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Protocols

Wastewater sample storage for physicochemical and microbiological analysis

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ABSTRACT

Wastewater-based epidemiology (WBE) is a crucial tool for health and environmental monitoring, providing real-time data on public health indicators by analysis of sewage samples. Ensuring the integrity of these samples from collection to analysis is paramount. This study investigates the effects of different cold-storage conditions on the integrity of wastewater samples, focusing on both microbiological markers (such as extractable nucleic acids, SARS-CoV-2, and crAssphage) and physicochemical parameters (including ammonium, orthophosphate, pH, conductivity, and turbidity). Composite samples from the combined raw wastewater influent from five wastewater treatment works in South Wales, UK, were stored at 4°C, −20°C, and −80°C, and subjected to up to six freeze-thaw cycles over one year. The study found significant effects of storage temperature on the preservation of certain WBE markers, with the best yield most frequently seen in samples stored at −80°C. However, the majority of WBE markers showed no significant difference between storage at −80°C or at 4°C, demonstrating that it may not always be necessary to archive wastewater samples at ultra-low temperatures, thus reducing CO_2 emissions and laboratory energy costs. These findings underscore the importance of optimized storage conditions to maintain sample integrity, while ensuring accurate and reliable WBE data for public health and environmental monitoring.

1. Introduction

Environmental monitoring through wastewater-based epidemiology (WBE) involves the assessment of sewage to track key public health indicators (Gagliano et al., 2023). This is achieved by systematically collecting and analysing samples from sewers or wastewater treatment works (WwTWs) to identify the presence of specific chemical markers or microbiological targets (Parra-Arroyo et al., 2023). WBE stands as a progressive framework in public health, offering swift and non-intrusive access to anonymous, population-scale data on both disease prevalence and chemical usage (Sims & Kasprzyk-Hordern, 2020). Interest in this field has predominantly focused on illicit drug use (Zuccato et al., 2008, Tscharke et al., 2016), xenobiotics (Gasser et al., 2014, Rousis et al., 2017), and human biomarkers (Gracia-Lor et al., 2017, Daughton, 2018, Vitale et al., 2021). Additionally, there has been a notable emphasis on monitoring infectious diseases, such as polio (Asghar et al., 2014, Klapsa et al., 2022) and typhoid (Sikorski & Levine, 2020). Perhaps the greatest resurgence in WBE has been seen in response to the COVID-19 pandemic

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where it gained widespread application (Martin et al., 2020, Medema et al., 2020, Hillary et al., 2021, Ando et al., 2023). Monitoring SARS-CoV-2 in wastewater was instrumental in providing governments with real-time information on prevalence and emerging SARS-CoV-2 variants of concern (Amman et al., 2022), relying on accurate detection of both microbiological and physicochemical markers to account for wastewater dilution (Wilde et al., 2022). Temporal or spatial analysis of changes in WBE targets (e.g., compounds, genes, or microbes) holds promise for offering early warnings of local and regional disease outbreaks (Sims & Kasprzyk-Hordern, 2020), while comparisons across different geographical regions can provide valuable insights into the environmental and socioeconomic factors contributing to outbreaks (Prado et al., 2023). For these reasons, WBE will also become pivotal in investigating the emergence and spread of antibiotic resistance (Huijbers et al., 2019, Prieto Riquelme et al., 2022, Bydalek et al., 2023, Knight et al., 2024), as well as the regulation and public scrutiny of untreated wastewater spills into the environment, and the risk this poses to public health in the context of One Health (Perry et al., 2024a).

To produce robust monitoring data via WBE, it is critical that wastewater samples are collected and stored appropriately. The integrity of samples must be protected from the point of collection through processing to analysis (Ahmed et al., 2020). Adequate storage conditions are essential for preserving the chemical and microbiological composition of the samples, preventing degradation or contamination (Baker & Kasprzyk-Hordern, 2011, Fedorova et al., 2014; Ahmed et al., 2020, Williams et al., 2024) through chemical, physical and biological mechanisms (Sliwka-Kaszyńska et al., 2003). For example, temperature, freeze-thaw cycles, exposure to oxygen and microbial degradation can all contribute to changes in wastewater composition (Fedorova et al., 2014, Ahmed et al., 2020, Guo et al., 2023).

Adherence to standard operating procedures (SOPs), including the use of chilled automated sampling systems, appropriate sample collection and transportation, controlled environmental conditions, and suitable long-term storage, ensure sample integrity and accurate analysis (O'Brien et al., 2019, Ahmed et al., 2020). In addition, appropriate storage practices facilitate the creation of a reliable historical archive (Thaler & Sakmar, 2021), bioarchive, or biobank, which not only serves as a reference for current analyses, but also allows for trend analyses to understand temporal variations in wastewater indicators and enables samples to be revisited as new technologies, public health biomarkers or pathogens emerge (Thaler & Sakmar, 2021). This approach ensures that the monitoring data derived from WBE is of high quality, provides valuable insights for public health, environmental trends, and the efficacy of interventions over time.

Despite the clear need for suitable storage of wastewater samples, there is a dearth of information on suitable temperature conditions for storing samples for both microbiological and physicochemical analyses (Ahmed et al., 2020). The aim of this study was to investigate wastewater cold-storage temperatures and their effect on different biological markers, such as extractable nucleic acids as well as SARS-CoV-2 and crAssphage gene levels, while also determining physicochemical parameters used routinely in wastewater-based monitoring (e.g. for normalisation of wastewater flow and sample dilution). In this study, we used composite wastewater samples collected and pooled from five WwTW sites in South Wales, UK, and analysed them for the concentration of DNA, RNA, ammonium, orthophosphate, pH, electrical conductivity, and turbidity, as well as SARS-CoV-2 nucleocapsid (*N*1 region) and the faecal marker virus crAssphage CPQ_056 marker gene copies. Importantly, analyses were conducted after the samples had been stored under standard cold-storage conditions at 4°C, −20°C, and −80°C, or after being subjected to a series of up to six freeze-thaw cycles over one year of storage.

2. Methods

2.1. Wastewater sample collection

Untreated wastewater samples were collected from Dŵr Cymru Welsh Water (DCWW) wastewater treatment works across South Wales at Cardiff, Newport, Swansea, Carmarthen, and Haverford West between 11 and 15 October 2021, taken as part of the Welsh National Wastewater Monitoring Programme (for background see Perry et al., 2024b). On each sampling occasion, one litre of wastewater influent was collected from each of the WwTWs by automated sampling using Bühler BU4010 (Hach UK Ltd.) or Aquacell S320H (Aquamatic Ltd.) water samplers. Samples were collected at 15 minute intervals over a 24 h period for each day and transported in a refrigerated Mobicool MT48W cooler box. However, at Haverford West spot samples were collected using a Buerkle™ telescopic rod (125–250 cm) and a 1 litre stainless steel pendulum beaker due to the autosampler at this site being out of commission at the time of collection. Temperature data for sample collection, transportation and initial laboratory handling was logged using an IButton DS1922L Thermochron Data Logger.

Samples (300 ml volumes) from each WwTW site each day were pooled together and mixed (collectively for 5 days) in a 10 litre Nalgene® round carboy with stopcock at 4°C to obtain a total volume of 7.5 litres of mixed raw wastewater. Aliquots of this well mixed wastewater were then dispensed in 50 ml volumes contained in 50 ml sterile polypropylene centrifuge tubes (Sarstedt AG and Co. KG) and stored at 4°C, −20°C, and −80°C until required. The remaining mixed wastewater was also stored at 4°C in a glass 2 litre-Duran bottle and used as the 'Bulk' storage sample (see sample storage experiment below).

2.2. Sample storage experiment

To compare long-term sample storage, different scenarios were investigated. (A) 'Standard' (ST) storage, samples stored long-term (for up to one year) in 50 ml volumes without being disturbed (4°C, −20°C, −80°C). (B) 'Freeze-thaw' (FT) storage, samples stored long-term (for up to one year) in 50 ml volumes but were all removed from storage (at 1, 2, 4, 8, 12 and 52 weeks) and therefore subjected to repeated (up to six for 52 weeks of storage) freeze-thaw cycles (-20°C, −80°C). (C) 'Bulk' storage, sample (1 litre, maintained at 4°C) stored in a 2 litre-Duran bottle and sampled at 0, 1, 2, 4, 8, 12 and 52 weeks (4°C only).

Wastewater samples from each storage condition were then analysed at 0, 1, 2, 4, 8, 12 and 52 weeks for different microbiological (nucleic acid content as well as SARS-CoV-2 *N*1 and crAssphage marker gene copies) and at 0 and 52 weeks for physicochemical (pH, ammonium, orthophosphate, and turbidity) parameters. Samples were stored in a Haier Biomedical HLR-198 laboratory refrigerator (4°C), Liebherr GG 5210 laboratory freezer (-20°C) and a Thermo Scientific TSX50086V ultra-low freezer (-80°C) for up to 52 weeks.

2.3. Physicochemical analysis

Wastewater ammonium ($NH4^{+}$) and orthophosphate (PO_4^{3-}) concentrations were determined by colorimetric analysis using the salicylic acid procedure (λ = 667 nm; Mulvaney, 1996) and the molybdate blue method (λ = 820 nm; Murphy & Riley, 1962), respectively. Analyses were performed on a 96-well plate format using a SPECTROstar Nano microplate reader (BMG Labtech). Electrical conductivity and pH were measured using a SevenCompact Duo S213 pH/Conductivity Meter (Metler-Toledo GmbH). Samples for turbidity were mixed thoroughly to resuspend particles and measured with a HI83414 turbidity meter (Hanna Instruments Ltd).

2.4. Viral concentration and nucleic acid extraction

Viral particles and nucleic acids were obtained from wastewater by polyethylene glycol (PEG) precipitation as described previously (Farkas et al., 2021). A 40 % (w/v) PEG solution (PEG8000, Sigma-Aldrich) with 8 % (w/v) NaCl (Sigma-Aldrich) was prepared and autoclaved at 121 °C for 30 min. Wastewater (3 ×50 ml aliquots) was poured into sterile 250 ml polypropylene centrifuge bottles (Nalgene) and centrifuged at 3000 x g for 15 min at 4°C in an Eppendorf 5920 R centrifuge. The supernatant (150 ml) was recovered, transferred to a new sterile 250 ml centrifuge bottle, and pH adjusted to 7.0–7.5 with 1 M NaOH or 0.5 M HCl. PEG-NaCl solution (50 ml) was then added to each wastewater sample, mixed, and incubated overnight at 4°C before centrifuging (10, 000 x g, 4°C, 30 min). Supernatant was then carefully removed with a SLS Lab Pro Safevac vacuum aspiration system and the concentrated pellet resuspended in 500 µl of phosphate buffered saline (PBS) and stored at 4°C until required for nucleic acid extraction and purification.

Nucleic acids (RNA and DNA) were extracted from PBS concentrates using the automated Bio-On-Magnetic-Beads (BOMB) protocol (Oberacker et al., 2019) and a GEN2 OT-2 pipetting robot (Opentrons). A volume of 100 µl PBS nucleic acid concentrate was added to a 96 deep-well-plate (Applied Biosystems) with 240 µl of magnetic bead solution (Magnacell Ltd) and 270 µl guanidine isothiocyanate (GITC) based lysis buffer (Oberacker et al., 2019). To aid adsorption of nucleic acids onto magnetic beads, the 96 deep-well-plate was placed onto an IKA MS 3 orbital shaker for 12 mins at 2000 rpm. The plate was then centrifuged for 1 min at 3000 rpm (Eppendorf 5920 R centrifuge) and placed on a magnetic rack for 10 mins to allow the beads to form pellets on the side of the wells. The magnetic beads were then washed three times with 200 µl 80 % (v/v) ethanol and dried at 60°C for 10 mins. Nucleic acids adsorbed onto the magnetic bead surface were eluted in 50 µl of RNAse-free water (Severn Biotech Ltd). To avoid transfer of any magnetic beads, a magnetic rack was used to separate the beads from the nucleic acid solution, with the nucleic acid solution subsequently transferred to 96 well plates for immediate downstream analysis or stored at −80°C for future use.

2.5. Determination of extractable DNA and RNA

Extracted nucleic acids were quantified using a Qubit™ 4 Fluorometer (Invitrogen) with the Qubit dsDNA HS Assay Kit for DNA and the Qubit RNA HS Assay Kit for RNA (Invitrogen). Samples (2 µl aliquots) were analysed in duplicate, and the mean value recorded as ng/µl of nucleic acid.

2.6. RT-qPCR analysis of SARS-CoV-2 and crAssphage

Quantitative-PCR for the SARS-CoV-2 nucleocapsid gene (*N*1 region) and crAssphage CPQ_056 marker was performed using the Luna® Universal Probe One-Step RT-qPCR Kit (New England BioLabs) on a QuantStudio™ 7 real-time PCR system (Applied Biosystems) in a multiplex PCR assay. Samples were run in a MicroAmp Endura optical 384 well clear plate (Applied Biosystems) with a reaction volume of 10 µl. The mastermix contained Luna® Universal Probe qPCR Master Mix, 10 pmol of each primer and 5 pmol probe, molecular grade water and 4 µl sample or standard (SARS-CoV-2 *N*1 or crAssphage). All plates were set up using a GEN2 OT-2 pipetting robot (Opentrons). Samples were run in triplicate, against synthetic ssRNA (SARS-CoV-2 *N*1 gene fragment; Twist Bioscience) or dsDNA (crAssphage CPQ_056 marker generated in house by PCR from wastewater) standard curve dilution series of the target sequence in the range of 1.0–1.0 $\times 10^6$ copies/µl. Both standards were quantified using a Qubit™ 4 Fluorometer (Invitrogen). Additionally, no template controls (molecular-grade water) were also run in triplicate. Standard curve slopes were −3.1 and −3.0, with intercepts of 41.1 and 35.7, efficiency of 109 % and 117 %, and R^2 value of 0.99 and 0.99 for SARS-CoV-2 *N*1 and crAssphage, respectively. Thermal cycler conditions were as follows: 55°C for 60 min (reverse transcription); 95°C for 5 min (reverse transcriptase inactivation); followed by 45 cycles of 95°C for 15 s, 60°C for 1 min, and 65°C for 1 min at increments of 1.6°C per second (Kevill et al., 2022). Primers and probes used were as follows: *N*1, forward primer GACCCCAAAATCAGCGAAAT, reverse primer TCTGGTTACTGCCAGTTGAATCTG, probe FAM-ACCCCGCATT ACGTTTGGTGGACC-NFQ-MGB (Lu et al., 2020), crAssphage, forward primer CAGAAGTACAAACTCCTAAAAAACGTAGAG, reverse primer GATGACCAATAAACAAGCCATTAGC, probe VIC-AATAACGATTTAC GTGATGTAAC-NFQ-MGB (Stachler et al., 2017).

2.7. Statistical analysis

Linear models were implemented for chemical measurements, and linear mixed effect models were implemented for extractable nucleic acid concentration and qPCR measurements using the R package lmer (Bates et al., 2015). For chemistry, the response variable for the models was the change in measurement before and after the experiment. Change was calculated by subtracting the measurement from each replicate in each of the temperatures and storage conditions after the experiment (post-experiment) from the mean measurement at the start of the experiment (pre-experiment). Time zero measurements were taken from three replicate samples of the mixed wastewater sample. For qPCR measurements and extractable nucleic acid concentrations, because there was a time series, change was not calculated, and gene copies/l and concentration were used as the response variable. Indeed, replicates of the same samples were measured throughout the time series, and therefore, to account for this, the unique sample identifier was included as a random effect.

All models contained the fixed terms temperature (levels: 4°C, −20°C, and −80°C) and storage condition (levels: FT and ST), along with an interaction term between these terms. The R package lmerTest (Kuznetsova et al., 2017) was used to calculate *p* values associated with the linear mixed effect model output. Finally, the estimated marginal means package emmeans (Lenth, 2016) was used to calculate pairwise differences between treatments with a Tukey multiple comparisons adjustment. Comparison of slopes for the DNA, RNA, *N*1 and crAssphage datasets was conducted using the emtrends function in the emmeans R package. For SARS-CoV-2 *N*1 and crAssphage, as well as RNA and DNA concentration, there was also a time series, which was included as a fixed term in the relevant models. Week 0 was included in the regression plots but was excluded from the models because of the focus on comparing means between treatment groups. The final models contained measurements from weeks 1, 2, 4, 8, 12, and 52.

3. Results

3.1. Wastewater sample collection

Sample integrity was ensured during collection, transportation from the WwTW site and initial laboratory handling by continuous recording of the sample temperature. All samples were consistently maintained at ≤8°C before sample pooling was undertaken (see Supplementary Figure S1). This included the use of refrigerated autosamplers, refrigerated sample storage boxes during transportation and rapid sample handling in the laboratory. Pooled wastewater samples were then maintained and processed at 4°C prior to initiation of experiments.

3.2. Storage effects on physicochemical properties of wastewater

3.2.1. Ammonium

No overall significant effect of treatment on ammonium concentrations was observed between pre- and post-experimental concentrations (Fig. 1A), but temperature had a significant effect ($F_{2,12} = 5.77$, Sum Sq = 888.59, $p = 0.02$), as well as the interaction term between treatment and temperature ($F_{2,12} = 4.04$, Sum Sq = 621.59, $p = 0.05$).

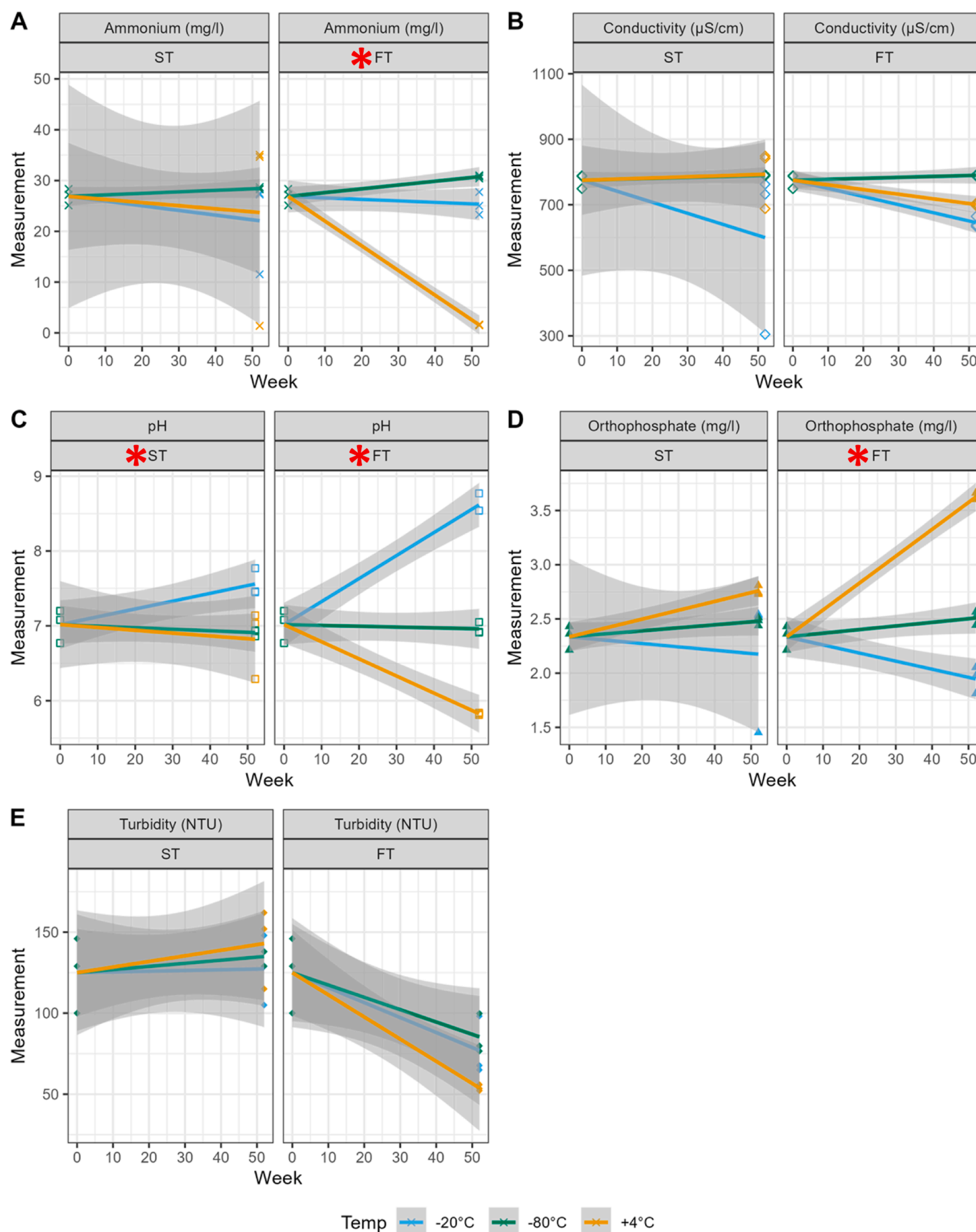

Fig. 1. Measurements of (A) ammonium concentration, (B) electrical conductivity, (C) pH, (D) orthophosphate concentration, and (E) turbidity before and after storage for 52 weeks at 4°C, −20°C and −80°C, while either having gone through standard (ST), or freeze-thaw (FT) storage conditions. Red asterisks highlight significant pairwise differences between pre- and post-experimental measurements for each storage condition. Samples stored under FT conditions experienced six freeze-thaw cycles, except samples at 4°C, these samples were stored as bulk (see Methods, Section 2.2).

Ammonium concentrations remained constant at ~25 mg N/l after 52 weeks of storage at all temperature and conditions with the exception of wastewater samples stored at 4°C under bulk conditions (Table 1). Samples stored at 4°C under bulk conditions showed a substantial decrease in ammonium concentrations with mean values recorded at 1.6 mg N/l at week 52. This was reflected in the significant pairwise comparisons, where samples stored at 4°C under bulk conditions had significantly greater differences in concentrations pre- and post-experiment than those stored at −80°C ($t = 4.07$, $p = 0.02$) and −20°C ($t = 3.32$, $p = 0.05$) under freeze-thaw conditions, caused by a decrease in concentration over time at 4°C under bulk conditions (Fig. 2A). In addition, samples stored at 4°C under bulk conditions also had a significantly greater difference in concentration pre- and post-experiment compared to samples stored at −80°C under standard conditions ($t = 3.75$, $p = 0.03$).

Table 1
Raw wastewater physicochemical parameters measured in pooled wastewater samples after storage for 52 weeks at different temperatures and conditions.

Storage temperature (°C)	Storage conditions	Length of time stored (weeks)	Physicochemical parameters (mean ± SD)				
			Ammonium (mg N/l)	Conductivity (μS/cm)	pH	Orthophosphate (mg P/l)	Turbidity (NTU)
None	None	0	26.9 ± 1.6	775 ± 22	7.0 ± 0.2	2.3 ± 0.1	125.0 ± 23.3
4	Standard	52	23.7 ± 19.3	793 ± 91	6.8 ± 0.5	2.8 ± 0.0	143.0 ± 24.8
−20	Standard	52	22.1 ± 9.1	600 ± 256	7.6 ± 0.2	2.2 ± 0.6	127.3 ± 21.5
−80	Standard	52	27.9 ± 0.2	790 ± 2	6.9 ± 0.0	2.5 ± 0.0	135.0 ± 5.2
4	Bulk	52	1.6 ± 0.1	701 ± 4	5.8 ± 0.0	3.6 ± 0.0	53.9 ± 1.9
−20	Freeze-thaw	52	25.3 ± 2.2	646 ± 17	8.6 ± 0.1	1.9 ± 0.1	77.0 ± 18.4
−80	Freeze-thaw	52	30.8 ± 0.3	790 ± 2	7.0 ± 0.1	2.5 ± 0.1	85.4 ± 12.6

Data values represent means ± standard deviation of three replicate samples for each analysis.
NTU = Nephelometric Turbidity Units.

3.2.2. Electrical conductivity

No significant effect of treatment, temperature, or their interaction term were observed between pre- and post-experimental conductivity measurements (Fig. 1B). This included all pairwise comparisons (Fig. 2B). Mean measurements of wastewater conductivity (specific conductance) at week 52 were similar (600–793 μS/cm) to that observed at time zero (775 ± 22 μS/cm) for all sample treatments and temperatures (Table 1). Interestingly, samples stored at −20°C at both standard and freeze-thaw conditions were the lowest recorded mean values of conductivity, both with values of <646 μS/cm, although no significant change was detected.

3.2.3. pH

No overall significant effect of treatment on pH was observed between pre- and post-experimental measurements. However, temperature had a significant effect ($F2,12 = 106.05$, Sum Sq = 9.64, $p < 0.01$), as well as the interaction term between treatment and temperature ($F2,12 = 34.68$, Sum Sq = 3.15, $p < 0.01$; Fig. 1C). Samples stored under standard conditions at 4°C and −80°C had very little changes in their pH (6.8–6.9) from that measured on raw wastewater at time zero (pH = 7.0 ± 0.2; Table 1). However, samples stored at −20°C under standard conditions did have significantly greater differences in concentrations pre- and post-experiment compared to those stored at −80°C ($t = 3.73$, $p = 0.03$) and 4°C ($t = 4.25$, $p = 0.01$) under standard conditions, caused by an increase in pH over time in samples stored at −20°C under standard conditions (Fig. 2C). Storage temperature at −80°C under freeze-thaw conditions showed no effect on pH. Whereas, samples stored under bulk conditions at 4°C (mean pH = 5.8) ($t = 6.51$, $p < 0.01$) or with freeze-thaw at −20°C (mean pH = 8.6) ($t = 16.03$, $p < 0.01$) had significantly greater differences in concentrations pre- and post-experiment compared to those stored at −80°C, caused by an increase in pH over time in samples stored at −20°C and a decrease in pH over time in samples stored at 4°C (Fig. 2C; Table 1).

3.2.4. Orthophosphate

No overall significant effect of treatment was observed between pre- and post-experimental orthophosphate concentrations; however, temperature had a significant effect ($F2,12 = 28.25$, Sum Sq = 3.91, $p < 0.01$), and the interaction term between treatment and temperature ($F2,12 = 7.08$, Sum Sq = 0.098, $p < 0.01$; Fig. 1D). Under standard storage conditions at −20°C and −80°C, orthophosphate concentrations remained relatively stable for one year, with mean values of 2.2 and 2.5 mg P/l, respectively compared with 2.3 mg P/l at time zero (Table 1), with no significantly greater differences in concentrations pre- and post-experiment observed between temperatures (Fig. 2D). However, when samples were stored under different temperatures with regular freeze-thaw cycles at −20°C or stored for one year in bulk at 4°C, orthophosphate showed either a slight decrease (1.9 mg P/l) or a marked increase (3.6 mg P/l) in concentration, respectively. This was reflected in the significant pairwise comparisons, where samples stored at 4°C under bulk conditions had significantly greater differences in concentrations pre- and post-experiment than those stored at −20°C ($t = 7.81$, $p < 0.01$) and −80°C ($t = 5.20$, $p < 0.01$) under freeze-thaw conditions, caused by an increase in concentration over time in samples stored at 4°C under bulk conditions (Fig. 2D). Wastewater samples stored at −80°C were similar in orthophosphate concentration to time zero samples with or without freeze-thaw storage conditions (mean value = 2.5 mg P/l). An increased orthophosphate concentration was also observed at 4°C under standard storage conditions, but the difference in concentration pre- and post-experiment did not significantly differ from other temperatures in the standard storage conditions.

3.2.5. Turbidity

No overall significant effect of temperature or the interaction between temperature and treatment were observed for pre- and post-experimental turbidity measurements, however, treatment had a significant effect ($F1,12 = 66.80$, Sum Sq = 17879.4, $p < 0.01$; Fig. 1E). Wastewater samples stored under standard conditions (4°C, −20°C or −80°C) had similar mean turbidity values to time zero (125 NTU; Table 1), and the difference in turbidity pre- and post-experiment did not significantly differ between temperatures (Fig. 2E). Samples that had undergone repeated freeze-thaw or stored at 4°C in bulk for one year all showed a decrease in turbidity over time (Table 1). This was reflected in the significant pairwise comparisons between treatments, where almost all pairwise comparisons between freeze-thaw and standard storage were significant, other than the differences in concentrations pre- and post-experiment between −80°C under freeze-thaw conditions and −20°C under standard storage conditions (Fig. 2E). Interestingly, wastewater stored in bulk conditions at 4°C had the lowest turbidity readings.

A full breakdown of significant pairwise comparisons for all physicochemical measurements, between temperatures and treatments, can be found in Supplementary Table S1.

3.3. Effects of sample storage on different microbiological markers

3.3.1. Extractable RNA and DNA concentrations

Temperature ($F2,24 = 6.80$, Sum Sq = 278.34, $p < 0.01$), treatment ($F1,24 = 18.30$, Sum Sq = 374.36, $p < 0.01$) and an interaction term with treatment and time ($F1,24 = 3.34$, Sum Sq = 136.82, $p = 0.05$) had a significant effect on extractable RNA concentration (Fig. 3A). Extractable RNA concentration from wastewater samples stored under standard storage conditions showed an increase in concentration over time for samples stored at both −20°C and −80°C (Fig. 3A; Table 2). This was reflected in the significant pairwise comparisons, where samples stored at 4°C under standard storage conditions had significantly lower concentrations than those stored at −20°C ($t = 3.94$, $p < 0.01$) and −80°C ($t = 3.74$, $p = 0.01$) standard storage conditions (Fig. 3C). For example, extractable RNA concentration increased from 7.0 ng/μl at time zero to 13.3 ng/μl and 17.1 ng/μl after one year of storage at −20°C and −80°C, respectively. However, samples stored under standard conditions at 4°C and at all other conditions (bulk and freeze-thaw) showed a rapid

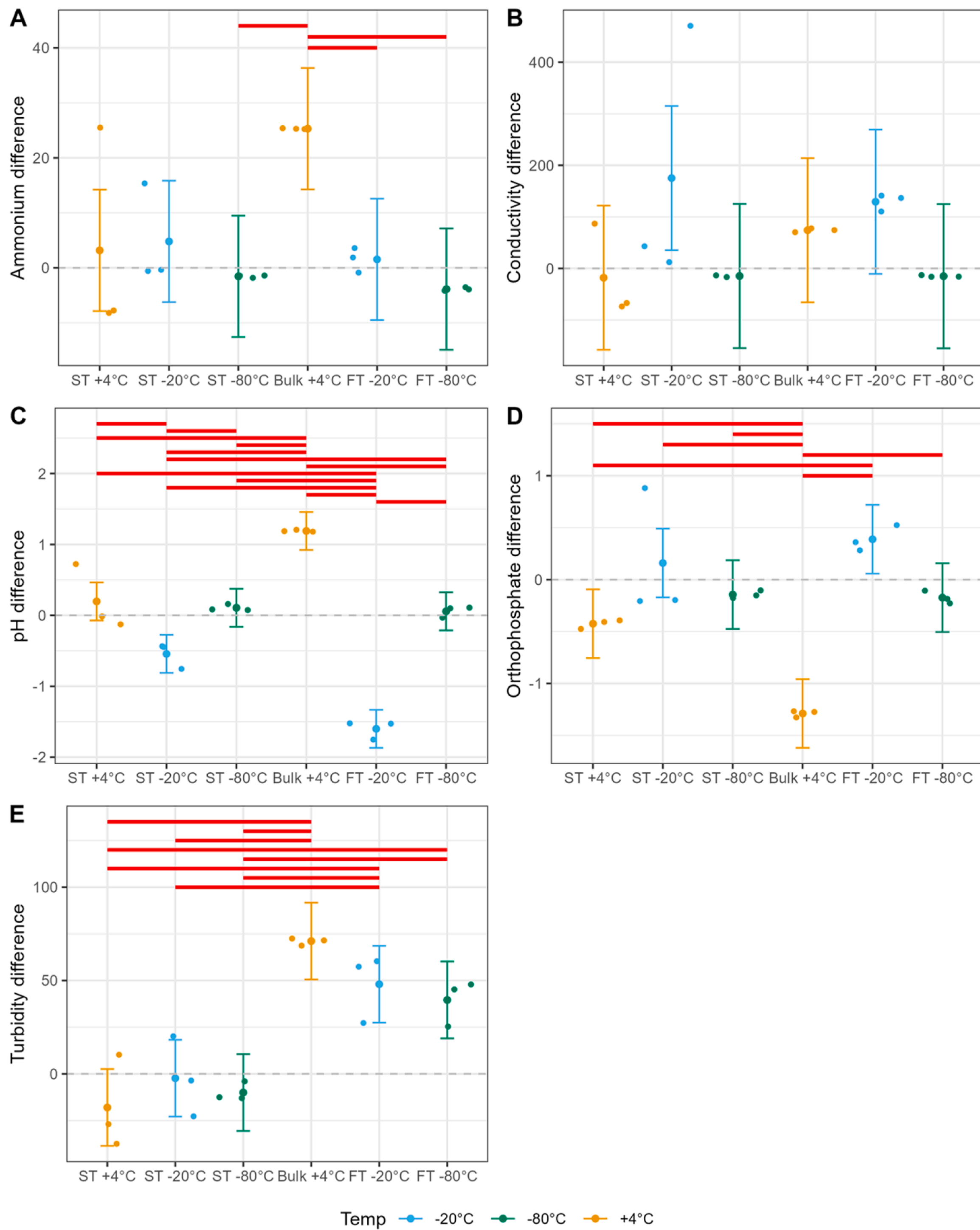

Fig. 2. Differences in (A) ammonium concentration, (B) electrical conductivity, (C) pH, (D) orthophosphate concentration, and (E) turbidity pre- and post-experiment, with manipulations including temperature (4°C, −20°C and −80°C), as well as the storage conditions standard (ST), freeze-thaw (FT), and bulk. The grey dotted line intercepting the y axis represents zero difference pre- and post-experiment calculated using a linear model. The red lines above each plot represent significant pairwise comparisons between experimental groups (i.e. the group mean pre- and post-experiment difference at the start of the bar and at the end of the bar are significantly different).

decline in extractable RNA concentrations and were below detection after one year of storage (Table 2), with no significant pairwise differences seen in overall concentration between these conditions (Fig. 3C).

There was no significant effect of temperature or storage conditions on extractable DNA, but there was a significant effect of time ($F_{1,24}$ = 10.57, Sum Sq = 33.33, $p < 0.01$; Fig. 3B & 3D). Concentrations of DNA steadily declined for all samples stored under standard storage conditions at all temperatures, decreasing from 4.1 ng/µl to ~1.0 ng/µl after 52 weeks (Table 2). Samples stored under bulk or freeze-thaw conditions similarly declined in extractable DNA, but the decay rate was more rapid, with very little DNA (0.5–0.8 ng/µl) being detected after 12 weeks of storage.

A full breakdown of significant pairwise comparisons for both RNA and DNA concentrations, between temperatures and treatments, can be

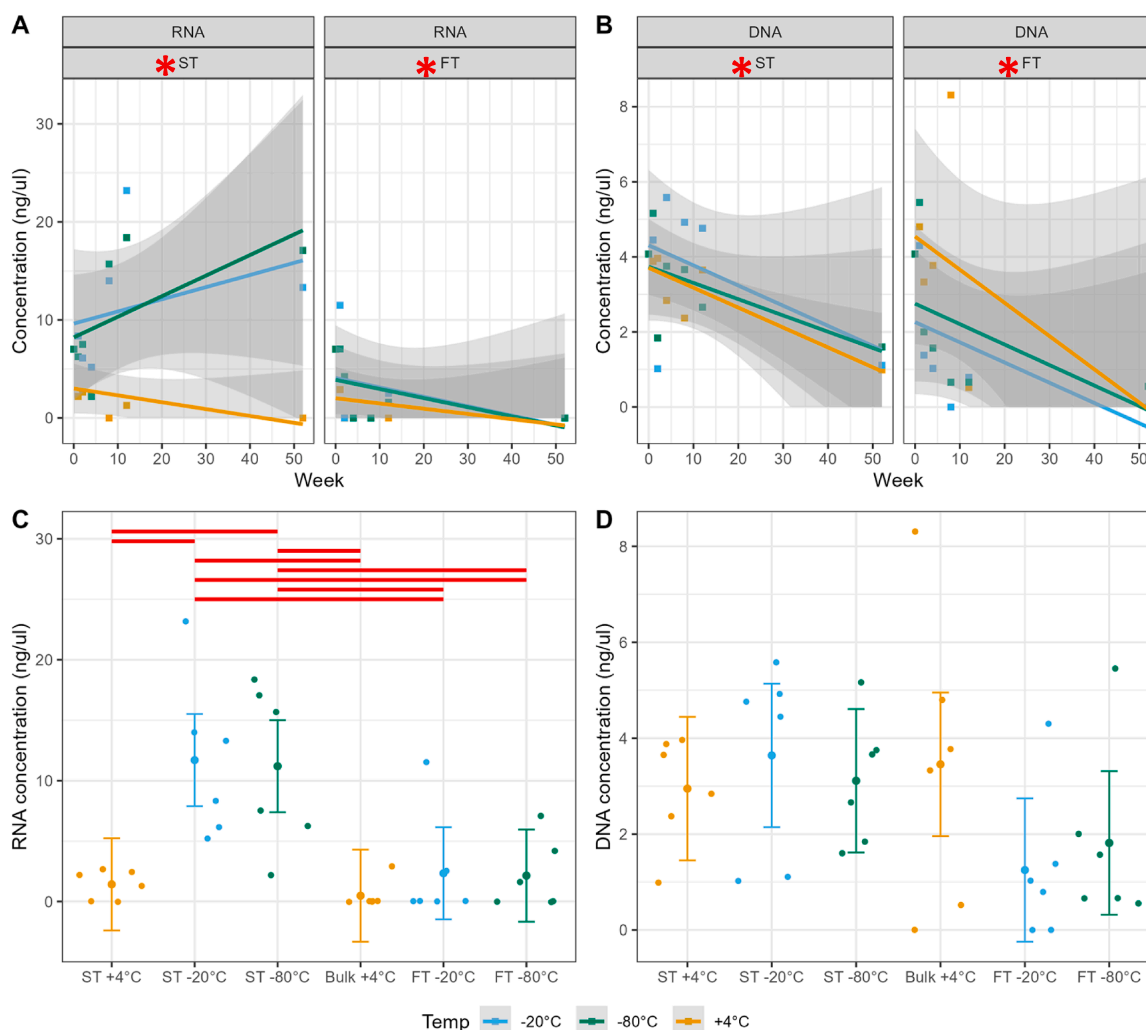

Fig. 3. Extractable nucleic acid concentration over the time course (52 weeks) of the wastewater storage experiment for (A) RNA and (B) DNA, as shown for different temperatures (4°C, −20°C and −80°C) and storage conditions, standard (ST), or freeze-thaw (FT) storage. Red asterisks highlight a significant effect of time (week). Samples stored under FT conditions experienced up to six freeze-thaw cycles, except samples at 4°C, these samples were stored as bulk (see Methods, Section 2.2). Statistical analysis calculated using a linear mixed effect model of (C) RNA and (D) DNA concentrations between temperature (4°C, −20°C and −80°C) and storage conditions (standard (ST), freeze-thaw (FT), and bulk). Included are the mean, 95 % confidence interval and jitter points showing the raw data. The red lines above each plot represent significant pairwise comparisons between experimental group means (i.e. the group mean at the start of the bar and at the end of the bar are significantly different).

found in Supplementary Table S1.

3.3.2. Quantitative PCR of SARS-CoV-2 N1 gene region

Temperature ($F2,24 = 30.68$, Sum Sq = 32.84, $p < 0.01$), storage condition ($F1,24 = 16.08$, Sum Sq = 8.61, $p < 0.01$), time ($F1,24 = 17.00$, Sum Sq = 9.10, $p < 0.01$) and an interaction term with temperature and time ($F2,24 = 9.17$, Sum Sq = 9.81, $p < 0.01$) all had a significant effect on SARS-CoV-2 *N*1 concentrations (Fig. 4A). Mean copy numbers of *N*1 genes in stored wastewater declined with time from 11.6 $\times 10^3$ copies/l at time zero to 1.0–4.1 $\times 10^3$ copies/l after one year of storage at all conditions and temperatures (Table 2). However, over the shorter storage time of 12 weeks, the abundance of *N*1 genes in samples stored at 4°C under standard conditions remained relatively high (10.9–8.5 $\times 10^3$ copies/l), with overall significantly higher copies than those stored at −20°C ($t = 3.49$, $p = 0.02$; Fig. 4C). Similarly, the abundance of *N*1 gene in samples stored at 4°C in bulk also remained relatively high (9.7–8.6 $\times 10^3$ copies/l) for the first 8 weeks of storage, with overall significantly higher copies than those stored at −20°C ($t = 5.07$, $p < 0.01$) and −80°C freeze-thaw ($t = 5.07$, $p < 0.01$). Comparison of the gradients between time (weeks), *N*1 copies and temperatures demonstrated that there was no significant difference between −80°C and −20°C stored samples, but there was a significant difference between 4°C and −80°C ($t = 3.45$, $p < 0.01$), as well as 4°C and −20°C ($t = 3.92$, $p < 0.01$; Fig. 4A & 4 C). This highlights that all conditions which included freezing (-20°C or −80°C) as a method of storage (standard or freeze-thaw) drastically reduced the copy numbers of SARS-CoV-2 enumerated by RT-qPCR and increased replicate variability (Supplementary Figure 2 A), even after only one freeze-thaw cycle (Table 2).

3.3.3. Quantitative PCR of crAssphage (CPQ_056 marker gene)

In contrast to the pattern observed for SARS-CoV-2, copy numbers for the non-enveloped DNA virus crAssphage remained relatively stable under most storage conditions for 52 weeks (with the exception of samples stored at 4°C), with significant terms including time ($F1,24 = 31.15$, Sum Sq = 0.85, $p < 0.01$) and an interaction term with temperature and time ($F2,24 = 10.13$, Sum Sq = 0.56, $p < 0.01$; Fig. 4B & 4D). This was reflected in the gradient comparisons between time, crAssphage copies and temperatures, where there was no significant difference between −80°C and −20°C, but there was a significant difference

Table 2
Microbiological markers analysed in pooled raw wastewater samples after long-term storage at different temperatures and conditions.

Storage temperature (°C)	Storage conditions	Length of time stored (weeks)	Microbiological markers (mean ± SD)[a]			
			RNA (ng/µl)	DNA (ng/µl)	SARS-CoV-2 *N1* gene (copies/l)	CrAssphage marker (copies/l)
None	None	0	7.0	4.1	11573 ± 540	10293 ± 514
4	Standard	1	2.2	3.9	9828 ± 95	9727 ± 129
		2	2.6	4.0	10967 ± 282	10254 ± 115
		4	2.4	2.8	7487 ± 2653	7788 ± 301
		8	<0.2	2.4	8975 ± 842	7027 ± 94
		12	1.3	3.7	8494 ± 1153	8568 ± 351
		52	<0.2	1.0	1017 ± 1762	2675 ±2376
−20	Standard	1	8.4	4.5	2942 ± 2676	8415 ± 103
		2	6.1	1.0	2614 ± 2424	7563 ± 116
		4	5.2	5.6	6006 ± 625	9508 ± 78
		8	14.0	4.9	7440 ± 785	9070 ± 5
		12	18.4	4.8	5000 ± 3448	8596 ± 160
		52	13.3	1.1	3545 ± 3071	7325 ± 90
−80	Standard	1	6.3	5.2	6049 ± 1035	9079 ± 143
		2	7.5	1.8	6428 ± 712	8265 ± 151
		4	2.2	3.8	6336 ± 1255	8762 ± 108
		8	15.7	3.7	5019 ± 4427	8994 ± 541
		12	18.4	2.7	5602 ± 1559	8946 ± 217
		52	17.1	1.6	4069 ± 3525	7494 ± 243
4	Bulk	1	2.9	4.8	9104 ± 320	9210 ± 109
		2	<0.2	3.3	9652 ± 450	8731 ± 116
		4	<0.2	3.8	8694 ± 712	8970 ± 127
		8	<0.2	8.3	8547 ± 1185	7969 ± 1370
		12	<0.2	0.5	2460 ± 4262	175 ± 303
		52	<0.2	<0.2	2480 ± 4296	0 ± 0
−20	Freeze-thaw	1	11.5	4.3	1773 ± 3072	8276 ± 1380
		2	<0.2	1.4	1773 ± 3070	7464 ± 93
		4	<0.2	1.0	3222 ± 1116	8458 ± 86
		8	<0.2	<0.2	0 ± 0	7904 ± 101
		12	2.5	0.8	4208 ± 3774	7527 ± 360
		52	<0.2	<0.2	1847 ± 3199	5048 ± 67
−80	Freeze-thaw	1	7.0	5.5	1052 ± 1822	9549 ± 249
		2	4.2	2.0	2941 ± 2702	7786 ± 170
		4	<0.2	1.6	2762 ± 2417	8841 ± 234
		8	<0.2	0.7	2276 ± 2101	7331 ± 412
		12	1.6	0.7	2250 ± 3898	7419 ± 108
		52	<0.2	0.6	1715 ± 2970	7371 ± 126

[a] Data values for RNA and DNA represent means of two replicates. Data values for SARS-CoV-2 and crAssphage are means ± standard deviation of three replicate RT-qPCR reactions.

between 4°C and −80°C ($t = 4.14$, $p < 0.01$), as well as 4°C and −20°C ($t = 3.61$, $p < 0.01$; Fig. 4B & 4D). Despite the near or complete loss of crAssphage signal at 52 weeks of storage at 4°C, the first 8–12 weeks of storage showed numbers of phage that were high and similar in comparison to time zero. Furthermore, the abundance of crAssphage was not affected by freeze-thaw cycles and similar values were reported for wastewater samples stored under either standard or freeze-thaw conditions at −20°C or −80°C (Table 2; Supplementary Figure 2B).

A full breakdown of significant pairwise comparisons for both *N*1 and crAssphage copy numbers, between temperatures and treatments, can be found in Supplementary Table S1.

4. Discussion

This study assessed the effects of cold-storage temperatures, freeze-thaw cycles, and storage time on selected microbiological and physicochemical markers in raw wastewater samples, taken as part of the Welsh national wastewater-based public health monitoring programme. The temperatures chosen to reflect common laboratory protocols used to store wastewater samples, such as in the refrigerator (4°C), freezer (-20°C), or the use of ultra-low temperature (ULT) freezers (-80°C). Additionally, we included multiple freeze-thaw cycles to simulate scenarios where samples undergo repeated retrieval for analysis.

4.1. Effect of storage on wastewater physicochemical markers

Analysis of the physicochemical markers (Table 1) revealed that electrical conductivity was the most stable, and not significantly affected by temperature or storage condition (Fig. 2B). Thus, storing wastewater samples for conductivity analysis at 4°C, in large (bulk) or small volumes (standard), is as effective as freezing for a 52-week period. This may be due to conductivity being directly related to the concentration of ions in wastewater, derived from dissolved salts and inorganic materials such as chlorides, sulphides and carbonates, compounds that are relatively stable in solution and not subject to biotransformation (Ali et al., 2012).

Ammonium concentrations also showed a high stability over time. All frozen samples (−20°C or −80°C) showed no significant difference with each other, with pre- and post-experimental differences close to zero (Fig. 2A). This was also true of undisturbed samples stored at 4°C under standard conditions with no headspace. Storing samples in vials with minimum headspace is important to restrict the effects of oxygen and reduce microbial activity (Bian et al., 2022). This is evident from samples stored in bulk at 4°C, which had a large headspace volume, in this case ammonium was significantly impacted by oxygen. Under these conditions, ammonium was removed by microbiological activity through ammonia oxidation (Su et al., 2021), which led to a decrease in pH due to the production of protons, often seen in wastewaters without sufficient alkalinity to buffer proton release (Fumasoli et al., 2017).

Unlike ammonium and conductivity, pH was not stable over different storage conditions and temperatures with time (Fig. 2C). Changes in pH

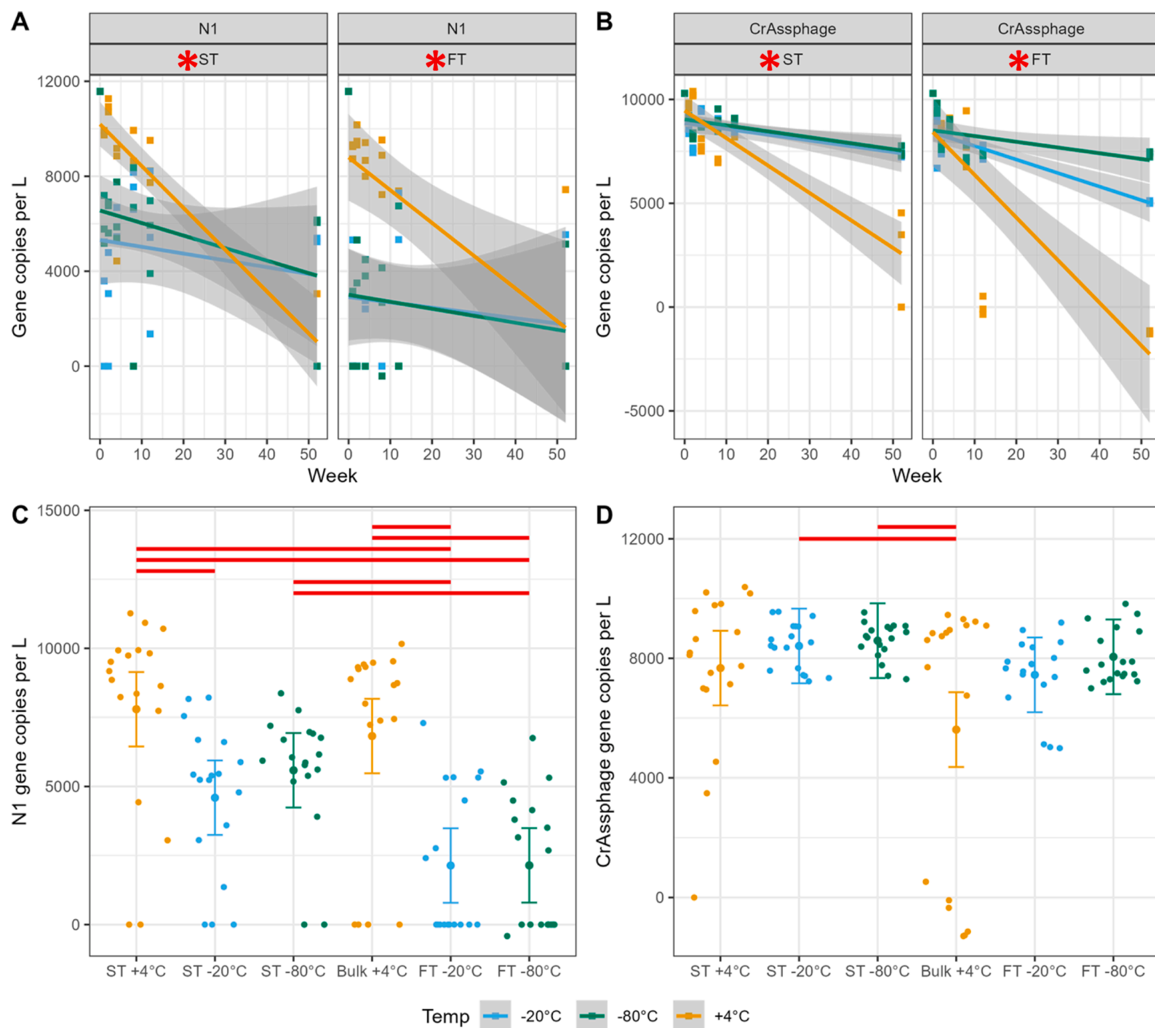

Fig. 4. Gene copies per litre of wastewater over the time course (52 weeks) of the experiment for (A) SARS-CoV-2 *N1* and (B) crAssphage markers, as shown for different temperatures (4°C, −20°C and −80°C) and storage conditions, standard (ST), or freeze-thaw (FT) storage. Red asterisks highlight a significant effect of time (week). Samples stored under FT conditions experienced up to six freeze-thaw cycles, except samples at 4°C, these samples were stored as bulk (see Methods, Section 2.2). Statistical analysis calculated using a linear mixed effect model of abundance of (C) SARS-CoV-2 *N1* and (D) crAssphage markers between temperature (4°C, −20°C and −80°C) and storage conditions (standard (ST), freeze-thaw (FT), and bulk). Included are the mean, 95 % confidence interval and jitter points showing the raw data. The red lines above each plot represent significant pairwise comparisons between experimental group means (i.e. the group mean at the start of the bar and at the end of the bar are significantly different).

occurred in wastewater samples stored in bulk at 4°C and in samples that had undergone freeze-thaw during storage at −20°C. These changes were indicative of microbiological activity as described above. However, in contrast to ammonia oxidation at 4°C in bulk samples, at −20°C with freeze-thaw, the increase in pH was probably due to low rates of free urease activity (Webster et al., 2005) which can be activated for short time periods during repeated thaw cycles. Similar findings have been observed during thawing of frozen soils fertilised with urea (Edwards & Killham, 1986), or in wastewater with high urea concentrations (Zaher & Shehata, 2021).

The concentration of orthophosphate was also not stable over the different storage conditions and temperatures, with the largest impact seen in samples stored in bulk at 4°C (Fig. 1D). This instability with storage at 4°C in bulk samples is ostensibly caused by mineralization and breakdown of organic matter (OM) by microbes into soluble orthophosphates (Witek-Krowiak et al., 2022). An increase in orthophosphate was also seen in samples stored under standard conditions at 4°C, but to a lesser extent. This may be related to the lack of headspace within the vials, and a more restricted amount of oxygen. Anaerobic digestion of organic phosphorous can also occur (Witek-Krowiak et al., 2022) and this slower process may have contributed to slight increases in orthophosphate under these conditions.

Turbidity indicates suspended particulates in wastewater and often serves as a proxy for organic matter (OM; Kitchener et al., 2017). Storage conditions that facilitate OM breakdown through biological or physicochemical mechanisms can affect turbidity measurements. This study observed turbidity instability across different storage conditions and temperatures over 52 weeks (Fig. 1E), with the most significant impact in bulk samples stored at 4°C, followed by freeze-thaw cycles at −20°C and −80°C when compared with samples stored under standard storage. The reduction in turbidity at 4°C is likely due to bacterial degradation of OM, while freeze-thaw cycles likely cause physical and thermal disruption of OM, a method routinely used as a pretreatment to make sewage sludges amenable to biodegradation (Machnicka et al., 2019).

Interestingly, the difference in turbidity in samples stored under standard conditions pre- and post-experiment (Fig. 2E) did not significantly differ between temperatures (4°C, −20°C and −80°C), and that turbidity was the only physicochemical marker where the difference in concentration pre- and post-experiment at −80°C with freeze-thaw significantly differed from samples stored at −80°C under standard

conditions. This highlights that samples stored for turbidity should be frozen immediately and used once.

4.2. Effect of storage on wastewater biological markers

Extractable DNA decreased significantly over time under all storage conditions and temperatures, reflecting DNA degradation. Degradation of DNA was far more rapid under freeze-thaw conditions, with little extractable DNA being detected after 52 weeks (Fig. 3B). Degradation of DNA occurs by enzymatic digestion via deoxyribonucleases (DNases) found within the wastewater matrix (Ahmed et al., 2020) or released during cell lysis (Romanazzi et al., 2015). Furthermore, spontaneous chemical decay of DNA during storage in water can also occur (Lindahl, 1993, Zupanič Pajnič et al., 2019). Modifications of the primary structure can be caused by several chemical reactions, such as hydrolysis of the phosphodiesteric and N-glycosilic bonds, deamination of bases and DNA oxidation, as well as physical stresses from the formation of ice crystals (Davis et al., 2000). Decreases in extractable DNA have been observed previously for WwTW samples stored at −20°C for 4 months, with estimated DNA losses of 65 % (Romanazzi et al., 2015). In our study we estimate that after 52 weeks at −20°C or −80°C under standard storage conditions, the loss in extractable DNA was 75 % and 69 %, respectively and was up to 96 % loss under freeze-thaw conditions (Table 2). However, storage of wastewater under standard conditions at −20°C for up to 12 weeks showed little or no DNA loss (Table 2). This highlights possible issues with long-term archiving of raw wastewater samples for DNA analysis, but for short-term storage most laboratory freezers are adequate. Furthermore, for very short-term storage (4 weeks), samples stored at 4°C, may also be suitable (Table 2).

Concentrations of extractable RNA also decreased over time under freeze-thaw conditions (Fig. 3A) and during storage at 4°C. Degradation of RNA under these conditions is well documented (Williams et al., 2024), and in raw wastewater can occur by enzymatic digestion via ribonucleases (RNases) from bacteria directly at 4°C or by RNases released by cell lysis during freeze-thaw after freezing at −20°C or −80°C. However, somewhat surprisingly when wastewater was stored undisturbed under standard conditions, at temperatures of −20°C and −80°C, there was a significant increase in extractable RNA over time (Fig. 3A). This could be due to a combination of lysis of bacterial cells during the single freeze-thaw cycle coupled with the gradual cryodamage from intra- and extracellular ice crystallization during storage resulting in increased bacterial cell membrane damage (Sarnaik et al., 2021) and release of bacterial RNA. Previously, freezing samples prior to RNA extraction has shown significantly improved yields of extractable RNA from bacterial cultures (Verbeelen et al., 2022) and environmental samples (Lever et al., 2015), and furthermore RNA concentrations from soil bacteria have been shown to increase after sample storage at −20°C or −80°C (Sessitsch et al., 2002). However, increased yields of RNA after prolonged storage can result in observed decreases in RNA integrity number (RIN). Studies on gene expression profiling in blood samples have shown that despite RNA yields being improved after long-term storage a loss in RIN and increased variability in genes counted by RT-qPCR occurred (Kim et al., 2007). Similarly, studies on bacterial RNA quantification have observed a decrease in RIN when obtaining higher bacterial RNA yields after including extra lysis steps (Jahn et al., 2008), whereas others have observed that extra lysis steps increased bacterial RNA but showed clear decreases in viral RNA (Babler et al., 2023). It should be noted that the nucleic acid extraction used in this study was developed to determine SARS-CoV-2 levels within wastewater (Nan et al., 2023) and may preferentially extract RNA over DNA and/or extracellular DNA over intracellular DNA and this could be a reason for the contrasting differences observed in the concentrations of extractable nucleic acids.

Consistent with the degradative effect on extractable RNA, freeze-thaw significantly reduced the levels of detectable SARS-CoV-2 *N*1 genes in stored wastewater samples, compared to samples stored under standard conditions (Fig. 4A & Supplementary Figure S2A). Moreover, all frozen wastewater samples, be it −20°C or −80°C, freeze-thaw or standard conditions, showed significantly lower mean *N*1 gene copies than samples stored at 4°C for 8–12 weeks, demonstrating that only one freeze-thaw cycle (as carried out during standard storage conditions) can significantly cause SARS-CoV-2 viral degradation resulting in low or variable gene abundance values (Ahmed et al., 2022). The benefits of storing raw wastewater samples at 4°C, at least in the short-term (10 days), has been demonstrated previously, with *N*1 gene copies significantly increasing over time (Williams et al., 2024), possibly due to SARS-CoV-2 RNA structural changes (Zhang et al., 2021); a trend only seen in raw wastewater, and not observed with processed (subjected to PEG precipitation) wastewater samples (Williams et al., 2024). The results in our study demonstrate that this increase in detectable gene copy numbers is short lived, but nonetheless, the relatively low level of degradation over a 12-week period stored at 4°C is compelling.

Low SARS-CoV-2 *N*1 gene copies detected in samples stored under standard conditions at −20°C or −80°C was curious, given that higher concentration of extractable RNA was obtained from these samples. This phenomenon may reflect increased specific RNase activity released from bacteria (Guo et al., 2023) during long-term wastewater storage, as a result of membrane cryodamage (Sarnaik et al., 2021), which can then inactivate viral RNA (Mahlknecht, 2022). Enveloped viruses like SARS-CoV-2 have been shown to have a shorter lifespan outside of their host (Sala-Comorera et al., 2021), and it is possible that the *N*1 gene region of SARS-CoV-2 RNA could be susceptible to microbial nuclease degradation (Williams et al., 2024). The SARS-CoV-2 genome has a secondary structure (Lan et al., 2022), with different regions likely to be exposed to nucleases and degrade at different rates due to structural differences (Simmonds, 2020). This demonstrates that freezing raw wastewater can adversely affects its use in WBE to determine the concentration of RNA viruses, like SARS-CoV-2, and should be avoided. Similar findings were reported for the storage of wastewater samples for quantification and sequencing of SARS-CoV-2 genomes (Williams et al., 2024). The decay of SARS-CoV-2 in wastewater is assumed to be mainly due to biological activity, however, chemical decay from the many pollutants in the wastewater matrix should not be dismissed (Núñez-Delgado, 2020).

Interestingly at 4°C, copy numbers of SARS-CoV-2 *N*1 genes were relatively stable for the first 8–12 weeks, and this is despite extractable RNA concentrations rapidly decreasing during this time (Table 2). This clearly demonstrates that the majority of extractable RNA detected in this study belonged to bacteria rather than RNA viruses and that low RNA concentrations do not necessarily reflect the abundance of the biological marker of interest. It also indicates that storing raw wastewater samples for SARS-CoV-2 analysis is more beneficial at 4°C for shorter periods of time than at −20°C or −80°C and agrees with other studies on the stability of SARS-CoV-2 RNA in wastewater (Islam et al., 2022, Williams et al., 2024).

The detection of crAssphage in stored wastewater samples by RT-qPCR behaved differently to SARS-CoV-2. No significant effect of storage condition was observed (Fig. 4D), suggesting that samples for crAssphage detection can be freeze-thawed without impact on phage abundance for up to one year (Table 2). This is likely due to crAssphage having a more stable circular DNA genome and being found in wastewater as a bacteriophage, sometimes protected within bacteria (Bacteroidota; Sabar et al., 2022). However, like other biological markers analysed in this study, samples stored for 52 weeks at 4°C had little or no crAssphage detected (Table 2). Highlighting that if samples are stored at 4°C then they should be analysed within 8–12 weeks. Accurate detection of crAssphage is crucial in WBE as it is often used as a faecal indicator (Sabar et al., 2022) and a normalisation marker (Wilde et al., 2022).

4.3. Environmental considerations for storage of wastewater samples

When samples were stored under standard conditions without freeze-thaw and had little or no headspace, eight out of the nine investigated

Table 3
Raw wastewater storage temperature which showed the best overall result (all timepoints combined) for each of the markers under standard storage and freeze thaw conditions.

Storage condition	Marker	Best storage temperature (°C)[a]	Storage temperatures (°C) that are not significantly different from best[b]	Highest storage temperature (°C) possible[c]
Standard	Ammonium	−80	−80, −20, 4	4
	Conductivity	−80	−80, −20, 4	4
	pH	−80	−80, 4	4
	Orthophosphate	−80	−80, −20, 4	4
	Turbidity	−20	−80, −20, 4	4
	RNA	−20 (−80)	−80, −20	−20
	DNA	−20 (−80)	−80, −20, 4	4
	SARS-CoV−2 *N*1	4 (−80)	−80, 4	4
	CrAssphage	−80 (−80)	−80, −20, 4	4
Freeze-thaw	Ammonium	−20	−80, −20, 4	4
	Conductivity	−80	−80, −20, 4	4
	pH	−80	−80, 4	4
	Orthophosphate	−80	−80, −20, 4	4
	Turbidity	4	−80, −20, 4	4
	RNA	−20 (−20)	−80, −20, 4	4
	DNA	4 (−80)	−80, −20, 4	4
	SARS-CoV−2 *N*1	4 (−20)	4	4
	CrAssphage	−80 (−80)	−80, −20, 4	4

[a] The best storage temperature for physicochemical markers is defined as the temperature with the smallest pre- and post-experiment difference. For microbiological markers, the best storage temperature is the temperature with the highest mean number of gene copies or concentration. Number in parentheses denotes the temperature with the highest gene copy or concentration after 52 weeks of storage.
[b] Temperatures which showed no significant difference from the best storage temperature. For direct comparison, all results for samples stored at 4°C shown here are those that were aliquoted into 50 ml volumes and stored under standard conditions, and not those that were stored in bulk.
[c] The highest storage temperature (most cost-effective) within those that showed no significant difference from the best result.

markers showed no significant difference between being stored at 4°C or at −80°C (Table 3). The one marker (extractable RNA) which did show a significant difference was not significantly different when stored at −20°C or −80°C. However, if samples underwent freeze-thaw during storage, the number of measured parameters showing a significant difference between 4°C (standard conditions) compared with −80°C (Freeze-thaw) was reduced to zero, demonstrating that if a sample is in frequent use and routinely removed from the freezer, then aliquoting and storing at 4°C is a more suitable option (Table 3).

These findings suggest that storing wastewater samples at −80°C for specific chemical or microbiological markers may not always be necessary (Table 3), potentially reducing environmental and economic impacts. The high greenhouse gas potential of refrigerants (Berchowitz & Kwon, 2012), along with the substantial energy consumption and financial costs of ULT freezers (Gumapas & Simons, 2013) highlights the importance of this consideration. ULT freezers consume 15–32 kWh/day (Faugeroux, 2016), which is 1.5–3 times more than the average UK household's daily electricity usage (Amin & Mourshed, 2024). In contrast, standard −20°C freezers or 4°C refrigerators require significantly less energy, ranging from 0.58 to 1.7 kWh/day (Dupret & Zimmermann, 2017). Storing samples at 4°C or −20°C instead of using a ULT freezer could save 990–2189 kg CO_2eq/year, reducing energy use by 93.5–96.9 %.

Our study concludes that wastewater samples can be stored at 4°C or −20°C for at least 12 weeks for some microbiological analyses (Table 2), and up to one year at 4°C for physicochemical parameters under standard conditions (Table 1). It is recommended to store samples as aliquots in reduced volume, devoid of headspace, and kept undisturbed in sealed tubes. However, for long-term storage of high-quality nucleic acids, particularly RNA, ULT freezers may be necessary. First processing raw wastewater and storing extracted RNA, as suggested by Williams et al. (2024), could be a viable strategy. We found that RNA from raw wastewater degrades over time or changes in integrity when stored at −20°C, affecting data consistency, as observed with SARS-CoV-2 (Table 2; Supplementary Figure 2 A).

Studies requiring high-quality DNA/RNA (e.g. for RNAseq) should consider operating ULT freezers at −70°C instead of −80°C, as this can reduce energy costs by ~36 % when combined with efficient practices, such as filling dead space with empty boxes (Leak et al., 2023). Adopting frameworks like the Laboratory Efficiency Assessment Framework (LEAF) and maintaining ULT freezers in good condition can further reduce energy and storage costs (Winter et al., 2023). Table 3 summarizes optimal storage temperatures to balance CO_2 emissions, costs, and sample integrity. These findings are crucial for both low-income countries conducting WBE surveys and high-income countries aiming for net-zero targets. Optimized storage conditions are vital for maintaining sample integrity, reducing costs, and ensuring reliable WBE data for public health and environmental monitoring.

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CRediT authorship contribution statement

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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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.115063.

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