Implications of long-term sample storage on the recovery of viruses from wastewater and biobanking

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ABSTRACT

Wastewater-based monitoring has been widely implemented worldwide for the tracking of SARS-CoV-2 outbreaks and other viral diseases. In many surveillance programmes, unprocessed and processed wastewater samples are often frozen and stored for long periods of time in case the identification and tracing of an emerging health threat becomes necessary. However, extensive sample bioarchives may be difficult to maintain due to limitations in ultra-freezer capacity and associated cost. Furthermore, the stability of viruses in such samples has not been systematically investigated and hence the usefulness of bioarchives is unknown. In this study, we assessed the stability of SARS-CoV-2, influenza viruses, noroviruses and the faecal indicator virus, crAssphage, in raw wastewater and purified nucleic aacid extracts stored at -80 °C for 6–24 months. We found that the isolated viral RNA and DNA showed little signs of degradation in storage over 8–24 months, whereas extensive decay viral and loss of qPCR signal was observed during the storage of raw unprocessed wastewater. The most stable viruses were noroviruses and crAssphage, followed by SARS-CoV-2 and influenza A virus. Based on our findings, we conclude that bioarchives comprised of nucleic acid extracts derived from concentrated wastewater samples may be archived long-term, for at least two years, whereas raw wastewater samples may be discarded after one year.

1. Introduction

Wastewater-based epidemiology (WBE) has proven to be an important surveillance tool during the COVID-19 pandemic and for other infectious agents, such as poliovirus, influenza viruses and noroviruses (Klapsa et al., 2022; Mabasa et al., 2018; Wolfe et al., 2022). The WBE approach assumes that the levels of pathogens in municipal wastewater would reflect the case numbers in the community, providing a rapid, affordable and unbiased tool for disease outbreak monitoring (Fuschi et al., 2021; Xagoraraki and O'Brien, 2020). During the COVID-19 pandemic, over 70 countries implemented WBE for SARS-CoV-2 in their surveillance programmes.

The analysis of archived wastewater samples suggested that SARS-CoV-2 was in circulation in France and Italy several weeks before COVID-19 was first detected using conventional clinical testing (Deslandes et al., 2020; La Rosa et al., 2021), suggesting that WBE can be useful as an early warning system for the source-tracking of emerging

and novel viruses in the future. Therefore, it is often desirable to archive some of the samples taken during routine viral monitoring programmes so the samples can be retrospectively tested for emerging pathogens. This type of bioarchive would also be useful for investigating long-term trends and seasonality of known pathogens (Brinkman et al., 2017). However, although an international standard for biobanking exists (ISO 20,387:2018), it is not directly applicable for wastewater samples and does not provide guidance on pathogen stability.

The stability of viral pathogens and their genetic material in wastewater during storage is understudied. Most research has focused on the stability of SARS-CoV-2 and other coronaviruses in wastewater during transport (i.e. chilled or ambient temperatures), or during the process of heat-inactivation to inactivate the viruses (Ahmed et al., 2020; Bivins et al., 2020; Casanova et al., 2009; Gundy et al., 2009; Markt et al., 2021; Wang et al., 2005). To date, only two studies have explored the stability of SARS-CoV-2 and norovirus during storage, suggesting some reduction in viral titres after storage at −20 °C for nine days but better relative

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stability when stored at −75 °C for 2–3 months (Hokajärvi et al., 2021; Markt et al., 2021). The stability of viruses in archived raw wastewater samples is also poorly understood.

Maintaining the stability of genetic material for long periods is important for future applications; for example, when analysing archived samples using molecular detection methods. The rate at which genetic material decays is dependent on the type of molecule and the storage conditions (Lee et al., 2013). Under most conditions in biological samples and generally, DNA is more stable than RNA, and genetic material tends to remain viable longer when stored at lower temperatures (Baoutina et al., 2019). It has therefore been recommended to keep such samples in a freezer set ≤ −70 °C freezer for long term storage beyond days (CDC, 2021). Alternative methods of storage exist such as liquid nitrogen, lyophilisation and preservation using high ionic RNA-stabilizing agents, and encapsulation (RNAshell, Imagene) (Casagrande et al., 2020; Liu et al., 2015; Mathay et al., 2012). The main cause of degradation of free RNA is enzymatic breakdown (e.g. by RNAse and ribozyme activity) which is enhanced in the presence of some moisture and can occur at subzero temperatures (Fabre et al., 2013; Ma et al., 2004; Seyhan and Burke, 2000). Therefore, any method of long-term storage must suppress enzymatic activity enough so that degradation is prevented.

As viral detection methods become ever more sensitive (Charre et al., 2020), biobanks become crucial to facilitate retrospective processing. The aim of this study was to assess the effect of long-term storage (6–24 months) at −80 °C on the stability of viral RNA/DNA in raw wastewater samples and wastewater-derived nucleic acid extracts. Samples were processed and viruses were quantified using RT-qPCR immediately upon arrival and then either re-processed or re-quantified in stored samples. The RNA and DNA concentrations of human respiratory viruses (SARS-CoV-2, influenza A and B viruses [FluA and FluB], respiratory syncytial virus [RSV]) enteric viruses (norovirus GI and GII [NoVGI and NoVGII], enteroviruses [EV]), and a faecal indicator virus (crAssphage) measured before and after storage were compared.

2. Methods

2.1. Sample collection and storage

Wastewater influent samples were collected at municipal wastewater treatment plants (WWTPs) as part of the Welsh Government's National Wastewater Monitoring programme for public health surveillance. The programme started with monitoring eight sites across North Wales in 2020 and then expanded to 47 WWTP sites (comprising urban, peri-urban and rural wastewater catchments) and eight hospital outlets by 2023. These samples were collected using refrigerated autosamplers generating 24-hour composites. In case of equipment failure, grab samples were collected. The samples were transported chilled to the virology laboratory within 24–48 h. Upon arrival, a 220 ml aliquot was used for virological and chemical analyses and a 45 ml aliquot was transferred to the bioarchive and stored at −80 °C. For this study, samples collected from WWTPs across Wales (including rural and urban sites, were processed upon arrival and then re-processed after 6, 8, 12, 19 and 24 months of storage with approximately 60 samples in each batch (Table 1).

2.2. Sample processing

Upon arrival, 20 ml of the wastewater samples were subject to physico-chemical analyses including pH, turbidity, ammonium, orthophosphate and electrical conductivity (EC) measurements as described previously (Hillary et al., 2021). A 200 ml aliquot of the samples were subject to polyethylene glycol (PEG) precipitation as described previously (Farkas et al., 2021). In brief, solid matter was removed by centrifugation at 10,000x*g* at 4 °C for 10 min and then 1 ml of the supernatant was taken for crAssphage testing for the samples collected

Table 1
Summary of samples subject to re-processing after storage at −80 °C.

n	Storage duration	Date of first sample process	Date of re-process	Target viruses
62	6 months	15–16 February 2023	15 August 2023	SARS-CoV-2, NoVGI, NoVGII, EV, FluA, FluB, RSV
61	8 months	21–22 December 2022	23 August 2023	SARS-CoV-2, NoVGI, NoVGII, EV, FluA, FluB, RSV
62	12 months	6–9 June 2022	5 June 2023	SARS-CoV-2, crAssphage
57	19 months	10–29 October 2021	23 May 2023	SARS-CoV-2, crAssphage
56	24 months	3–13 August 2021	8 August 2023	SARS-CoV-2, crAssphage

before July 2021. The pH of a 150 ml aliquot of the supernatant was adjusted to 7.0–7.5 and spiked with a process control virus Phi6 bacteriophage (Kevill et al., 2022). The supernatant was then mixed with PEG 8000 and NaCl to reach the final concentration of 10 % and 2 %, respectively. The samples were then stored at 4 °C for 16 h followed by precipitation at 10,000x*g* at 4 °C for 30 min. The pellet was subject to viral nucleic acid extraction. Each batch of samples contained a negative (ion exchanged water) and a positive control (ion exchanged water spiked with phi6). The archived samples were thawed at 4 °C and subject to PEG precipitation in the same manner with 37.5 ml supernatants being spiked and concentrated.

2.3. Viral RNA/DNA extraction

The pellet concentrates and 0.2 ml aliquots of the supernatants taken prior to concentration were mixed with 0.8 ml NucliSens Lysis Buffer (BioMerieux, France) and viral nucleic acids were then extracted using the NucliSens extraction system on a KingFisher 96 Flex purification system (Thermo Scientific, USA) as described previously (Kevill et al., 2022). Each extraction plate contained a negative control (0.2 ml phosphate saline buffer [PBS], pH 7.4 mixed with 0.8 ml Lysis Buffer) and a positive control (0.2 ml PBS spiked with phi6 and mixed with 0.8 ml Lysis Buffer). The final volume of the extracts was 0.1 ml. The extracts were stored at 4 °C prior to viral detection and then transferred to −80 °C.

2.4. Detection and quantification of viruses

In this study, we quantified a Class I dsDNA virus (crAssphage), a Class III dsRNA virus (phi6), Class IV ssRNA(+) viruses (SARS-CoV-2, EV, NoVGI and NoVGII) and Class V ssRNA(-) viruses (FluA and FluB). For the quantification of viral RNA and DNA in the original, reprocessed and re-PCR-ed samples, existing protocols were used (Farkas et al., 2022). SARS-CoV-2 N1 gene fragment with Phi6 RNA were quantified in a duplex RT-qPCR assay, NoVGI – NoVGII – EV and FluA – FluB – RSV were quantified in triplex assays using the TaqMan Virus Fast qRT-PCR mix.

CrAssphage DNA was quantified in unconcentrated and concentrated wastewater samples using a singleplex qPCR assay with the QuantiNova Probe qPCR mix (Qiagen, Germany). Details of the qPCR conditions can be found in Table S1. The assays were carried out using a QuantStudio 6 Flex real-time PCR machine (Applied Biosystems, USA). As samples were tested in duplicates and each run contained 2 non-template controls and a 6-point 10x dilution series of standards.

2.5. Sample types

The following samples were included in this study:

- **Original samples:** wastewater samples (150 ml) processed and analysed upon arrival.
- **Reprocessed samples:** wastewater samples (37.5 ml) processed after storage at −80 °C for 6, 8, 12, 19 or 24 months.
- **Re-PCR-ed samples:** wastewater samples (150 ml) processed upon arrival and the RNA/DNA extracts were tested (by PCR re-amplification) for viruses after storage at −80 °C for 6, 8, 12, 19 or 24 months.
- **Raw samples:** wastewater sample where the DNA/DNA was extracted after the initial clarification using centrifugation, before PEG precipitation.
- **Concentrated samples:** wastewater sample where the DNA/DNA was extracted after PEG precipitation.

2.6. Data analyses

Real-time PCR data was initially analysed and quality controlled using the QuantStudio real-time PCR software v1.7 (Applied Biosystems, USA). Standard curve slope was between −3.1 and −3.6 and efficiency was between 90 % and 110 %. The viral nucleic acid concentrations were expressed at genome copies (gc) /μl RNA/DNA extract. The gc/l wastewater concentrations were calculated as:

$$\frac{\text{Concentration of the nucleic acid extract} \times \text{Extract volume}}{\text{Volume of sample supernatant processed}} \times 1000 \quad (1)$$

Virus stability was calculated as:

$$\frac{\text{Concentration measured after storage}}{\text{Concentration measured before storage}} \quad (2)$$

Virus recovery was calculated as:

$$\frac{\text{Concentration of the concentrated samples}}{\text{Concentration of the spiked process control}} \times 100\% \quad (3)$$

The calculated viral concentrations and recoveries are shown in Table S2.

As the data were not normally distributed (Shapiro-Wilk test, $p < 0.001$) Friedman's Two-Way Analysis of Variance by Ranks test and Wilcoxon Signed Rank test were used to explore the differences in concentrations of samples tested. Significance values were adjusted by the Bonferroni correction for multiple tests. Correlation between virus stability (Eq. (2)) and wastewater physico-chemical properties were calculated using Spearman's Rank correlation. Analyses were performed using SPSS v27 (IBM, USA).

3. Results

Throughout this study, RSV was not detected in any of the samples, while the other targets were noted. The most frequently detected and most relatively abundant viruses in qPCR assays were noroviruses, crAssphage and SARS-CoV-2, whereas enteroviruses and influenza viruses were less so (Table 2).

3.1. Stability of process control and indicator viruses

Both the process control virus Phi6 and the faecal indicator virus crAssphage were detected in the majority of the samples (Table 2) suggesting that viruses were successfully recovered. The Phi6 concentrations were approximately one order of magnitude higher in the reprocessed samples, probably due the different batch of virus used for spiking due to spiking with a different batch of Phi6 after thawing the samples (Fig. 1, Table S2). Interestingly, in the samples re-PCR-ed after 6 and 8 months, the Phi6 concentrations were significantly higher than in the original samples processed upon arrival. No such difference in Phi6 concentrations were observed after 12–24 months of storage.

Table 2

Virus detection rates in the original samples (0 h, pre-storage) and in samples reprocessed after storage (i.e. reprocessed samples) and in the nucleic acid extracts after storage (i.e. re-PCR-ed samples) after 6, 8, 12, 19 and 24 months of storage at −80 °C. The total number of samples tested is shown in brackets.

Virus	Storage duration	Original	Reprocessed	*Re*-PCR-ed
Phi6	6 months	98.39 % (62)	100 % (62)	98.39 % (62)
	8 months	96.72 % (61)	100 % (61)	98.36 % (61)
	12 months	83.87 % (62)	100 % (62)	82.26 % (62)
	19 months	100 % (57)	100 % (57)	98.25 % (57)
	24 months	83.93 % (56)	100 % (56)	86.54 % (52)
CrAssphage concentrate	12 months	100 % (62)	100 % (62)	100 % (58)
	19 months	80.7 % (57)	100 % (57)	100 % (57)
	24 months	100 % (56)	100 % (56)	100 % (54)
CrAssphage raw	12 months	100 % (62)	100 % (62)	100 % (62)
	19 months	100 % (57)	94.74 % (57)	98.21 % (57)
	24 months	100 % (56)	100 % (56)	100 % (54)
SARS-CoV-2	6 months	100 % (62)	88.71 (62)	100 % (62)
	8 months	100 % (61)	100 % (61)	100 % (61)
	12 months	100 % (62)	83.87 % (62)	93.44 % (62)
	19 months	96.49 % (57)	66.67 % (57)	94.74 % (57)
	24 months	100 % (56)	35.71 % (56)	100 % (52)
Influenza A virus	6 months	1.61 % (62)	0 % (62)	1.61 % (62)
	8 months	100 % (61)	19.67 % (61)	98.36 % (61)
Influenza B virus	6 months	0 % (62)	0 % (62)	0 % (62)
	8 months	1.64 % (61)	0 % (61)	0 % (61)
Norovirus GI	6 months	98.39 (62)	95.16 (62)	98.36 (61)
	8 months	100 % (61)	100 % (61)	100 % (59)
Norovirus GII	6 months	100 % (62)	96.77 (62)	98.36 (61)
	8 months	100 % (61)	93.44 % (61)	89.83 % (59)
Enteroviruses	6 months	6.45 (62)	3.23 % (62)	0 % (62)
	8 months	1.64 (61)	0 % (61)	0 % (59)

Both the raw and concentrated wastewater samples were subject to nucleic acid extraction and qPCR targeting crAssphage prior to and following storage at −80 °C for 12–24 months (Fig. 2). Approximately one order of magnitude difference in the crAssphage concentrations was observed in the concentrates vs raw wastewater indicating that the PEG method enabled ~10 % viral recovery. In both sample types, crAssphage concentration was lower when the sample was processed after 12–24 months storage. The difference was significant in almost all cases (Fig. 2). The crAssphage DNA concentration in the nucleic acid extracts was stable over 12 months of storage, however, it was significantly lower in concentrated sample extracts stored for 19–24 months and in the raw sample extracts stored for 24 months.
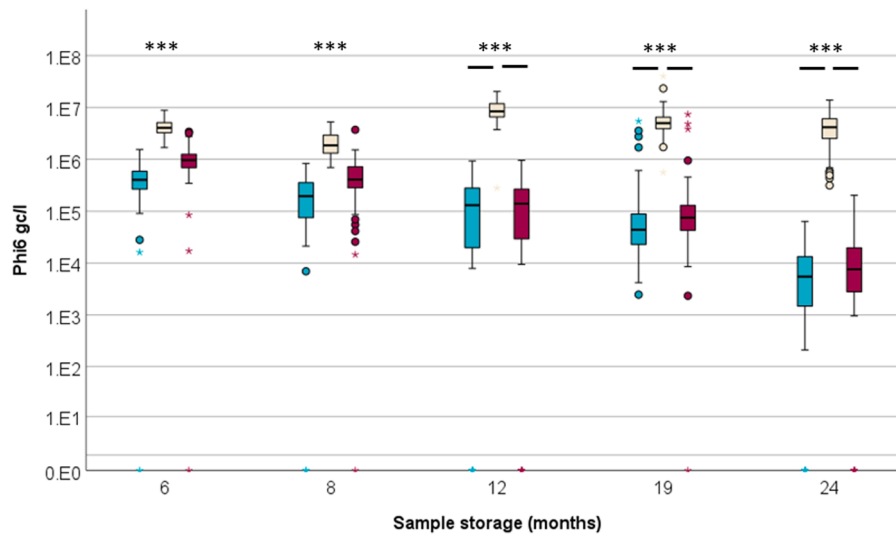
Fig. 1. Process control Phi6 bacteriophage concentrations (genome copies (gc)/l) in wastewater. Blue bars indicate the concentration/recovery in the original samples processed upon arrival, beige bars indicate the concentration/recovery in reprocessed samples stored at −80 °C and the red bars indicate concentration/recovery in re-PCR-ed samples. Reprocessed samples were spiked with Phi6 after storage. No recovery percentile is available of the samples re-PCR-ed after 24 months due to the lack of positive controls. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.
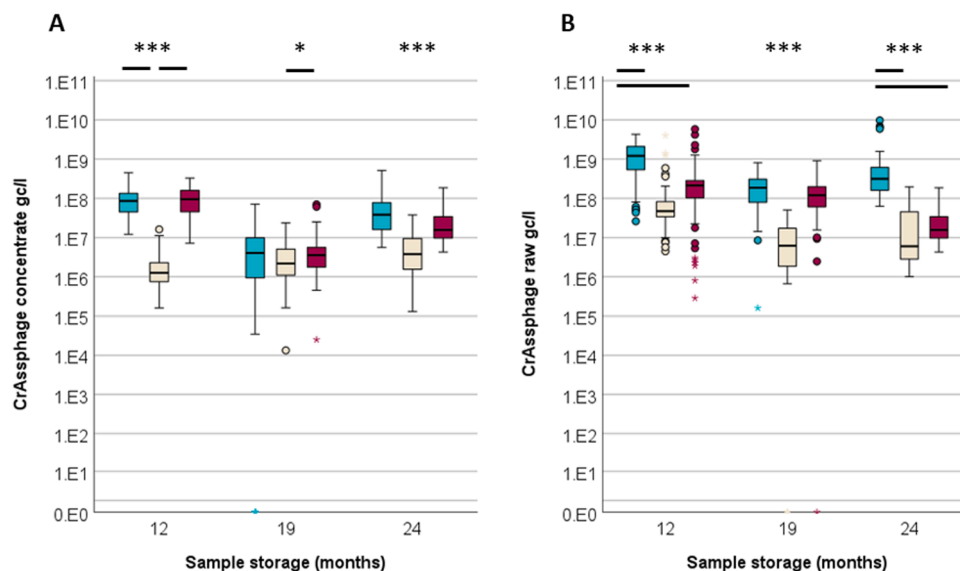

Fig. 2. CrAssphage concentrations (A) in concentrated samples and (B) in raw wastewater samples. Blue bars indicate the concentration in the original samples processed upon arrival, beige bars indicate the concentration in reprocessed samples after storage at −80 °C and the red bars indicate concentration in re-PCR-ed samples. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.2. Stability of respiratory viruses

SARS-CoV-2 was detected in the majority of the original samples, however, the detection rates and concentrations significantly reduced when the wastewater samples were reprocessed after storage (Table 2, Fig. 3). The decay of SARS-CoV-2 RNA in stored wastewater was more apparent after 12–24 months of storage when the samples, which originally had low SARS-CoV-2 concentrations, tested negative after reprocessing. Similar to Phi6, the concentrations of SARS-CoV-2 RNA in the re-PCR-ed samples were significantly higher after 6–19 months of storage than when originally processed (Table S2).

In the batch of samples stored for 6 months, FluA was detected in one original sample (983 gc/l). The sample was still positive in the re-PCR-ed concentrate sample (4661 gc/l), but it was negative in the reprocessed sample. All samples in the batch stored for 8 months were positive

for FluA in the original samples (Table 2), but the detection rates and concentrations in the reprocessed samples was significantly lower than what was noted upon arrival (Fig. 4). The viral concentrations in the re-PCR-ed samples were stable during storage for 8 months.

FluB was only detected in one sample upon arrival at low concentration (239 gc/l) and was not detected after storage for 8 months in either sample type.

3.3. Stability of enteric viruses

Noroviruses were detected at relatively high rates before and after 6–8 months of storage in all samples. Interestingly, the reprocessed sample concentrations were slightly but significantly higher than measured in the original samples before storage. For NoVGI, higher concentrations were observed in the re-PCR-ed samples than in the
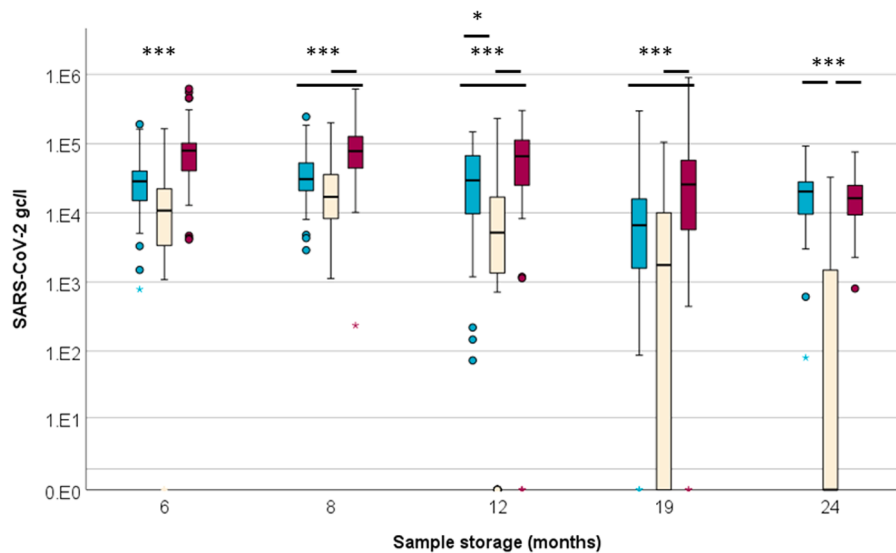
Fig. 3. SARS-CoV-2 concentrations in concentrated wastewater samples. Blue bars indicate the concentration in the original samples processed upon arrival, beige bars indicate the concentration in reprocessed wastewater samples after being stored at −80 °C and the red bars indicate concentration in re-PCR-ed samples. *: $p <$ 0.05, **: $p < 0.01$, ***: $p < 0.001$.
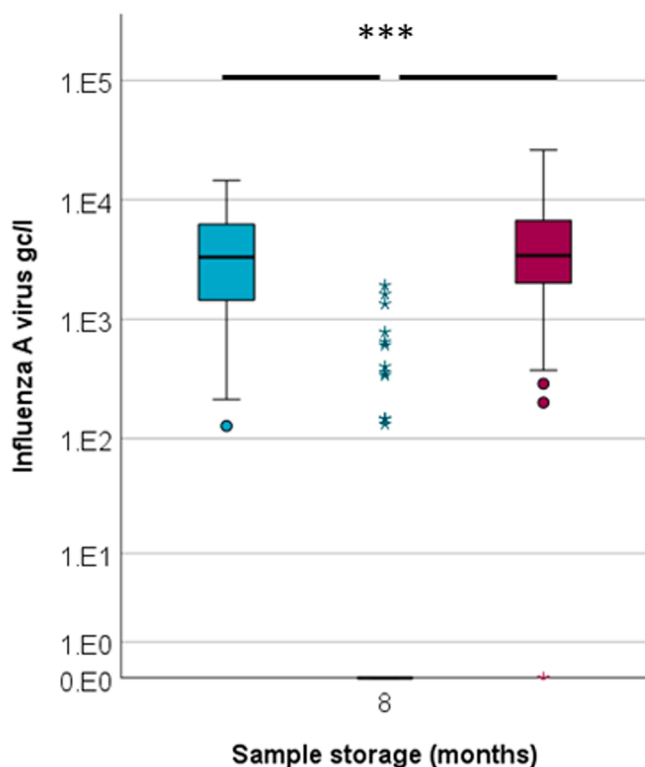

Fig. 4. Influenza A virus concentrations in concentrated wastewater samples after eight months of storage. Blue bars indicate the concentration in the original samples processed upon arrival, green stars indicate the concentration in reprocessed samples after being stored at −80 °C and the red bars indicate concentration in re-PCR-ed samples. ***: $p < 0.001$.

original samples. A similar trend was only observed for NoVGII after 8 months of storage (Fig. 5, Table S2).

Enteroviruses were only detected sporadically in the wastewater samples (Table 2). After 6 months storage, EV was detected in two reprocessed samples (2227 gc/l and 6585 gc/l) out of the four samples that tested positive before storage (560–30,996 gc/l). The one sample that tested positive at low concentration (141 gc/l) before 8 months

storage was negative when reprocessed. The re-PCR-ed samples were all negative for EV after 6–8 months storage.

3.4. Correlations between viral signal and sample physico-chemical properties

Weak positive and negative correlations between wastewater physico-chemical properties and virus stability were observed (Table 3). Ammonium levels correlated with crAssphage and NoVGII stability, whereas a negative correlation was observed with SARS-CoV-2 stability. Both negative and positive correlations were noted between crAssphage stability and orthophosphate and EC levels while NoV and orthophosphate showed positive correlations. Turbidity and pH showed positive correlations with noroviruses. SARS-CoV-2 and crAssphage stability negatively correlated with turbidity and SARS-CoV-2 showed positive correlation with pH.

4. Discussion

In this study, we explored the stability of viruses in wastewater and wastewater-derived nucleic acid extracts when stored at −80 °C for 6–24 months. Overall, we found good recovery rates of the process control virus Phi6 and of the faecal indicator virus crAssphage, suggesting that the PEG method and subsequent nucleic acid elution method enables the recovery of viruses from wastewater, in accordance with previous findings (Farkas et al., 2022; Kevill et al., 2022; Lu et al., 2020; Sapula et al., 2021; Zheng et al., 2023). Well-established virus quantification methods (Farkas et al., 2022) which enable the accurate, repeatable and sensitive quantification of the target viruses, hence the data reported here are reliable.

This is the first study exploring the effect of long-term, up to two years, storage of wastewater samples on virus concentrations. Overall, the results suggested that respiratory virus RNA, such as SARS-CoV-2 and FluA, and crAssphage DNA are more stable in nucleic acid extracts than in wastewater, while enteric noroviruses are relatively stable in both matrices. The difference in observations may partially be due to differences in the wastewater matrix. Here, we aimed to investigate the effect of physico-chemical properties of the wastewater on virus stability. However, only weak negative and positive correlations between viral rates and pH, EC, turbidity, ammonium and orthophosphate levels were observed, and the results were inconsistent (Table 3). This may
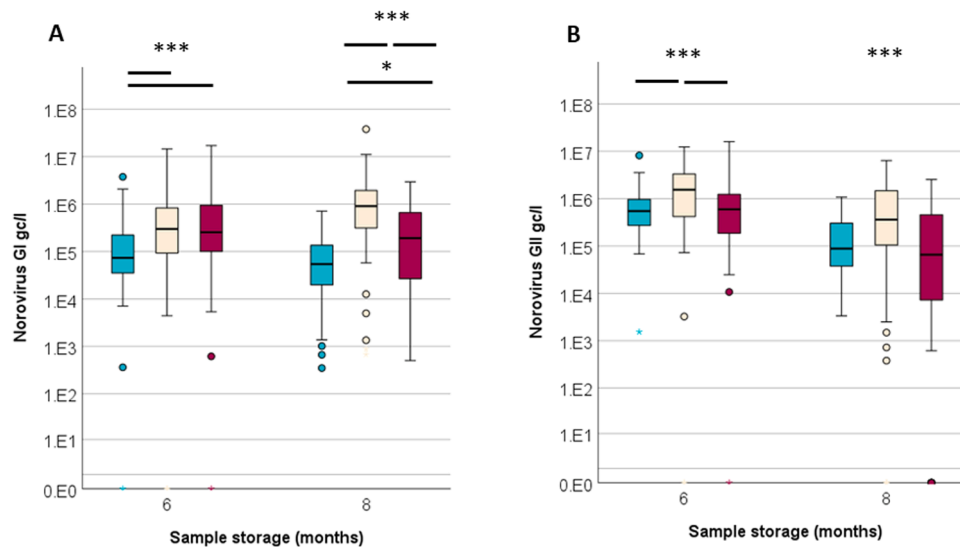
Fig. 5. (A) Norovirus GI and (B) norovirus GII concentrations in raw wastewater samples. Blue bars indicate the concentration in the original samples processed upon arrival, beige bars indicate the concentration in reprocessed samples after being stored at −80 °C and the red bars indicate concentration in re-PCR-ed samples. *: $p <$ 0.05, ***: $p < 0.001$.

Table 3

Spearman correlations between virus stability and wastewater physico-chemical properties. RNA/DNA indicated the stability estimated in the re-PCR-ed samples. *: $p < 0.05$, **: $p < 0.01$.

Wastewater property	Virus	Time in storage	Rho$_s$
Ammonium	CrAssphage DNA	24 months	+0.331*
Ammonium	NoVGII RNA	6 months	+0.346**
Ammonium	NoVGII RNA	8 months	+0.472**
Ammonium	SARS-CoV-2	6 months	−0.340**
Ammonium	SARS-CoV-2	8 months	−0.296*
Electrical conductivity	CrAssphage	24 months	−0.452**
Electrical conductivity	CrAssphage-raw DNA	24 months	+0.312*
Electrical conductivity	SARS-CoV-2 RNA	12 months	−0.303*
Orthophosphate	CrAssphage	19 months	+0.320*
Orthophosphate	CrAssphage-raw DNA	12 months	−0.306*
Orthophosphate	NoVGI	8 months	+0.277*
Orthophosphate	NoVGII	8 months	+0.380**
Orthophosphate	NoVGII RNA	8 months	+0.459**
pH	NoVGI	8 months	+0.399**
pH	NoVGII	8 months	+0.366**
pH	SARS-CoV-2	8 months	+0.301*
Turbidity	CrAssphage-raw	19 months	−0.263*
Turbidity	NoVGI RNA	8 months	+0.296*
Turbidity	NoVGII RNA	8 months	+0.470**
Turbidity	Phi6 RNA	12 months	−0.284*
Turbidity	SARS-CoV-2 DNA	12 months	−0.270*
Turbidity	SARS-CoV-2 DNA	6 months	−0.267*

suggest the chemical properties of wastewater rather affect viral recovery efficiency than viral stability. A more comprehensive investigation estimating wastewater sample parameters, including biological oxygen demand, the characterization of organic matter, ionic strength and composition, disinfectant and detergent concentrations would be necessary to better understand the effect of sample matrix on viral stability.

To date, few studies, limited in scope, have been conducted that report on the stability of viruses in wastewater stored in bioarchives as most research has been focusing on viral stability during transport and short-term storage at +4 °C and at ambient temperatures. These studies showed rapid viral decay under refrigerated or room temperature conditions, concluding that these temperatures are not suitable for the long-term storage of wastewater for viral quantification (Ahmed et al., 2020;

Bivins et al., 2020; Casanova et al., 2009; Gundy et al., 2009; Sherchan et al., 2023; Ye et al., 2016). Therefore, exploring the effect of long-term freezing and subsequent thawing on the stability of viruses is necessary for the establishment of bioarchives.

In our study, SARS-CoV-2 nucleic acids were recovered from wastewater with approximately 10 % loss of signal after 6–12 months of storage at −80 °C. The signal significantly reduced after one year of storage, such that many samples initially positive for SARS-CoV-2 at low concentrations were negative after storage. The same phenomenon was observed for FluA virus; virus detection significantly reduced after wastewater was stored at −80 °C for 8 months, whereas RNA was stable in nucleic acid extracts during the same period of time (Fig. 4). Overall, the results suggested that SARS-CoV-2 is more stable in wastewater when stored at −80 °C than influenza viruses (Table 2). The observed differences may be due to the different structure of the viruses and the differences in their decay mechanisms. For example, to date, no infectious SARS-CoV-2 viruses have been detected in wastewater (Albert et al., 2021; Rimoldi et al., 2020), suggesting that the viral proteins expressed on the envelop decay rapidly. However, the nucleocapsid may remain stable in wastewater, protecting the RNA for longer periods of time. While influenza viruses are also enveloped, their genome is segmented and not protected by a nucleocapsid and hence they may decay more rapidly than SARS-CoV-2.

In agreement with our results, it has previously been shown that SARS-CoV-2 RNA is stable in wastewater at −75 °C for up to 84 days, whereas significant reduction in SARS-CoV-2 RNA was noted when wastewater was stored −20 °C for 1–3 months (Fernandez-Cassi et al., 2021; Hokajärvi et al., 2021) and at 5 °C for 1 month (Ahmed et al., 2020). No longer-term studies on influenza virus stability have been performed to date. In our study, higher concentrations of SARS-CoV-2 were measured in stored nucleic acid extracts than before storage. Previous studies have also reported an increased SARS-CoV-2 signal when filtration-concentrated samples were stored frozen (Beattie et al., 2022). This may be due to the decay or co-precipitation of organic matter (e.g. potential PCR inhibitors) during storage which could result in more sensitive viral detection.

Similar stability patterns were observed for crAssphage in wastewater stored at −80 °C for 12–24 months (Fig. 2), where the viral DNA was more stable in DNA extracts than in wastewater. The observed stability may be due to the structure of the dsDNA genome of crAssphage. To date, no studies on the long-term stability of crAssphage in

wastewater have been reported, however, frequent detection this virus at high concentration in untreated and treated wastewater and in wastewater-polluted water suggests high stability (Sabar et al., 2022).

Interestingly, higher concentrations of noroviruses were noted in reprocessed samples stored for 6–8 months than in original samples. For NoVGI, slightly higher concentrations were noted in re-PCR-ed samples as well, whereas the concentration of NoVGII was similar in original and re-PCR-ed samples. This contradicts previous findings where approximately 1 log reduction in NoVGII concentration was observed in 84 days when the wastewater samples were stored at $+4$ °C, -20 °C and -75 °C (Hokajärvi et al., 2021). However, that study utilised ultrafiltration for sample processing, which may have excluded free RNA of viruses which decayed during storage, whereas the PEG precipitation method applied in this study concentrated free RNA along with intact viruses. The differences in viral recoveries in reprocessed samples may be due to the difference in the rate of capsid damage during sample thawing. While some viruses would decay during thawing, exposing the viral RNA to RNases, resulting in RNA decay, noroviruses may be better preserved and able to shield their genomes from enzymatic attack. More research is needed to better understand the processes that affect viral particles occurring during the freezing and thawing wastewater samples.

In our study, nly a few samples were positive for EV, providing limited information on the stability of EV in wastewater during storage. The results suggest that EV may be stable during storage for up to 6 months, but more data would be necessary to evaluate its long-term stability. Enteric viruses, such as EV and NoV are known to be generally stable in aquatic environments and wastewater (Gantzer et al., 1999; Stobnicka-Kupiec et al., 2022), similar to crAssphage, and have been considered persistent pathogens. These findings also suggest that non-enveloped viruses are often more stable in wastewater than enveloped viruses.

In summary, we found that the nucleic acids of faecal indicator (crAssphage), respiratory (SARS-CoV-2 and FluA) and enteric viruses (NoVGI and NoVGII) in concentrated wastewater-derived RNA/DNA extracts remain stable for 8–24 months at -80 °C, whereas viral decay was more rapid (6–12 months) when wastewater was stored under the same conditions. Therefore, we recommend for viral wastewater monitoring purposes, that archived wastewater is reprocessed within 12 months for comparable results to the original sample, whereas wastewater-derived RNA and DNA can be stored for at least two years and still provide valuable historical data.

The storage of RNA/DNA extract instead of wastewater is also cost-effective considering the space and energy required, as well as the associated costs (capital, recurrent and maintenance) for the storage of high volume (45 ml wastewater vs 0.1 ml extract) samples at -80 °C. Viral stability in archived samples may be extended by the use of preservation reagents, such as ethanol or RNAlater solution. A recent study also suggested that wastewater can be filtered and the membrane can also be preserved at either -20° or -80 °C to be analysed for viruses and bacteria for at least four years (Ando et al., 2023). This approach may be more suitable and cost effective for the long-term storage of wastewater samples than archiving unprocessed wastewater.

CRediT authorship contribution statement

Kata Farkas: Conceptualization, Methodology, Formal analysis, Supervision, Writing – review & editing. **Jessica Fletcher:** Investigation, Formal analysis, Writing – review & editing. **James Oxley:** Investigation, Writing – review & editing. **Nicola Ridding:** Writing – review & editing, Methodology, Investigation. **Rachel C. Williams:** Writing – review & editing, Methodology. **Nick Woodhall:** Writing – review & editing, Methodology, Investigation. **Andrew J. Weightman:** Writing – review & editing, Funding acquisition. **Gareth Cross:** Funding acquisition, Conceptualization. **Davey L. Jones:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

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.

Data availability

Data provided in supplementary material

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122209.

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