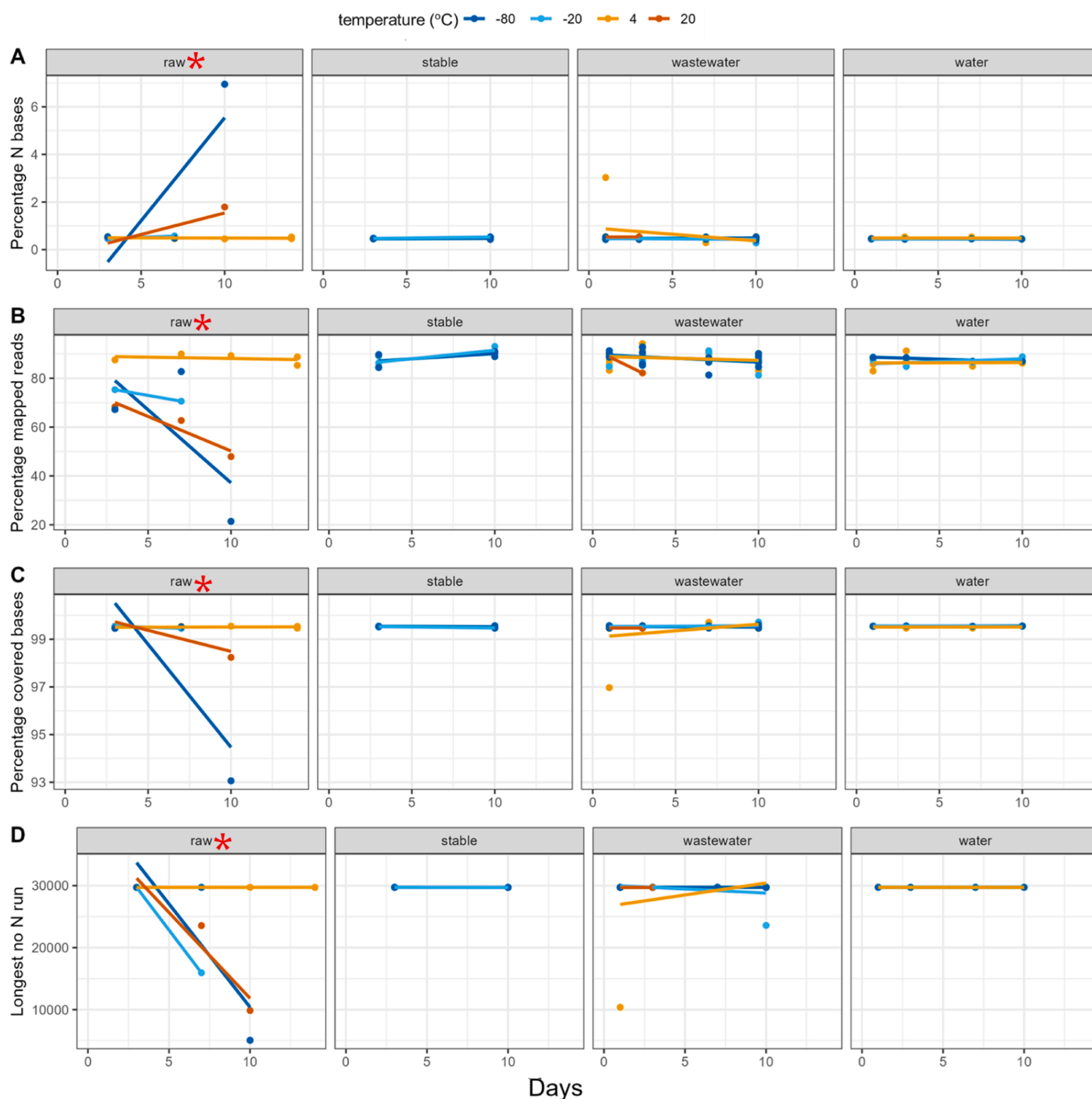

Fig. 4. Sequencing metrics for SARS-CoV-2, including (A) percentage of N bases, (B) percentage of mapped reads, (C) percentage of covered bases and (D) length of the longest run without Ns, over time under different experimental treatments and temperatures, with a linear regression over time for each of the experimental groups and temperatures. Red asterisks represent treatments where at least one of the temperatures has a significant effect on the slope between days and the sequencing metric, assessed using a linear model. Samples are: raw - unprocessed wastewater; Stable - processed and extracted wastewater not subjected to daily freeze-thaw cycles; wastewater - processed and extracted wastewater; water - processed and extracted distilled water.

Sequencing data showed no within treatment differences in mean percentage N bases, percentage covered bases or longest number of Ns between temperatures. However, temperature did have a significant effect on percentage mapped reads in the raw treatment group, with 4 °C showing significantly higher values than −80 °C and 20 °C (Fig. 5B and F).

3.4. *Effect of freeze-thaw cycles on the quality of RNA*

A subset of samples from the wastewater and raw treatment groups showed low (≤6) RNA Integrity Numbers (RIN) for samples kept at 20 °C when run on the TapeStation (Table S1). RIN did not decline over time, but the patterns remained consistent (i.e., lower starting RIN for −20 °C at both three and ten days). On average, 4 °C and −80 °C showed the highest RNA quality, with the highest individual RIN (8.8) coming from a sample stored at 4 °C for one day. Raw unprocessed wastewater samples corroborate the RT-qPCR results; the genetic material degrades in the sample when freeze-thawed and showed increased RIN for samples held at 4 °C. Comparing three freeze-thaw cycles to ten cycles showed no large difference for each condition over time.

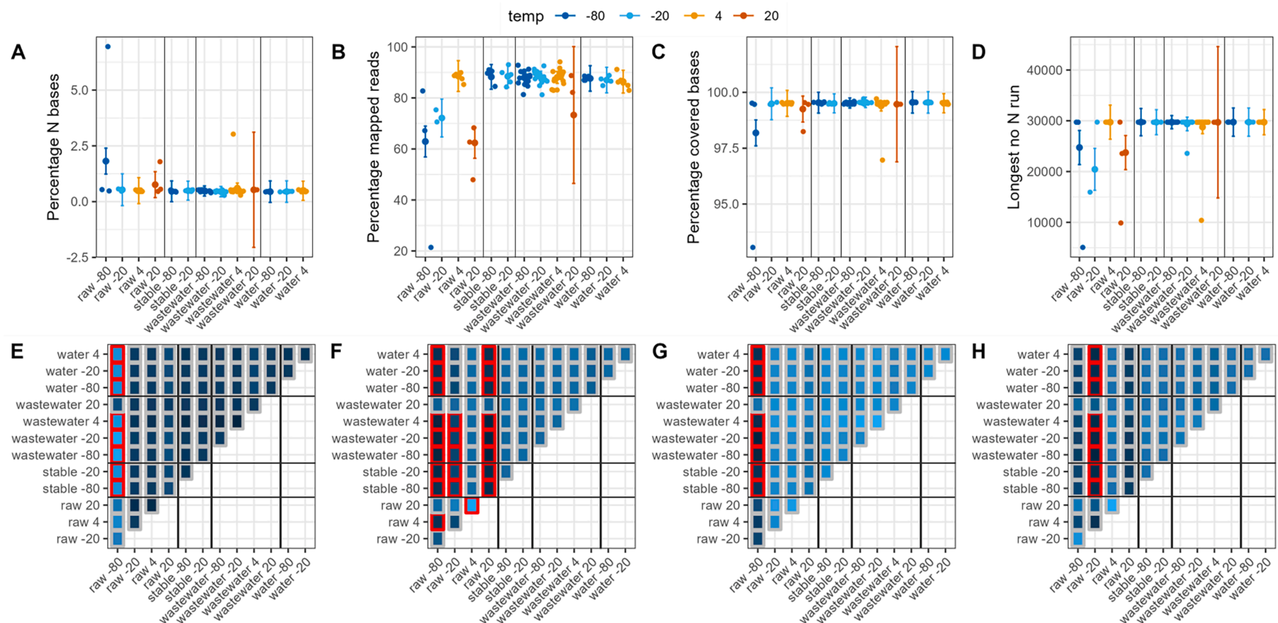

Fig. 5. Mean sequencing metrics for SARS-CoV-2, with jitter points and 95 % confidence intervals, including (A) percentage of N bases, (B) percentage of mapped reads, (C) percentage of covered bases and (D) length of the longest run without Ns under different experimental treatments and temperatures, with all time points merged (apart from time zero). Pairwise comparisons between temperatures and treatment groups are also shown for (E) percentage of N bases, (F) percentage of mapped reads, (G) percentage of covered bases and (H) longest number of Ns under. For the pairwise comparisons, tiles are coloured by t ratio (the smaller the value, the darker the blue) and red outlines represent significant comparisons. Samples are: raw - unprocessed wastewater; Stable - processed and extracted wastewater not subjected to daily freeze-thaw cycles; wastewater - processed and extracted wastewater; water - processed and extracted distilled water.

3.5. *Effect of viral gene abundance on archived wastewater samples*

Historical samples were re-quantified 12, 14 and 16 months after original quantification (Fig. 6). When viewing change in viral gene abundance, samples showed no strong association to increasing or decreasing over time, although low concentrations were more likely to increase, and higher concentrations were more likely to decrease. The largest changes were seen in samples that increased viral copies over time, with several samples showing >100 % increase.

4. Discussion

4.1. *Differential decay of SARS-CoV-2 genes*

Here, we evaluated the effects of storage time, temperature and freeze-thaw cycles on the concentration and quality of SARS-CoV-2, Phi6 and crAssphage derived RNA and DNA in wastewater. The integrity and detectability of RNA and DNA in wastewater samples can be compromised by various mechanisms of degradation during storage. Temperature fluctuations, freeze-thaw cycles, and duration of storage can all contribute to nucleic acid degradation via hydrolytic depurination, oxidative damage, or enzymatic digestion by nucleases (Ahmed et al., 2020; Guo et al., 2023). We observed that patterns in the relative abundance of extracted DNA/RNA under the different conditions were not consistent across viruses or markers measured using RT-qPCR. For all SARS-CoV-2 markers in raw sewage, one of the most obvious significant trends was an increase in detectable gene copy numbers over time after day 7 when stored at 4 °C. Wastewater and distilled water samples showed similar patterns to each other, indicating that increased quantification values are not solely the result of the SARS-CoV-2 RNA structure relaxing over time (Zhang et al., 2021). From an epidemiological standpoint, this trend has implications for WBE and sensitivity testing; increased sensitivity detection would benefit early detection of pathogens in this or a future pandemic. The capacity to detect low levels of circulating viruses and estimate infection rates within communities, particularly during the initial phases of an outbreak, becomes crucial to implementing appropriate mitigation measures (McMinn et al., 2023).

Overall, the Envelope protein (*E*) gene and Nucleocapsid protein (*N*) gene showed similar trends, with no significant relationship with storage time under any of the temperature regimes in the stable or freeze-thawed wastewater extract treatment groups. They also both showed a significant relationship in the freeze-thawed water treatment group under certain temperatures, indicating that these results may be more associated with the RNA itself, rather than the wastewater matrix. The SARS-CoV-2 genome has a secondary structure (Lan et al., 2022), which likely degrades at a different rate to their underlying coding sequences due to structural differences (Simmonds, 2020). With the *E* gene, this was demonstrated with −80 °C, −20 °C and 4 °C storage, which showed an initial increase in gene copies at day 3 followed by a decrease. With the *N* gene, this was demonstrated with −80 °C storage which showed a decrease in gene copies at day 3 followed by an increase in gene copies at day 10.

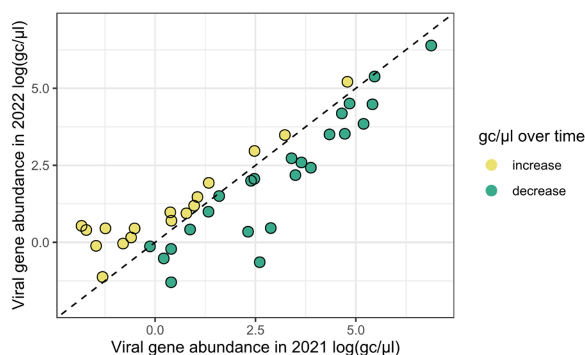

Fig. 6. Viral gene abundance of archived wastewater samples re-quantified after a year of −80 °C storage. The log of viral abundance measured in 2021 (the original quantification) is shown against the log of viral abundance measured from the same samples in 2022, with increase or decrease in gc/μl indicated. Dashed line shows a 1:1 relationship.

The SARS-CoV-2 Spike protein (*S*) gene behaved differently from *E* and *N*, due to it being the only marker where time significantly impacted copy number under stable conditions, showing a decrease over time. This suggests that the *S* gene may degrade at a faster rate than the other regions of the SARS-CoV-2 genome. Similar observations were made by Kumar et al. (2021) and Srivastava et al. (2021) who found consistently higher *N* gene concentrations compared to *S* gene concentrations in corresponding wastewater samples in their national WBE programme. Due to freeze thaw cycles not having a significant effect on *S* gene concentrations in wastewater, this may demonstrate the effect of mutation accumulation, or the wastewater matrix, and PCR amplification inhibitors breaking down or coalescing at a faster rate than the RNA (Murrell and Dorman, 2020). Microbes present in the wastewater will undergo the same impacts of freeze-thaw cycles but with unknown consequences (Guo et al., 2023). The nuclease activity present in the wastewater matrix can inactivate viruses (Mahlknecht, 2022), and with enveloped viruses like SARS-CoV-2 having a shorter lifespan outside of their host (Sala-Comorera et al., 2021), these agents of nucleic acid degradation may play a larger role (Guo et al., 2023), and the speed of this activity may vary in 24 h composite samples, compared to the grab samples presented here. The stability of the *S* gene in wastewater is particularly interesting because it is the target region in most COVID-19 vaccines (Jia and Gong, 2021), yet the common mutations in the spike protein undergoing positive evolutionary selection have given rise to new viral variants with greatly increased overall fitness (Magazine et al., 2022). The pattern of degradation in water is also surprising, as following decreases in concentration at day 3, this is followed by an increase at day 7 and a further decrease at day 10. Due to a lack of inhibitors in this treatment group, and the fact the same trend is seen in all temperature groups (and to some extent in the wastewater treatment group), regardless of freeze-thaw, could suggest that the inherent positioning of the *S* gene as a more internal gene on the SARS-CoV-2 genome away from terminal regions could be playing a role.

4.2. Differential impact of storage conditions on SARS-CoV-2 control viruses

The Phi6 bacteriophage has been commonly used as a surrogate virus for SARS-CoV-2 in clinical and environmental studies (Fedorenko et al., 2020; Gomes et al., 2022; Serrano-Aroca, 2022), making its comparison in this study with SARS-CoV-2 genes relevant. Trends in Phi6 gene copy number under raw, stable and wastewater conditions were dwarfed by those stored in water, where there was a significant effect of time. This variation potentially highlights differences observed in cultured viruses vs naturally occurring viruses; the SARS-CoV-2 already present in the wastewater, may have somewhat bridged the difference between the water and the wastewater values. We acknowledge that heat inactivation of the spiked SARS-CoV-2 may also cause RNA degradation, and that we would need to explore further methods of inactivation to eliminate this. Phi6 is often used as a suitable extraction and recovery control when quantifying SARS-CoV-2 in the laboratory (Farkas et al., 2023; Morvan et al., 2022; Scott et al., 2021; Tandukar et al., 2022), and in this context, the use of a virus that mirrors coronavirus degradation closely would be highly beneficial. Increased water values compared to wastewater values across viruses confirm that the wastewater matrix (including inhibitors) does inhibit RT-qPCR and can result in lower values of quantification. Our results show that with an almost identical degradation profile in water to the *N* gene under all temperatures, Phi6 is an excellent candidate to quantify SARS-CoV-2 loss during sample processing.

For crAssphage, significant effects of time were only seen in raw and a freeze-thawed wastewater extract treatment. Like most other markers, there was no significant effect of time when stored under stable conditions. Studying the degradation of crAssphage is crucial given its potential role as a faecal normalisation indicator in wastewater testing; crAssphage is commonly used as a normalisation marker in many WBE programmes (Heijnen et al., 2021; Hillary et al., 2021; Wilder et al., 2021). Although crAssphage shedding rates vary between people, crAssphage loads per person per day are constant over time at a population level (Langeveld et al., 2023), making it a valuable part of data interpretation for schemes that choose this method of normalisation. SARS-CoV-2 sewage data that have not been normalised can misrepresent actual short-term trends of SARS-CoV-2 circulation in populations connected to combined sewer networks, due to the impact of dilution caused by surface runoff (e.g. rain and snowmelt; Wilde et al., 2022). Discrepancies between raw and normalised data are considerable in short-term (1–2 week) trends (Langeveld et al., 2023), and short-term data are most relevant for informing public health actions. It is therefore of value to understand the stability of crAssphage in storage.

Storage conditions for downstream applications

For sequencing metrics, the effect of temperature over days was only seen to impact the raw treatment group, which was characterised by samples stored at 4 °C remaining stable, with those stored at −80 °C showing the most deviation. This was also reflected in mean difference of percentage mapped reads, where samples stored under −80 °C and 20 °C in the raw treatment group were significantly lower than those stored at 4 °C. This demonstrates that freezing unprocessed wastewater adversely affects its use in WBE applications and should be avoided. Applying these results to logistical arrangements in a laboratory setting highlights the need to process samples immediately, although both wastewater and water extracts appear relatively resilient to time and freeze-thaw cycles, both of which negatively impacted all sequencing metrics in the raw treatment. We acknowledge the limited sample size, particularly within the raw treatment groups, but aim to provide initial guidelines that can serve as a foundation for future expansion. For low-income countries, or surveillance programmes that involve transporting samples long distances, these logistic guidelines are challenging to follow (De Araujo et al., 2021). Many low-income countries lack widespread sewage networks, and keeping samples chilled throughout transport requires mobile refrigeration. Resolving this would require increased financial and infrastructure investment but would provide the early warning benefits afforded by conventional WBE (Adhikari and Halden, 2022). It could also be remedied by pre-processing samples close to source before transporting to a sequencing hub.

Logistical constraints would also impact RNA stability. An RNA integrity number (RIN) above 7 generally indicates good quality RNA suitable for demanding downstream applications like sequencing. While we acknowledge that RIN numbers typically refer to mammalian samples (Schroeder et al., 2006), lower RIN values under 6 indicate increasing degrees of RNA degradation, but this degraded RNA may still work for applications like RT-qPCR. Similar to the sequencing metrics, the RIN of samples here showed low values for wastewater samples stored at 20 °C, indicating degraded RNA quality for wastewater extracts stored at 20 °C, and likely explaining the large variation shown across all mean sequencing metrics for 20 °C. However, samples tested on day 3 and day 10 showed consistent RIN patterns over time at temperatures −80 °C, −20 °C and 4 °C. The highest RIN were observed 4 °C and −80 °C. For raw wastewater samples, we see similar pattens in both quantification and sequencing of RNA degradation associated with freeze-thaw cycles relative to storage at 4 °C. Comparing 3 versus 10 freeze-thaw cycles showed no significant decline in RNA quality over time for each storage condition. Following total nucleic acid extraction, samples remained relatively stable and suitable for quantification in a range of temperatures and after many freeze-thaw cycles. This provides the opportunity for laboratories to retrospectively analyse archived samples to establish a timestamp on the emergence of new variants or new and emerging viruses, detected both in real-time and post hoc. The re-quantification of historical samples after 12, 14, and 16 months of storage showed no clear pattern of increasing or decreasing viral gene abundance with storage time. Viral content is still capable of being quantified, but results should be interpreted with caution until the process of degradation is better understood. Whilst some samples did

exhibit large increases in viral copies, with several showing greater than 100 % growth, these increases did not correlate with duration of storage. This adds to the case of degradation not being a linear process, but instead a multifaceted one that incorporates the behaviour of inhibitors (Murrell and Dorman, 2020).

5. Conclusion

In agreement with procedures in the English wastewater monitoring programme (Wade et al., 2020), UK, our findings confirm that raw wastewater samples should be stored at or below 4 °C during collection by autosamplers and subsequent transport to the laboratory, and processed as soon as possible after collection to minimise loss of viral genetic material. The choice between freeze-thaw cycles and warmer temperature storage really depends on the virus of interest and the end goal of a specific project, such as quantification or sequencing data. Unexpected patterns in recovery of RNA and RNA viruses have been documented previously (Huang et al., 2017), and patterns of degradation observed here indicates that the RNA does not simply degrade linearly over time, but that more complex processes are occurring.

While an increasing number of biobanks are established and populated around the world, there is a need to develop standardised guidelines for wastewater sample handling, processing, and archiving. By understanding patterns of degradation for specific viruses and in different regions of the genome, it may be possible to account for them when re-screening historical samples; we show that historical wastewater samples archived at −80 °C can be reliably quantified even after 12 months of storage. It would be beneficial to the field, however, to expand our understanding of extended storage effects, freeze-thaw cycles, and processing stage by assessing the stability of other viruses, biomarkers, and antimicrobial resistance genes. Further, there is need to investigate impacts of preservatives or stabilised media on wastewater sample integrity. RNase inactivating agents or inhibitors of general microbial activity may have a role to play in the long-term storage of archivable samples.

CRediT authorship contribution statement

Rachel C. Williams: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **William B. Perry:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Kathryn Lambert-Slosarska:** Writing – review & editing, Conceptualization. **Ben Futcher:** Writing – review & editing, Data curation. **Cameron Pellett:** Formal analysis. **India Richardson-O'Neill:** Data curation. **Steve Paterson:** Writing – review & editing, Funding acquisition, Data curation. **Jasmine M.S. Grimsley:** Writing – review & editing, Conceptualization. **Matthew J. Wade:** Writing – review & editing. **Andrew J. Weightman:** Writing – review & editing, Funding acquisition. **Kata Farkas:** Writing – review & editing, Funding acquisition, Conceptualization. **Davey L. Jones:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Davey Jones reports financial support was provided by UK Government Accelerated Capability Environment. Steve Paterson reports financial support was provided by Department of Health and Social Care DHSC UK. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121879.

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