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Improved recovery of SARS-CoV-2 from wastewater through application of RNA and DNA stabilising agents

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Significance and Impact of the Study

Wastewater based epidemiology has been an important component of the response to Covid-19. As wastewater is a complex matrix containing numerous inhibitors that adversely impact molecular diagnostic techniques such as qPCR, virus levels within the community can be underestimated. The results of this study provide an understanding of how commercially available nucleic acid stabilising agents can be used to improve qPCR detection of SARS-CoV-2 from wastewater, without impacting Next Generation Sequencing.

Abstract

Wastewater Based Epidemiology (WBE) has become an integral part of the public health effort to track the levels of SARS-CoV-2 within communities. Detection of SARS-CoV-2 in wastewater can be challenging due to relatively low levels of virus within the sample. The wastewater matrix is also comprised of commercial and domestically derived contaminants, as well as RNases, all of which can adversely affect RT-qPCR analysis. To improve SARS-CoV-2 detection within wastewater samples we investigated both the effect of template dilution (as a means to reduce RT-qPCR inhibition) and sample stabilisation via addition of DNA/RNA Shield™ and / or RNA Later™ (to prevent RNA degradation via RNases) as a means to improve viral fragment detection. Using both methodologies a significant improvement in SARS-CoV-2 detection from wastewater samples was observed. No adverse effects of stabilising agent addition on downstream Next Generation Sequencing workflows were detected.

Keywords: SARS-CoV-2, COVID-19, wastewater virus surveillance, improved detection

Abbreviations

CP Select- Concentrating Pipette Select
DNA- Deoxyribose nucleic acid
gc- gene copies
LLC- Limited Liability Company
NGS- Next generation sequencing
NI- Northern Ireland
p.e.- population equivalents
PEG- Polyethylene Glycol Precipitation
PHA- Public Health Agency
WBE- Wastewater based epidemiology

Introduction

Wastewater-based epidemiology (WBE) has long been established as an effective tool for pathogen detection (Corpuz et al., 2020). WBE has thus been effectively utilised by numerous countries to track SARS-CoV-2 levels and provide early outbreak indications at a community level during the COVID-19 pandemic (Ai et al., 2021; Izquierdo-Lara et al., 2021; Wade et al., 2022). Globally the establishment of SARS-CoV-2 surveillance programmes has allowed for policy creation and the management of clinical responses (Ahmed et al., 2020; Farkas et al., 2020; Hemalatha et al., 2021; Kirby et al., 2021; Wade et al., 2022).

Concentrating or precipitating virus present in wastewater samples is critically important to ensure maximum virus recovery, as SARS-CoV-2 levels within wastewater can be low, negatively impacting target detection (Gibson et al., 2012; Ahmed et al., 2020). Many of the published WBE studies have focused on optimising viral recovery, with numerous different concentration and precipitation methodologies described (Ahmed et al., 2020; Farkas et al., 2021; Jafferali et al., 2021). It is however important to address the issues caused by the presence of inhibitors (Schrader et al., 2012; Gibson et al., 2012; Ahmed et al., 2020; Monteiro et al., 2022) and / or RNases (Khan et al., 2021; Kumblathan et al., 2021) present within the wastewater matrix that can have an adverse impact on viral fragment detection (Keenum et al., 2021). Compounds such as surfactants - found in domestic and commercial cleaning products - form a significant proportion of wastewater and have been demonstrated to negatively impact PCR analysis, whilst the presence of RNases can lead to RNA degradation (Corpuz et al., 2020; Alhama et al., 2022; Robinson et al., 2022). Ultimately the presence of such inhibitors within the wastewater matrix contributes to an underestimation of virus levels and false negatives (Keenum et al., 2021), leading to underestimation of SARS-CoV-2 concentrations across communities, which in turn has potential public health consequences.

Whilst there are a number of established methods for reducing the effect of inhibitors on qPCR (reviewed by Schrader et al., (2012) one of the most simple and cost effective strategies is to dilute nucleic acid samples prior to molecular analysis. Indeed, using smaller volumes of nucleic acid in PCR reactions has been shown to improve pathogen detection through inhibitor dilution (Schrader et al., 2012; Wang et al., 2017; Chavarria-Miró et al., 2021; Monteiro et al., 2022). In addition to inhibitor removal commercially available products for the storage of biological samples such as DNA/RNA Shield™ and RNA Later™

are also available for stabilising nucleic acid within samples (Pedersen et al., 2019; Magzal et al., 2022). These products ensure nucleic acid integrity by protecting against RNase degradation (Pedersen et al., 2019; Magzal et al., 2022).

With the number of national COVID-19 wastewater surveillance programmes expanding globally there is a need to develop better methodologies for SARS-CoV-2 wastewater monitoring. As yet however no studies have investigated the effect of the addition of compounds to prevent RNA degradation on SARS-CoV-2 detection within wastewater. To this end, we describe the impact of template dilution, addition of RNA Later™ (Sigma-Aldrich, Denmark) and DNA/RNA™ Shield (Zymo Research, USA) on SARS-CoV-2 detection when using qPCR diagnostics, and the improvements so observed. We further investigate whether the addition of such stabilising agents impacts downstream sequence analysis. These results will thus be of interest to any laboratories involved in the monitoring of SARS-CoV-2 prevalence within wastewater. Indeed, as cases of Covid-19 subside the ability to detect SARS-CoV-2 at lower levels within wastewater will become more important, to act as a sentinel system for new infection waves and variants.

One strategy to counter the effects of wastewater inhibitors on qPCR efficiency would be to dilute nucleic acid samples prior to molecular analysis. However, there is a lack of investigation into commercially available products and their ability to alleviate the impact of qPCR inhibitors. Commercially available products for the storage of biological samples such as DNA/RNA Shield™ (Zymo Research, USA) and RNA Later™ (Sigma-Aldrich, Denmark) are also available for stabilising DNA/RNA within samples (Pedersen et al., 2019; Hamilton et al., 2021). These products ensure both nucleic acid integrity whilst also inhibiting the activity of RNases present within samples (Pedersen et al., 2019; Hamilton et al., 2021). We thus hypothesised that the addition of stabilising agents to wastewater samples could improve SARS-CoV-2 viral detection and therefore more accurately reflect community level infection levels. Indeed, as cases of Covid-19 subside the ability to detect SARS-CoV-2 at lower levels within wastewater will become ever more important, to act as a sentinel system for new infection waves and variants. Protocols which increase our ability to detect SARS-CoV-2 and circumvent inhibitor catalysed signal loss are thus required.

Materials and Methods

Sample collection and concentration

Wastewater was sampled from a range of 15 rural and urban wastewater treatment works across Northern Ireland (NI) (Table 1). At each site a composite 1000 mL sample of screened primary, untreated influent was collected using an Isco Glacier autosampler (Isco; Lincoln,

USA) over a 24 h period. Samples were collected by NI Water Ltd., delivered to the laboratory by the NI Environment Agency and stored at 4°C overnight before analysis. For each of the 15 wastewater sites, 50 mL of sample with 0.005% v/v Tween® 20 (Merk KgaA, Germany) were centrifuged at 4,000 rpm for 10 mins at 4°C before subsequent concentration of the supernatant using a CP select™ (InnovaPrep LLC, Drexel, Mo, USA) with hollow fibre-based ultrafiltration tips (Pore size not specified; InnovaPrep LLC, Drexel, Mo, USA). Ultrafiltration tips were purged twice using an elution buffer containing 0.075% Tween 20 in 25 mM Tris buffer.

Comparison of stabilisation agents

Concentratedsamples were treated prior to extraction and PCR amplification.Samples were treated as follows, 1) control sample (concentrated and extracted sample with no amendments); 2) concentrated sample with DNA/RNA Shield™ (Zymo Research, USA) added at four concentrations (0.5x, 1x, 1.5x, and 2.5x (v/v)); 3) concentrated sample with RNA Later™ (Sigma-Aldrich, Denmark) added at four concentrations (0.5x, 1x, 1.5x, and 2.5x (v/v)); 4) concentrated sample with nuclease free water added at four concentrations (0.5x, 1x, 1.5x, and 2.5x (v/v)).

Nucleic Acid Extraction and qPCR analysis

Nucleic acid extraction and qPCR analysis was carried out using the FLOW Classic workflow (Roche Diagnostics Limited). Processing cartridges containing eluate from CP Select™ (InnovaPrep LLC.) were prepared using a Hamilton, Flow Primary Sample Handling Unit (Roche Diagnostics Limited). Nucleic acid was extracted from each sample using a MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostic Limited). AgPath-ID™ One-Step RT-PCR Reagents (ThermoFisher Scientific) and SARS-CoV-2 N1 + N2 Assay Kits (Qiagen) were used to amplify RNA. A LightCycler 480 (Roche Diagnostics Limited) was used for RT-qPCR analysis, using the following thermal cycling conditions, one cycle of 50°C for 10 mins, followed by 45 cycles of 95°C for 10 mins and 60°C for 30 s. For each sample qPCR assays were run in duplicate. Each 25 µL mastermix included, 12.5 µL 2x RT-PCR Buffer, 1 µL 25x RT-qPCR enzyme (AgPath-ID™ One-Step RT-PCR Reagents (ThermoFisher Scientific), 0.25 µL DEPC (7.25 µL added when 3 µL of template is used), 1 µL primer/probe (SARS-CoV-2 N1=N2 Assay Kit), 0.25 µL Bovine Serum Albumin and either 3 µL or 10 µL of template. qPCR plates containing mastermix and nucleic acid were prepared using a Hamilton, FLOW PCR Setup Instrument (Roche Diagnostics Limited).

Results are the means of duplicates at 3 µL and 10 µL respectively, for all method optimisation and comparison studies at the fifteen wastewater sites.

Sequencing

For each site, three different treatments were taken forward for sequencing: 1) control sample, 2) concentrated sample with DNA/RNA shield at four concentrations and 3) concentrated sample with RNA later at four concentrations. SARS-CoV-2 amplicon whole-genome sequencing and following data analysis were performed using the Mini-XT SARS-CoV-2 protocol (Fuchs et al., 2022). In brief, viral nucleic acid extracts and no-template controls were reverse transcribed with LunaScript RT SuperMix Kit (New England Biolabs). The cDNA samples were amplified by tiled PCR using two separate primer pools from the ARTIC SARS-CoV-2 v4 panel (IDT). The amplicons were purified using KAPPA magnetic beads (Roche) and quantified using an Invitrogen Quant-iT dsDNA broad-range assay kit with a PHERAstar FS multimode microplate reader (BMG Labtech). The quantified RT-PCR product was normalised to a concentration of 0.2 ng/µL using the Echo 525 Liquid Handler (Beckman Coulter). 384 libraries were multiplexed using Nextera XT DNA Library Preparation Kit (Illumina) and sequenced paired-end 250 nucleotides on an Illumina MiSeq instrument. The "connor-lab/ncov2019-artic-nf" nextfow-based open-source pipeline (Connor-Lab) was then used to analyse the data.

Statistical Analysis

Kruskal–Wallis $\chi 2$ tests followed by Dunn's test for multiple pairwise comparisons were used to compare the difference in the means of RT-qPCR Cp values and genome coverage (percentage covered bases) between treatments. Statistical analysis and data visualisation were performed in R (R Core Team, 2020).

Results and Discussion

1. Effect of template volume SARS-CoV-2 detection

To determine if the dilution of template volume (and thus potential inhibitor concentration) led to an increase in the detection of SARS-CoV-2 two different volumes of template (3 µL and 10 µL respectively) were processed. Across all fifteen wastewater sites, we found that significantly more virus was recovered when using 3 µL nucleic acid template compared to 10 µL in RT-qPCR reactions (chi-squared = 125.36, d.f. = 1, $p < 0.001$). Our results are thus in agreement with Wang et al., (2017) who, whilst working on soils, observed that smaller template volumes increased PCR detection efficiency due to the dilution of inhibitors present within the soil matrix. Although template dilution significantly improved SARS-CoV-2

detection across all sites, reduction of template volume could ultimately dilute samples to below the limit of detection (LOD), especially when levels of SARS-CoV-2 in wastewater are low (Gibson et al., 2012; Ahmed et al., 2020). For our surveillance workflow the LOD for SARS-CoV-2 RNA detection in wastewater was 6.25 copies per qPCR reaction.

2. Addition of stabilising agents to wastewater

After the addition of DNA/RNA Shield™ (Zymo Research, USA) or RNA Later (Sigma-Aldrich, Denmark) at four concentrations (0.5x, 1x, 1.5x and 2.5x v/v), significantly more virus was detected across all concentrations using 10 µL of template than when using 3 µL of template (when used in the absence of DNA/RNA Shield™ or RNA Later™) ($\chi 2 = 8.7225$, df = 1, $p < 0.001$ & $\chi 2 = 30.592$, df = 1, $p < 0.001$ respectively). This was a reversal of the trend seen previously when no stabilising agent was added.

The concentration at which both stabilising agents were added to concentrated wastewater had an effect on virus detection. When DNA/RNA Shield™ was added at the concentrations, 0.5x, 1x and 1.5x v/v, virus detection significantly improved ($p > 0.001$, $p > 0.001$ and $p = 0.007$ respectively) compared to a control with no DNA/RNA Shield™, using 10 µL of template (Figure 1; Table S1). The addition of RNA Later™ also improved virus detection at the concentrations, 0.5x and 1x v/v ($p = 0.008$ and $p = 0.016$, respectively) when compared to a control with no RNA Later™, using 10 µL of template. RNA Later at the concentration, 2.5x also improved virus detection when 3 µL of template was tested ($p = 0.002$). DNA/RNA Shield™ did not improve detection at any concentration when 3 µL of template was tested.

To ascertain if viral detection was improved by DNA/RNA Shield™ and RNA Later™ addition, and not solely as a result of sample dilution, distilled H_2O was also added at the same concentrations (0.5x, 1x, 1.5x, 2x and 2.5x v/v). The addition of H_2O did not significantly improve virus detection when compared with samples containing the equivalent concentration of DNA/RNA Shield™ or RNA Later™ (Figure 1; Table S1). This highlighted that sample dilution was not the reason for improved SARS-CoV-2 detection in wastewater when DNA/RNA shield and RNA Later were used. Stabilising agents such as DNA/RNA Shield™ and RNA Later™ are typically used for long term preservation of nucleic acid integrity when a sample will not be extracted immediately or will not be frozen to -80°C upon return to the laboratory (Mutter et al., 2004; Najafi, 2014; Pavlovska et al., 2021).

We further assessed the impact of DNA/RNA Shield™ and RNA Later™ at a concentration of 0.5x on 15 wastewater samples as the optimal concentration of both DNA/RNA Shield and RNA Later™ required to significantly improve viral detection. This further analysis

reinforced that both stabilising agents were able to significantly improve viral detection at this concentration and can both therefore be used during qPCR wastewater analysis.

Sequencing

Next generation sequencing (NGS) is an important WBE tool that has been used in SARS-CoV-2 variant analysis (Martin et al., 2020; Izquierdo-Lara et al., 2021; Pechlivanis et al., 2022; Wilhelm et al., 2022). As both DNA/RNA Shield™ and RNA Later™ improved SARS-CoV-2 detection using qPCR analysis we sought to determine if the addition of these stabilising agents had any impact on NGS workflows. DNA/ RNA Shield™ had no adverse effect at any concentration used on the percentage base coverage when compared to controls and other concentration of DNA/RNA Shield™ (0.5x, 1x, 1.5x and 2.5x v/v; Figure 2A; Table S2). RNA Later™ when added at the concentrations, 0.5x and 1x had no adverse effect on NGS workflows (Figure 2B; Table S2). However, RNA Later™ concentrations of 1.5x and 2.5x did lead to a significant increase in sequencing failure (Figure 2B; Table S2). Although 0.5 x DNA/RNA Shield™ does not significantly reduce the percentage of covered bases during NGS, it is lower than the control and other concentrations. To this end, we suggest 0.5 x RNA Later™ as an effective stabilising agent since it did not adversely affect NGS workflows and can therefore be adapted and used for any wastewater virus detection protocol.

Conclusion

The optimisation of viral detection using reduced template volume and DNA/RNA stabilising agents outlined in this study supplements current protocols that exist for viral concentration and precipitation. We have shown that both template dilution and the addition of the stabilising agents, DNA/RNA Shield™ and RNA Later™ can significantly improve SARS-CoV-2 detection in wastewater using RT-qPCR, without negatively impacting subsequent NGS sequencing. As such the inclusion of either strategy into a SARS-CoV-2 wastewater detection workflow could significantly improve viral detection rates for SARS-CoV-2 wastewater surveillance laboratories. Given that a number of other strategies exist for inhibitor removal from environmental samples prior to PCR, including increased extractions, Bovine Serum Albumin addition and magnetic beads (Schrader et al., 2012), further studies specifically focussing on inhibitor removal to improve SARS-CoV-2 detection in wastewater are warranted.

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Conflict of Interests

No conflict of interest declared.

Data availability

The data in this article will be shared on reasonable request to the corresponding author.

Authors' Contribution

Stephen H Bell: Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Writing- original draft, Writing- review & editing. **Danielle M. Allen:** Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Writing- review and editing. **Marina I Reyne:** Data curation, Investigation, Writing- review & editing. **Jonathan F W Lock:** Investigation. **Arthur Fitzgerald:** Investigation. **Ashley Levickas:** Investigation. **Andrew J Lee:** Investigation, Methodology, Writing- review & editing. **Connor G G Bamford:** Investigation, Methodology, Writing- review & editing. **Deirdre F Gilpin:** Conceptualisation, Funding acquisition, Supervision, Writing- review & editing. **John W McGrath:** Conceptualisation, Funding acquisition, Methodology, Supervision, Writing- review & editing.

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Table and table legend

Table 1. Location of Wastewater Treatment Works and population equivalents that they service.

Location	Population equivalent
Antrim, Co. Antrim	41735
Ballyclare, Co. Antrim	14933
Banbridge, Co. Antrim	18803
Belfast, Co. Antrim	228939
Coalisland, Co. Tyrone	9312
Cookstown, Co. Tyrone	12797
Craigavon, Co. Armagh	78899
Culmore, Co. Londonderry	94655
Dungannon, Co. Tyrone	18079
Lurgan, Co. Armagh	29302
Newry, Co. Armagh	34042
North Down, Co. Down	73384
Strabane, Co. Tyrone	13251
Warrenpoint, Co. Down	12766
Whitehouse, Co. Antrim	68952

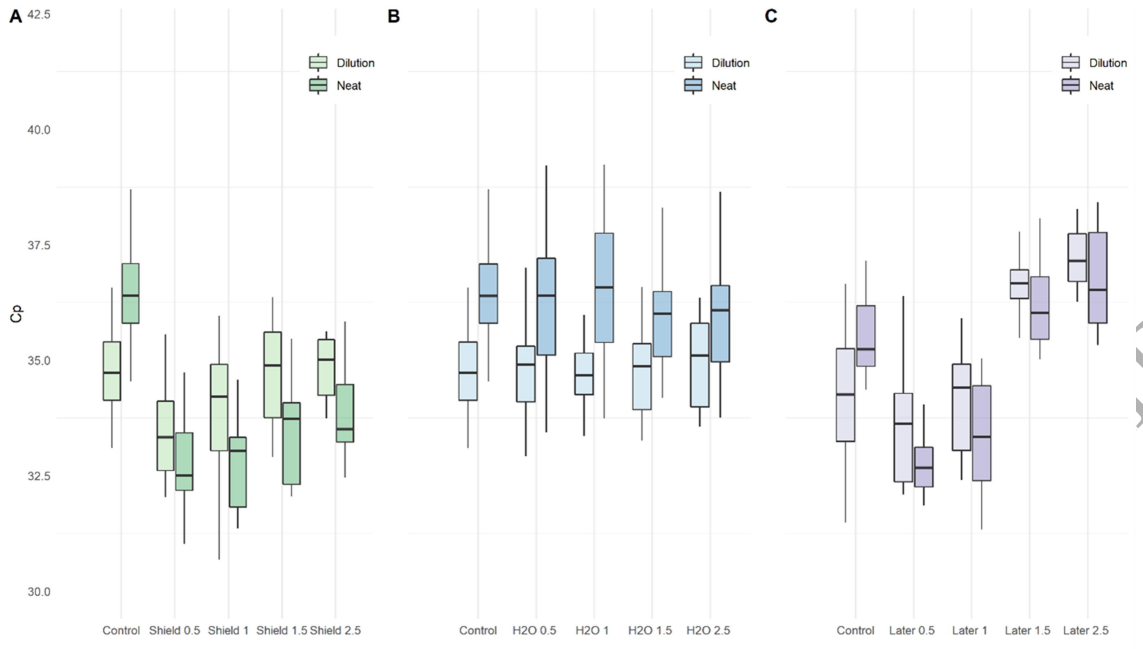

Figure 1. SARS-CoV-2 assay Cp values after the addition of stabilising agents at different concentrations across fifteen wastewater sites. A) DNA/RNA shield™; B) H_2O and C) RNA Later™. Boxplots represent the median (horizontal lines within each box), 25% and 75% quartiles (box), 10% and 90% percentiles (whiskers). Neat denotes 10 µL of template and dilution denotes 3 µL of template being analysed in qPCR analysis.

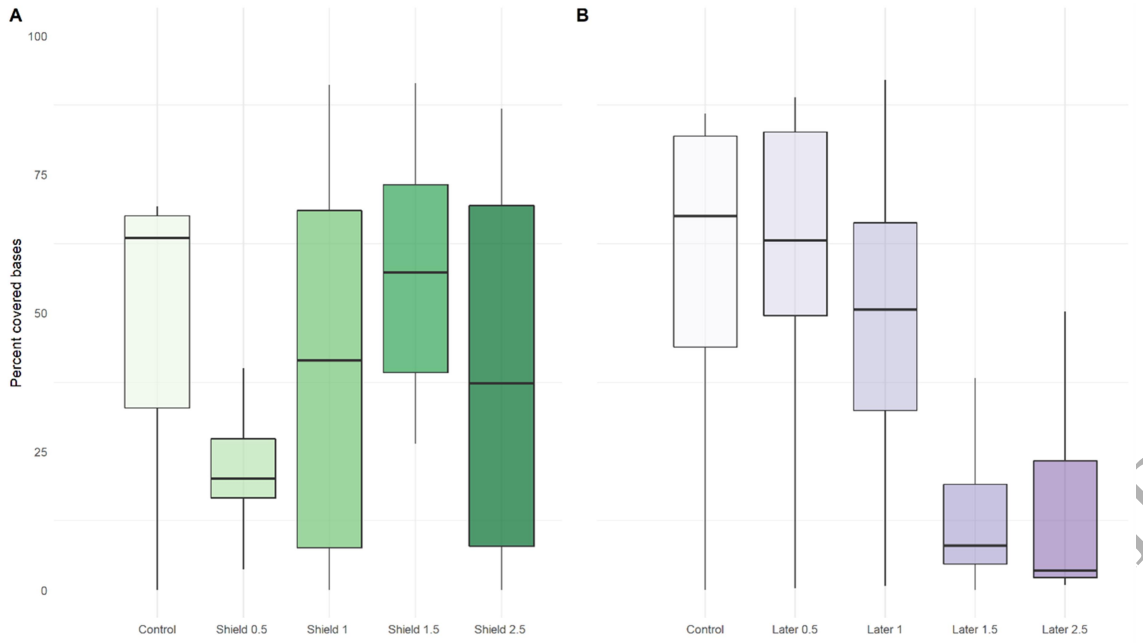

Figure 2. Whole-genome sequencing coverage of SARS-CoV-2 using RNA and DNA stabilizing agents: A) RNA/DNA shield™ and B) RNA later™. Samples with coverage under 50% fail quality control (QC).