[Instruction: There are significant formatting issue with the proof so I need to have another llok before it can be published. Can you please send the amenden proof to Warish.Ahmed@csiro.au for final approval?]RT-qPCR and ATOPlex sequencing for the sensitive detection of SARS-CoV-2 RNA for wastewater surveillance

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Abstract

During the coronavirus disease 2019 (COVID-19) pandemic, wastewater surveillance has become an important tool for monitoring the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) within communities. In particular, reverse transcription-quantitative PCR (RT-qPCR) has been used to detect and quantify SARS-CoV-2 RNA in wastewater, while monitoring viral genome mutations requires separate approaches such as deep ~~genomic~~ sequencing. A high throughput sequencing platform (ATOPlex) that uses a multiplex tiled PCR-based enrichment technique has shown promise in detecting ~~viral~~ variants of concern (VOC) while also providing virus quantitation data. However, detection sensitivities of both RT-qPCR and sequencing ~~analyses~~ can be impacted through losses occurring during ~~sample processing (e.g.,~~ sample handling, virus concentration, nucleic acid extraction, and RT-qPCR.~~),~~ ~~t~~Therefore, process limit of detection (PLOD) assessments are required ~~needed~~ to estimate the gene copies~~y~~ ~~numbers~~ of target molecule ~~required~~ to attain specific probability of detection. In this study, we compare the PLOD ~~estimates~~ of four ~~commonly used~~ RT-qPCR assays (US CDC N1 and N2, China CDC N and ORF1ab) for ~~quantification~~ detection of SARS-CoV-2 ~~(US CDC N1 and N2, China CDC N and ORF1ab)~~ to that of ATOPlex sequencing~~e analyses through~~ by seeding known concentrations of gamma-irradiated SARS-CoV-2 into wastewater. Results suggest that among the RT-qPCR assays, US CDC N1 was the most sensitive, especially at lower SARS-CoV-2 seed levels. However, when results from all RT-qPCR assays were combined, it resulted in ~~the~~ greater detection rates than individual assays, suggesting that application of multiple assays is better suited for the trace detection of ~~trace levels of~~ SARS-CoV-2 from wastewater samples. Furthermore, while ATOPlex offers a promising approach to SARS-CoV-2 wastewater surveillance, this approach ~~technology is~~ appears~~ed~~ to be less sensitive compared to RT-qPCR under the experimental conditions of this study, and may require~~s~~ further refinements. ~~for routine monitoring and quantification of SARS-CoV-2.~~ Nonetheless, the combination of ~~both approaches (~~RT-qPCR and ATOPlex~~)~~ may be a powerful tool to simultaneously detect/quantify SARS-CoV-2 RNA ~~the viral load in wastewater~~ and monitor emerging VOC in wastewater samples.~~variants of concern.~~

Keywords:

SARS-CoV-2, COVID-19, Detection limit, Recovery, Concentration method, Enveloped virus, Wastewater

Abbreviations

No keyword abbreviations are available

1 Introduction

Over the past two years, wastewater surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has grown to become a valuable tool for tracking coronavirus disease 2019 (COVID-19) at the population level in many countries and regions [Instruction: can you provide a hyperlink?](https://arcg.is/1aummW). Furthermore, many studies have described the potential of wastewater surveillance to provide early warning of COVID-19 in the community (~~Randazzo et al., 2020;~~ Medema et al., 2020; La Rosa et al., 2020; ~~Nemudry et al., 2020;~~ Peccia et al., 2020; ~~Ahmed et al., 2021a;~~ Hata et al., 2021), making it a valuable tool for public health agencies. For the detection and quantification of SARS-CoV-2 in wastewater, reverse transcription quantitative PCR (RT-qPCR) and digital PCR (RT-dPCR) have been widely applied (Ahmed et al., 2020a; Ciesielski et al., 2021; D'Aoust et al., 2021; Pecson et al., 2021; Gonzalez et al., 2020). The advantages of PCR-based assays include high sensitivity, specificity, and speed (results can be obtained in ~1 to 1.5 h). A well-optimized RT-qPCR assay can theoretically detect a single DNA/RNA molecules~~fragment~~ in a sample (Bustin et al., 2009). However, the assay limit of detection (ALOD) of currently used RT-qPCR assays for wastewater surveillance varies widely, sometimes up to two orders of magnitude, within and between laboratories (Gonzalez et al., 2020; Bivins et al., 2021; Chik et al., 2021; Gerrity et al., 2021)~~.; Randazzo et al., 2021).~~

Since the first publication of the SARS-CoV-2 genome in January 2020~~ January~~, many RT-qPCR assays have been developed including gene targets (67–158 bp fragments) within nucleocapsid (N), envelope protein (E), RNA-dependent RNA polymerase (RdRP), open reading frame (ORF), membrane protein (M), and surface protein (S) regions of the SARS-CoV-2 genome (~30,000 bp) (Kitajima et al., 2020). Despite the high analytical and diagnostic sensitivities and specificities of these assays in the clinical context, their success in early detection of SARS-CoV-2 RNA circulating in community wastewater is somewhat mixed. The high dilution and fragmentation of viral RNA in wastewater pushes this technology to the limit and there is an ongoing need to improve method detection sensitivity and minimize false-negative results (Ahmed et al., 2020b)~~. (Ahmed et al., 2021a). RT-qPCR assay limitations~~Limitations of RT-qPCR include potentially reduced efficiency if mutations occur in the gene target region as was previously observed for assays targeting the S gene for the alpha variant in the UK (Grint et al., 2021). Another limitation of RT-qPCR is low throughp~~o~~ut, only one genomic target ~~(i.e., fragment of a genome)~~ can be analysed at a time. To help overcome these limitations, ~~many~~ studies have used multiple RT-qPCR assays for the detection of SARS-CoV-2 RNA in wastewater (Ahmed et al., 2020a; Medema et al., 2020), however,~~but~~ this can be time consuming~~requires increased analysis time~~. While the issue of time could be resolved by developing multiplex RT-qPCR assays, that requires additional and more complex method optimization~~.~~ Furthermore, ~~and the~~ multiplex assay may not be as sensitive as a simplex assay (Parker et al., 2015).

Several recent studies have highlighted the potential application of genome sequencing for SARS-CoV-2 and its variant~~s~~ of concern (VOC) detection in wastewater. For example, Fontenele et al. (2021) analysed wastewater using high-throughput sequencing and single-nucleotide variant analysis of sequences to describe SARS-CoV-2 genetic lineage variations and population structure circulating within a community. The SARS-CoV-2 sequence data generated from wastewater indicated that there were more lineages circulating across communities than identified in the clinical data. Similarly, it was ~~Crits-Cristoph et al. (2021)~~ demonstrated that metagenomic sequencing of wastewater samples could not only identify SARS-CoV-2 and other viruses, but track VOC~~variants~~ of the former concomitantly with those detected by clinical surveillance in California, USA (Crits-Christoph et al., 2021).

This~~at~~ study also detected SARS-CoV-2 VOC~~variants~~ in California wastewater not yet identified clinically in the state (but present in other jurisdictions) as well as completely novel VOC~~variants~~, indicating that wastewater sequencing can provide evidence for ~~recent introductions of~~ viral lineages before they are detected by ~~local~~ clinical sequencing (Crits-Christoph et al., 2021). Izquierdo-Lara et al. (2021) ~~study~~ used nanopore sequencing of wastewater samples to evaluate the diversity of SARS-CoV-2 at the community level in the Netherlands and Belgium. P~~Their p~~hylogenetic analysis showed the presence of the most prevalent clades and clustering of wastewater samples with clinical samples from patients in the same region. The authors~~study~~ also identified 57 unique mutations that were not present in the global database, that like the other studies indicates heterogeneity of SARS-CoV-2 variation in wastewater is greater than in clinically derived samples~~ is greater in the clinic~~. This might reflect the presence of defective viral particles in feces and/or infections with novel virions. Along similar lines, Lin et al. (2021) applied targeted metagenomic sequencing of SARS-CoV-2 in wastewater and observed that the frequency and daily load of mutations associated with ~~variants of~~

~~concern (~~VO~~o~~C~~s)~~ were highly correlated with clinical incidence rates within the region of British Columbia, Canada. Taken together, ~~Based on these analyses,~~ it is apparent that genomic sequencing of wastewater samples can be used to investigate the diversity of SARS-CoV-2 circulating in a community and potentially identify new outbreaks.

Genomic sequencing of wastewater may not only shed light on the evolution of SARS-CoV-2 during an outbreak by identifying viral mutations, but it could also be applied as an approach for quantifying genomic fragments of SARS-CoV-2 as well. A recent study used a high-throughput sequencing platform (ATOPlex) that uses a multiplex tiled PCR-based enrichment technique and reported~~claimed~~ that ATOPlex is capable of quantifying SARS-CoV-2 RNA in wastewater at concentrations that are at least one order of magnitude lower than RT-qPCR quantitation (Ni et al., 2021). This~~at proof-of-concept~~ study compared the detection sensitivities~~achieved by~~ of RT-qPCR assays (i.e., US CDC N1 and N2) ~~RT-qPCR assays with~~and ATOPlex, using a dilution series of cDNA samples generated from a commercially available SARS-CoV-2 RNA positive control, rather than seeding SARS-CoV-2 in wastewater samples. Therefore, the impacts of wastewater matrix interference on the ATOPlex assay limit of detection (ALOD) are not known. Collectively, these studies demonstrate the potential application of sequencing approaches for monitoring the presence and allelic frequencies of SARS-CoV-2 RNA in wastewater. Along with clinical data, ~~wastewater-based~~ ~~metagenome~~ sequencing approaches can potentially identify emerging ~~variants/lineages~~VOC of clinical importance within a community~~population~~. However, benchmarking RT-qPCR and sequencing approaches has not yet been assessed~~performed~~ and, requires further investigations. ~~is required to understand and quantify the sensitivities and of these two detection methods.~~

The process limit of detection (PLOD) represents the analytical sensitivity of a sampling method after incorporating the efficiency of all the processing steps (e.g., sample handling, concentration, nucleic acid extraction, and PCR assays). The PLOD estimates the copy number of a target molecule required in the wastewater sample matrix to achieve a specific probability of detection. ~~(Ahmed et al., 2022 under review)~~. The primary objective of this study was to evaluate the PLOD of four RT-qPCR assays (US CDC N1 and N2, China CDC N and ORF1ab (CCDC N and CCDC ORF1ab) and ATOPlex sequencing for the detection of SARS-CoV-2 RNA in wastewater. This was achieved by seeding a dilution series of known concentrations of gamma-irradiated SARS-CoV-2 virions into wastewater followed by primary concentration, nucleic acid extraction, and RT-qPCR and ATOPlex sequencing ~~analysis~~. To the best of our knowledge, this is the first study to ~~assess the SARS-CoV-2 PLOD for wastewater and~~ provide~~s~~ important insights on the analytical limitations for trace detection of SARS-CoV-2 RNA in wastewater using RT-qPCR and ATOPlex sequencing.

2 Materials and methods

2.1 Gamma-irradiated SARS-CoV-2 stock

Gamma-irradiated SARS-CoV-2 stock used in this study was kindly provided by our colleagues from the Australian centre for Disease Preparedness, CSIRO. Gamma radiation process to minimize the potential risk associated with handling SARS-CoV-2 during experiments has been reported in our previous study. [Instruction:
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Ahmed, W., Bivins, A., Metcalfe, S., Smith, W.J.M., Verbyla, M.E., Symonds, E.M., Simpson, S.L., 2022a. Evaluation of process limit of detection and quantification variation of SARS-CoV-2 RT-qPCR and RT-dPCR assays for wastewater surveillance, Water Res. 213, 118132,](Ahmed et al., 2022a). ~~(Ahmed et al., 2022).~~ Immediately prior to seeding experiments, t~~T~~he concentration of the SARS-CoV-2 stock was determined from three aliquots of the stock suspension using the CDC N1 RT-dPCR assay, as described elsewhere (Ahmed et al., 2022a). ~~(Ahmed et al., 2022).~~ The concentration determined to be $4.60 \pm 2.50 \times 10^6$ GC/μL

~~Immediately prior to seeding experiments, (Ahmed et al., 2022).~~

2.2 Wastewater samples

For seeding experiments, archived wastewater samples were used in this study. For Trial A, 36 wastewater samples (WW3 – WW20 and WW23 – WW40) were used representing nine wastewater treatment plants (WWTPs). The same number of samples were also used for Trial B (WW5 – WW40) representing the same nine WWTPs. These wastewater samples were RT-qPCR negative for SARS-CoV-2 RNA determined using the US CDC N1 assay~~.~~ (Ahmed et al., 2020a).

2.3 SARS-CoV-2 seeding experiments

Two trials (A and B) were conducted to determine the detection sensitivity of SARS-CoV-2 in wastewater samples by RT-qPCR and ATOPlex sequencing workflows. A dilution series with varying concentrations of gamma-irradiated SARS-CoV-2 were seeded into wastewater. The dilution series had 10-fold decrements and were prepared by ~~serial~~ diluting the stock suspension using DNase and RNase free water, and then seeding these serial dilutions into 50-mL wastewater samples. For the trials A and B, the seeded SARS-CoV-2 concentrations ranged from $\sim 2.32 \times 10^5$ to

2.32×10^2 GC/50 mL and ~1.79×10^5 to 1.79×10^2 GC/50 mL, respectively along a serial dilution in 10-fold decrements.

2.4 Virus concentration

Adsorption extraction (AE) method was used to concentrate SARS-CoV-2 from wastewater samples (Ahmed et al., 2020a; Juel et al., 2021). Briefly, 25 mM dissolved $MgCl_2$ was added to each 50 mL wastewater. Wastewater samples were filtered through a 0.45-µm pore-size, 47-mm diameter electronegative HA membrane (HAWP04700; Merck Millipore Ltd, Sydney, Australia) using a magnetic filter funnel apparatus (Pall Corporation). ~~(Ahmed et al., 2020a).~~ The membrane was removed from the filtration apparatus, rolled, and inserted into a 5-mL-bead-beating tube with two sterile tweezers (Qiagen, Valencia, CA) for nucleic acid extraction.

2.5 Nucleic acid extraction

RNeasy PowerWater Kit (Cat. No. 14,700–50-NF) (Qiagen, Valencia, CA) was used to extract nucleic acid from the HA membranes (Ahmed et al., 2022a). ~~(Ahmed et al., 2022).~~ Briefly, mixture of 990 µL of buffer PM1 + 10 µL of β-Mercaptoethanol (Sigma-Aldrich; M6250–10 mL) was added into each 5 mL bead tube. A Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, FR) was used for the homogenization of the sample. Precellys was set for 3 × 15 s at 10,000 rpm at a 10 s interval. The bead-beating tubes were centrifuged at 4000 *g* for 5 min to separate the lysate from the pellet debris. The resultant sample lysate was used for the nucleic acid extraction with two minor ~~slight~~ modifications, (i) the use of DNase I solution was omitted to isolate both RNA and DNA; (ii) 200 µL of DNase and RNase free water was used to eluate nucleic acid instead of 100 µL. A DeNovix Spectrophotometer & Fluorometer (Wilmington, DE, USA) was used to determine the purity of extracted nucleic acid by measuring 260/280 ratio.

2.6 Inhibition assessment

Known quantities (1.5×10^4 GC) of murine hepatitis virus (MHV) were seeded into each homogenized lysate as an inhibition process control. The same quantity of MHV was also added to a distilled water extraction control followed by nucleic acid extraction. An MHV RT-qPCR assay was used to determine PCR inhibition in nucleic acid samples extracted from wastewater (Besselsen et al., 2002). ~~(Besselen et al., 2002).~~ The reference quantification cycle (Cq) values obtained for nucleic acid samples (MHV seeded into the distilled water) were compared with the Cq values of the MHV seeded into wastewater lysate to assess potential RT-qPCR inhibition (Ahmed et al., 2022a). ~~(Ahmed et al., 2022).~~ If the Cq value resulting from the sample was greater than two cycles different from the reference Cq value for the distilled water control, the sample was interpreted as inhibited (Ahmed et al., 2018).

2.7 RT-qPCR analysis

For MHV (Besselsen et al., 2002)~~(Besselen et al., 2002)~~ detection and SARS-CoV-2 RNA quantification (US CDC, 2020; China CDC, 2020), ~~China CDC, 2020),~~ previously published RT-PCR and RT-qPCR assays were used. For the MHV positive control, gBlocks gene fragment was purchased from Integrated DNA Technologies (Integrated DNA Technology Coralville, IA, US), while gamma-irradiated SARS-CoV-2 was used as an RT-qPCR standard for all four RT-qPCR assays. For each RT-qPCR assay standard curve dilutions ranged from 5×10^5 to 0.5 GC/reaction. Primer and probe sequences, reaction concentrations, and thermal cycling conditions are listed in Table 1. All RT-qPCR analyses were performed in 20-µL reaction mixtures using TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystem, California, USA). MHV RT-qPCR mixture contained 5 µL of Supermix, 300 nM of forward primer, 300 nM of reverse primer, 400 nM of probe, and 5 µL of template RNA. US CDC N1 and N2 RT-qPCR mixture contained 5 µL of Supermix, 500 nM of forward primer, 500 nM of reverse primer, 125 nM of probe, and 5 µL of template RNA. CCDC N RT-qPCR mixture contained 5 µL of Supermix, 400 nM of forward primer, 400 nM of reverse primer, 250 nM of probe, and 5 µL of template RNA. CCDC ORF1ab RT-qPCR mixture contained 5 µL of Supermix, 300 nM of forward primer, 300 nM of reverse primer, 300 nM of probe, and 5 µL of template RNA. The RT-qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Richmond, CA, USA) using manual settings for threshold and baseline.

alt-text: Table 1

Table 1

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[Instruction: Can you please add a footnote for Table 1? Please see below.
CCDC: China CDC][Instruction: MC: Please check comment section.]RT-qPCR performance characteristics.

Assay	Performance characteristic (range)

	Efficiency (E) (%)	Linearity (R^2)	Slope	Y-intercept
US CDC N1	97.7	0.993	−3.378	36.40
US CDC N2	95.9	0.989	−3.424	39.12
CCDC N	100	0.982	−3.314	37.00
CCDC ORF1ab	98.0	0.991	−3.370	37.41

2.8 ATOPlex sequencing and bioinformatics

The ATOPlex SARS-CoV-2 full-length genome panel (MGI, Shenzhen, China) was used to construct libraries of short amplicons (159–199 bp) according to the manufacturer's instructions. The wastewater RNA sample was converted to cDNA using reverse transcriptase (RT) with random hexamers (5′-NNNNNN-3′) (MGI~~, China~~). The 20-μL RT reaction mixture contained 10 μL of RNA template (5% of extracted volume), 4 μL of N6 buffer, 5 μL of RT buffer, 12.5 μM of random hexamers, and 1 μL of RT enzyme mix. The RT reaction was performed in a C1000 thermal cycler (Bio-Rad, USA) using the program: 10 min at 25 °C, 30 min at 42 °C, 15 min at 70 °C. Lambda phage DNA (200 GC) was added into each sample as a spike-in control to ensure each sample generated sufficient amplification products for sequencing and relative quantifying SARS-CoV-2 RNA (> 4 ng/μL). ~~Next,~~ Lambda phage DNA and SARS-CoV-2 primers were co-amplified in the same reaction as follows:

DNA/cDNA samples were subjected to two rounds of PCR for target enrichment (first round) and addition of dual barcode (second round). In the first round the PCR amplification mixture contained 25 μL of PCR Enzyme Mix (proprietary products), 0.5 μL of PCR Clean Enzyme, 4 μL of PCR Primer Pool, and 20 μL of the wastewater-derived cDNA. The first-round PCR cycling parameters were 5 min at 37 °C, 10 min at 95 °C, 13 cycles of 95 °C for 10 s, 64 °C for 1 min, 60 °C for 1 min and 72 °C for 10 s, followed by a final extension step at 72 °C for 2 min performed on a C1000 thermal cycler. The first-round PCR products were then purified using 1.2 × 60 μL clean magnetic beads. In the second round the PCR amplification mixture contained 25 μL of PCR Enzyme Mix, 0.5 μL of PCR Clean Enzyme, 1 μL of PCR additive, 2 μL of PCR block (259 sets of barcoded SARS-CoV-2 primers each targeting a different ~ 200 bp region to encompass the entire genome (accession MN908947.3) (Wu et al., 2020), 10 sets of Lambda Phage DNA primers; and four sets of primers targeting the human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene for human DNA/RNA contamination control into the purified PCR products from the first round. The second-round PCR was performed under the same cycling parameters as that of the first-round PCR, except 27 PCR cycles were used. The second-round PCR products were also purified using 0.9 × 45 μL clean magnetic beads. After bead-based purification, the second-round PCR products were quantified with the Qubit dsDNA High Sensitivity Assay kit ((Thermo Fisher Scientific, Waltham, MA, USA) to confirm the required concentration of ≥4 ng/μL.

Short amplicons libraries from each sample were pooled at equimolar levels and subjected to single-stranded circular DNA ~~(ssDNA)~~ library preparation with the MGIEasy Dual Barcode Circularization kit (MGI~~, Shenzen, China~~) to obtain circularized DNA molecules. These molecules were subsequently digested to form circularized single strand DNA (ssCirDNA) and then subjected to rolling circle amplification to generate DNA nanoballs (DNBs) based libraries. The DNBs were added to a silicon slide that contains a grid-like pattern of binding sites, which enables the DNBs to self-assemble into a dense grid of spots for sequencing. The bases (A, C, T or G) of the DNBs were identified through digital imaging during each cycle of sequencing, when complementary and fluorophores-nucleotide containing nucleotides were ligated to the DNBs (Drmanac et al., 2010). The DNB libraries were sequenced on a DNBSEQ-G400 instrument at BGI Australia with pair-end 100 sequencing set (MGI~~, Shenzhen, China~~).

In this study, the read processing was carried out by following the SARS-CoV-2_MultiPCR_v1.0 workflow (https://github.com/MGI-tech-bioinformatics/SARS-CoV-2_Multi-PCR_v1.0). A total of 2459,029,098 paired end reads (100 bp) were generated from the 72 samples for a total of 491,805,819,600 bp of sequencing data. For samples with a total read number >20 million, each sample was randomly subsampled to 20 million reads. Across 72 samples, 90.6% of the reads were above 99.9% accuracy (Phred score ≥ 30). Filtering of reads was conducted based on read quality, adaptor content and rate of unknown bases (“N” s), from which an average of 98.3% of reads were kept. The primer-trimmed, mapped reads were used to obtain the numbers of reads that map to either the SARS-CoV-2 genome, Lambda phage DNA (NC_001416.1), or GAPDH (NM_001289745.3).

2.9 Quality control

To minimize RT-qPCR contamination, nucleic acid extraction and RT-qPCR set up were performed in separate laboratories. A sample negative control was included during the concentration process. An extraction negative control was also included during nucleic acid extraction to account for any contamination during extraction. All sample and extraction negative controls were negative for the analyzed targets.

2.10 Data analysis

For RT-qPCR, samples were considered positive (SARS-CoV-2 detected) if amplification was observed in at least one of the four replicates and no amplification occurred for negative controls. For ATOplex, samples were classified as either positive or negative for SARS-CoV-2 by first normalizing the number of SARS-CoV-2 reads to those of a spike-in lambda phage DNA control (18,000 GC) according to the [Instruction: I see the full equation below but in the PDF proof half of the equation is missing. This needs to be fixed as this equation is important.]Equation below. This normalization is required to account for the randomness of the ~~number produced~~ reads during sequencing by calibrating to a target seeded at a known quantity, which ~~and~~ minimizes the bias from variation in sequencing depth across samples (Ni et al., 2021).

$$\text{Nomalized SARS} - \text{CoV} - 2 \text{ reads} = \frac{\#\text{ of SARS} - \text{CoV} - 2 \text{ reads}}{\#\text{ of lambda phage reads}} \times \frac{1,400 \text{ bases (lambda phage genome length)}}{29,871 \text{ (SARS} - \text{CoV} - 2 \text{ genome length}}$$

When normalized SARS-CoV-2 reads were ≥to 0.01 (i.e., 21 SARS-CoV-2 reads/100 lambda phage reads), it was classified as positive, and when normalized SARS-CoV-2 reads were <0.001 (i.e., 2.1 SARS-CoV-2 reads/100 lambda phage reads), it was classified as negative. Normalized read values between 0.001 and 0.01 were interpreted as low depth samples. The low depth samples were classified as positive when number of mapped amplicon tiles were >5. The proportion of samples positive by each RT-qPCR assay and all RT-qPCR assays pooled together were compared to the proportion positive by ATOPlex at each seeding level using Fisher's exact test (Fisher, 1922).

3 Results

3.1 Assay performance and relevant QA/QC

The RT-qPCR standard curves prepared from gamma-irradiated SARS-CoV-2 had a linear dynamic range from 6×10^5 to 6 GC/reaction (1.2×10^5 to 1.2 GC/µL). The slopes of the standard curves ranged between −3.314 (CCDC N) and −3.424 (US CDC N2) (Table 1). The ranges for amplification efficiencies (94.0 to 100%) and y-intercepts ~~(~~36.40 (US CDC N1) to 39.12 (US CDC N2) were within the prescribed range of MIQE guidelines (Bustin et al., 2009). The squares of the correlation coefficient~~s~~ (r^2) ranged from 0.982 (CCDC N) to 0.993 (US CDC N1). All method, extraction and RT-qPCR negative controls were negative. All positive controls or standard curves were successfully amplified in each PCR run. PCR inhibition was not identified in any RNA samples based on the seeded GC of MHV (all well within 2-Cq values of the reference Cq value) (Supplementary Tables ST4 and ST5). The measured 260/280 ratio of nucleic acid >1.8 for wastewater RNA sample was considered acceptable RNA quality (Supplementary Tables ST2 and ST3). ~~(Sambrook et al., 1989).~~

Wastewater samples, corresponding dilutions and ATOPlex-specific generated total number of quality-filtered reads, SARS-CoV-2 mapped reads, genome depth and coverage, and mapping rates are shown in Table 2. During both trials, the total number of quality-filtered reads across all samples and replicates at all SARS-CoV-2 seeding concentrations ranged from 17 to 20 million; however, the total number of SARS-CoV-2 mapped reads decreased with decreasing seeding concentration, with means of 290,000 to 48,000 at the highest titer ($\sim 2 \times 10^5$ GC/50 mL) and 33 to 94 at the lowest ($\sim 2 \times 10^2$ GC/50 mL) during trials A and B, respectively. At the highest seeding level in Trials A and B, the breadth of genome coverage (at or above 30 times depth) was 93.9 - 99.9% and 71.4 - 99.3%, respectively. As expected, this breadth of coverage decreased with decreasing seeding concentrations to minimums of 0 – 1.65% and 0 – 8.40% during the two trials (Table 2).

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Table 2

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[Instruction: In the last column, the values are spilling over to the next line. Can you please fix this issue. It looks awkward. There is enogh space in between the column to fix this issue.]Genome depth, coverage, and mapping rates of known concentrations of SARS-CoV-2 seeded in wastewater in Trials A and B. Average and range values were generated from each batch of dilution (9 samples each) in Trials A and B.

Concentrations of SARS-CoV-2 seeded/50 mL of wastewater	No. of wastewater samples analyzed	Total no.of quality-filtered reads(mean ± SD)	Total no. ofSARS-CoV-2 mapped reads(mean ± SD)	Range of SARS-CoV-2 mapping rates (%)	Average genome depth (range)	Range of genomic coverage breadth at ≥30 times the depth (%)	Mapping rate (%)
Trial A							
2.32×10^5 GC	9	$1.93 \pm 0.06 \times 10^7$	289,875 ± 341,950	0.36 –	222 –	93.9 – 99.9	92.4 –

				5.73	3214		93.8
10^{-1} Dilution	9	$1.96 \pm 0.03 \times 10^7$	4383 ± 2702	0.00 – 0.05	1.39 – 27.9	13.5 – 52.9	92.3 – 93.9
10^{-2} Dilution	9	$1.95 \pm 0.04 \times 10^7$	1271 ± 1913	0.00 – 0.30	0.01 – 19.4	1.30 – 15.6	91.6 – 94.0
10^{-3} Dilution	9	$1.91 \pm 0.19 \times 10^7$	33 ± 78	0.00 – 0.00	0.00 – 0.78	0.00 – 1.65	93.0 – 93.5
Trial B							
1.79×10^5 GC	9	$1.71 \pm 0.44 \times 10^7$	48,013 ± 90,543	0.02 – 3.27	12.2 - 945	71.4 – 99.3	95.5 – 96.5
10^{-1} Dilution	9	$1.79 \pm 0.40 \times 10^7$	912 ± 1499	0.00 – 0.03	0.00 – 16.1	0.00 – 50.8	96.0 – 97.4
10^{-2} Dilution	9	$1.88 \pm 0.18 \times 10^7$	304 ± 321	0.00 – 0.00	0.00 – 2.89	0.00 – 9.70	95.7 – 99.1
10^{-3} Dilution	9	$1.69 \pm 0.47 \times 10^7$	94 ± 178	0.00 – 0.00	0.00 – 1.65	0.00 – 8.40	94.8 – 97.0

CCDC: China CDC; SD = standard deviation.

In Trial A, SARS-CoV-2 RNA was detected in all 18 wastewater samples (nine samples for 2.32×10^5 GC and nine samples for 10^{-1} dilution (i.e., containing ~2.32×10^4 GC/50 mL of wastewater) using any of the four RT-qPCR assays and technical replicates ($n = 3$ per assay) and ATOPlex amplicon sequencing ($n = 1$ replicate/sample). Similar results were also observed for Trial B (Table 3), where all wastewater samples were RT-qPCR (all four assays) and ATOPlex positive for 1.79×10^5 GC and 10^{-1} dilution (i.e., containing ~1.79×10^4 GC/50 mL of wastewater).

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Table 3

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[Instruction: First few columns, the values are spilling over to the next line. Can you please fix this issue. It looks awkward. There is enogh space in between the column to fix this issue. So the values shoould be like 9/9 (100). I am a bit concerned about the quality of the proof you guys produce considering we pay hefty $$ for OA.]Proportion of samples positive for SARS-CoV-2 RNA in Trials A and B of wastewater seeded at four concentrations using four RT-qPCR assays and ATOPlex sequencing.

Concentrations of SARS-CoV-2 seeded/50 mL of wastewater	**No. of wastewater samples positive/No. of samples tested (%)**					
	US CDC N1	**US CDC N2**	**CCDC N1**	**CCDC ORF1ab**	**RT-qPCR all assays combined**	**ATOPlex**
Trial A						
2.32×10^5 GC	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)
10^{-1} Dilution	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)
10^{-2} Dilution	8/9 (88.9)	1/9 (11.1)	9/9 (100)	7/9 (77.8)	9/9 (100)	6/9 (66.6)
10^{-3} Dilution	6/9 (66.6)	1/9 (11.1)	3/9 (33.3)	1/9 (11.1)	8/9 (88.9)	1/9 (11.1)
Trial B						
1.79×10^5 GC	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)
10^{-1} Dilution	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)	9/9 (100)	8/9 (88.8)
10^{-2} Dilution	8/9 (88.8)	6/9 (66.6)	4/9 (44.4)	6/9 (66.6)	8/9 (88.8)	6/9 (66.6)
10^{-3} Dilution	5/9 (55.5)	2/9 (22.2)	1/9 (11.1)	5/9 (55.5)	7/9 (77.7)	3/9 (33.3)

CCDC: China CDC.

For the 10^{-2} dilution in Trial A, the SARS-CoV-2 RNA detection rate (100%) of CCDC N1 RT-qPCR assay was slightly greater than US CDC N1 (88.9%) followed by CCDC ORF1 (77.8%). US CDC N2 assay detection rate was much lower (11.1%) than the other three RT-qPCR assays. When unique positive and negative results from all four RT-qPCR assays were combined for the 10^{-2} dilution, all nine samples were positive for SARS-CoV-2 RNA. In contrast, six (66.6%) of nine samples were positive for SARS-CoV-2 by ATOPlex sequencing, which outperformed the US CDC N2 assay (detection rate is 11.1%). In Trial B at the 10^{-2} dilutions, the SARS-CoV-2 RNA detection rate (88.9%) by the US CDC N1 assay was greater than other three assays (44.4 to 66.6%). Combined, the RT-qPCR results from all four assays did not increase the detection rate compared to the US CDC N1 assay but the rate was greater than the US CDC N2, CCDC N and CCDC ORF1ab assays individually. ATOPlex sequencing produced six positives (66.6%) of nine seeded wastewater samples with a detection rate similar to the US CDC N2 and CCDC ORF1ab (both 66.6%), but greater than CCDC N1 (44.4%) RT-qPCR assays.

The frequency of SARS-CoV-2 RNA detection at the 10^{-3} dilution (containing $\sim 1.79 \times 10^2$ GC/50 mL of wastewater) by all assays were lower than the 10^{-2} dilution. At the 10^{-3} dilution, in Trial A the detection rate (66.6%) by the US CDC N1 was greater than US CDC N2 (11.1%), CCDC N1 (33.3%) and CCDC ORF1ab (11.1%). However, combined unique positive and negative results from all four RT-qPCR assays increased the detection rate (88.9%) compared to the detection rates of single assays. The ATOPlex detection rate (11.1%) at this dilution was similar to the US CDC N2 and CCDC N1 RT-qPCR assays.

In Trial B at 10^{-3} dilution detection rates of the US CDC N1 (55.5%) and CCDC ORF1ab (66.6%) assays outperformed US CDC N2 (22.2%) and CCDC N1 (11.1%) assays. An increased detection rate (77.7%) was observed when results from all RT-qPCR assays were combined in comparison to results from any single RT-qPCR assay (which ranged from 11.1 to 55.5%). ATOPlex sequencing produced three positives of nine seeded wastewater samples, and the detection rate (33.3%) was greater than the US CDC N2 (22.2%) and CCDC N1 (11.1%) assays but lower than US CDC N1 and CCDC ORF1ab (both 55.5%).

Between Trials A and B, the US CDC N1 assay detection rates were relatively consistent, while the US CDC N2 and CCDC ORF1ab detection rates were greater for Trial B than A, and the CCDC N1 detection rate was greater for Trial A than B. Fisher's exact test (Table 4) indicated no difference ($p > 0.999$) in the SARS-CoV-2 RNA positivity rate between RT-qPCR assays (individual and combined) and ATOPlex when seeding concentrations were greater than 4 $\log_{10}$ GC/50 mL. At the 3 $\log_{10}$ GC/50 mL seeding concentration, positivity rates between individual RT-qPCR assays and ATOPlex sequencing were still not significantly different ($p \geq 0.121$). However, when unique detections were combined across all assays, SARS-CoV-2 RNA positivity by RT-qPCR was significantly greater than by ATOPlex ($p = 0.041$). At the lowest seeding concentration (~2 $\log_{10}$ GC/50 mL), SARS-CoV-2 RNA positivity was significantly greater by the US CDC N1 ($p = 0.041$) assay individually and all RT-qPCR assays combined ($p < 0.001$) than by ATOPlex. Conversely, the US CDC N2, CCDC N1, and CCDC ORF1ab RT-pPCR assays did not yield significantly different positivity from ATOPlex sequencing ($p \geq 0.711$).

alt-text: Table 4

Table 4

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Table 4, would be better if column 1 (left) was less wide (narrowed so the column title text could spread over 3-4 lines OK) to allow for the > symbols not to disconnect from the 0.999 etc]Fisher's exact test p-values to compare the positivity rate of each RT-qPCR assay and all RT-qPCR assays combined with ATOPlex sequencing.

Concentrations of SARS-CoV-2 seeded/50 mL of wastewater	Fisher's exact p-value for the proportion of samples positive by RT-qPCR versus ATOPlex				
	US CDC N1	US CDC N2	CCDC N1	CCDC ORF1ab	RT-qPCR all assays combined
5 $\log_{10}$ GC	>0.999	>0.999	>0.999	>0.999	>0.999
4 $\log_{10}$ GC	>0.999	>0.999	>0.999	>0.999	>0.999
3 $\log_{10}$ GC	0.121	0.318	0.725	0.725	0.041
2 $\log_{10}$ GC	0.041	>0.999	>0.999	0.711	<0.001

CCDC: China CDC.

For the lowest two dilutions (10^{-2} and 10^{-3}) of the nine SARS-CoV-2-seeded wastewaters in each Trial, the mean Cq values of RT-qPCR assays were compared to the ATOPlex-positive samples and the number of ATOPlex-mapped sites (Table 5). In Trial A at 10^{-2} dilution, the ATOPlex ~~method~~ produced positive results when the Cq values of US CDC N1 and CCDC N1 RT-qPCR assays ranged between 32.4 and 41.5 (mean Cq = 36.7) and 35.3 to 37.9 (mean Cq = 36.8), respectively. In the Trial B at 10^{-2} dilution, ATOPlex yielded positive results when the Cq values of the RT-qPCR assays ranged between 34.4 and 39.6 (mean Cq = 36.3). At this dilution, three samples were classified as negative by ATOPlex when the RT-qPCR assay in both trials, being Cq values ranged between 34.6 and 40.3 (average Cq = 36.4) (most instances Cq values were greater than 35) in Trial A and Cq values were >35 in Trial B.

alt-text: Table 5

Table 5

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WWTP samples	US CDC N1	US CDC N2	CCDC N1	CCDC ORF1ab	ATOPlex positive samples (number of mapped amplicon sites)
	Mean Cq values				
Trials A					
10^{-2} Dilution					
WW32	+ (32.4)	ND	+ (35.7)	+ (41.1)	+ (32)
WW33	+ (34.6)	+ (44.5)	+ (37.0)	+ (41.4)	+ (160)
WW34	+ (39.1)	ND	+ (36.9)	+ (41.6)	+ (93)
WW35	+ (37.8)	ND	+ (37.9)	+ (43.8)	+ (28)
WW36	ND	ND	+ (35.7)	+ (44.5)	ND (4)
WW37	+ 41.5	ND	+ (37.8)	+ (42.5)	+ (14)
WW38	+ 34.8	ND	+ (36.1)	+ (41.6)	ND (3)
WW39	+ 36.8	ND	+ (36.5)	ND	ND (2)
WW40	+ 34.8	ND	+ (35.3)	ND	+ (40)
10^{-3} Dilution					
WW23	ND	ND	ND	ND	ND (3)
WW24	ND	+ (37.1)	ND	ND	ND (0)
WW25	+ (42.8)	ND	ND	ND	ND (0)
WW26	+ (41.1)	ND	+ (35.5)	ND	ND (0)
WW27	+ (41.1)	ND	+ (36.9)	ND	ND (3)
WW28	ND	ND	ND	ND	ND (0)
WW29	+ (41.1)	ND	+ (40.4)	ND	ND (2)
WW30	+ (41.7)	ND	ND	ND	ND (2)
WW31	+ (42.4)	ND	ND	+ (44.5)	+ (6)
Trials B					
10^{-2} Dilution					
WW23	+ (34.7)	+ (37.0)	+ (34.4)	+ (36.1)	+ (44)
WW24	+ (35.2)	ND	ND	ND	+ (35)
WW25	+ (35.8)	+ (40.3)	+ (34.7)	+ (34.6)	ND (4)
WW26	+ (35.3)	+ (38.1)	ND	ND	+ (39)
WW27	+ (35.2)	+ (38.9)	ND	+ (38.1)	+ (6)

WW28	+ (34.9)	+ (37.1)	+ (35.5)	+ (36.5)	+ (28)
WW29	ND	ND	ND	ND	ND (0)
WW30	+ (35.7)	ND	+ (36.6)	+ (37.3)	ND (0)
WW31	+ (35.3)	+ (39.6)	ND	+ (35.2)	+ (42)
10^{-3} Dilution					
WW32	+ (35.6)	+ (38.0)	ND	ND	ND (0)
WW33	ND	ND	ND	ND	ND (2)
WW34	ND	ND	ND	+ (37.4)	ND (0)
WW35	ND	ND	ND	+ (38.5)	ND (0)
WW36	ND	ND	ND	ND	+ (12)
WW37	+ (36.4)	ND	ND	+ (35.9)	+ (10)
WW38	+ (35.8)	ND	ND	+ (37.2)	ND (0)
WW39	+ (35.6)	ND	ND	ND	ND (0)
WW40	+ (34.5)	+ (36.9)	+ (33.2)	+ (35.4)	+ (40)

For the Trial A 10^{-3} dilution, the majority of the samples were classified as negative by ATOPlex compared to RT-qPCR and the Cq vales of these positive samples were >35. For the Trial B 10^{-3} dilution, the majority of the samples were classified as negative by ATOPlex compared to RT-qPCR with Cq vales of these RT-qPCR positive samples >35. Interestingly, sample WW36 was negative by all RT-qPCR assays but ATOPlex mapped to 40 SARS-CoV-2 genomic loci~~sites~~ and was classified as positive.

4 Discussion

Multiple studies have reported the application of ~~2nd and 3rd generation~~ sequencing methods to detect and quantify the SARS-CoV-2 virus and its VOC~~variants~~ in wastewater. These methods were developed for clinical use but have been applied for the analysis of wastewater samples. These include tiled amplicon approaches such as a Nanopore-based method utilizing 89 primer sets (Izquierdo-Lara et al., 2021; Oude Munnink et al., 2020), or ones that employ ARTIC primer sets (Lin et al., 2021; ~~Nemudryi et al., 2020;~~ Rios et al., 2021; Swift et al., 2021). Other tiling amplicon approaches include ATOPlex, utilizing 259 primer sets (Ni et al., 2021), or Illumina sequencing with the Swift Nomalase® Amplicon SARS CoV-2 Panel (SNAP) panel (Fontenele et al., 2021). An oligo-based enrichment/capture approach has also been used (Crits-Christoph et al., 2021).

Most of these studies were conducted in countries with high COVID-19 prevalence such as USA, Canada, Belgium, France, and Netherlands. However, analysis of wastewater-based SARS-CoV-2 detection sensitivities between sequencing (i.e., targeting multiple genomic loci) and RT-qPCR (i.e., targeting a single genomic locus) has not been performed. In view of this, we compared diagnostic sensitivities from several RT-qPCR assays and ATOPlex amplicon-based sequencing by seeding serially-diluted gamma-irradiated SARS-CoV-2 in wastewater. We present detection results by RT-qPCR and ATOPlex sequencing workflows for scenarios when the seeded numbers of SARS-CoV-2 in wastewater samples are moderate to low (10^5 to 10^2 GC/50 mL).

While the data from this study allows a cross-comparison among RT-qPCR assays, however, making a direct comparison between RT-qPCR assays and ATOPlex sequencing is difficult due to several differences in processing and worflow; namely, the RT-qPCR assays used in this study are one-step (RT and PCR included in the same tube), while ATOPlex sequencing is a two-step multiplex PCR which amplifies the RNA target region in a single tube, and sequencing involved preparation of circularized single strand DNA from RNA. Another significant difference between these two strategies is that RT-qPCR assays target a small fragment of the genome (~60 to 160 bp), while ATOPlex utilizes 259 primer sets along the SARS-CoV-2 genome with amplicon tiles ranging in size from 159 to 199 bp. There are also differences in input nucleic acid concentrations, kits, and in the designation of samples to positive or negative detections.

This study was carefully designed to include a number of wastewater samples to capture the inherent variations in the wastewater matrix, rather than using a bulk wastewater. Two trials~~trails~~ of experiments were conducted to obtain confirmatory results. ~~For SARS-CoV-2 concentrations, we used an adsorption-extraction method which is reported to be less variable for concentrating SARS-CoV-2 from wastewater (Ahmed et al., 2021a).~~ After seeding SARS-COV-2 in wastewater, sample processing, RNA extraction and analysis were undertaken within 48 h to avoid RNA degradation. The purity of extracted nucleic acid was checked and RT-PCR inhibition was assessed.

For both trials and seeding dilutions, consistent SARS-CoV-2 detections was achieved for RT-qPCR assays and ATOPlex at the two higher seeding levels, suggesting that detection using the adsorption-extraction concentration method is quite robust when the numbers of seeded SARS-CoV-2 on the order of 10^4 to 10^5~~.~~ GC/50 mL. However, detection rates decreased at dilutions 10^{-2} and 10^{-3} by both RT-qPCR and ATOPlex sequencing. This is probably due to sub-sampling error which can introduce errors in RNA detection at low viral concentrations (Taylor et al., 2019). After concentration, some wastewater RNA samples may contain 10–50 GC in a volume of 100 µL RNA. Since we analyzed only a portion of the 200 µL RNA (5 µL per RT-qPCR and 10 µL for ATOPlex sequencing), this will result in stochastic detection. The impacts of sub-sampling error in wastewater samples with low target RNA concentrations have been discussed thoroughly (Ahmed et al., 2022b). While MHV seeding analysis suggested the absence of PCR inhibitors, however, the presence of low levels of inhibition could not be ruled out~~. and may have masked the positive detection.~~

Among the RT-qPCR assays, the detection rate of US CDC N1 assay was greater than other assays, suggesting application of this assay may be advantageous when the level of SARS-CoV-2 is low or near the limit of detection in wastewater. However, combining results from multiple RT-qPCR assays produced a greater detection rate than the individual assays alone. Multiple assays, including US CDC N1, should be used for trace detections and to avoid potential false negative results due to mismatches in the primer target sequence from mutations. Interestingly, ATOPlex sequencing was not as sensitive as the US CDC N1 nor combined RT-qPCR assay results, despite its use of 259 multiplexed primer sets to amplify the SARS-CoV-2 genome and despite 2-fold greater RNA input in this study design.

A recent study has highlighted the potential application of ATOPlex sequencing for wastewater surveillance and reported that ATOPlex sequencing was capable of quantifying SARS-CoV-2 RNA at concentrations at least one order of magnitude lower than the detection limit of RT-qPCR (Ni et al., 2021). However, ATOPlex and RT-qPCR comparison was undertaken using a commercial RNA positive control diluted in water and did not account for matrix interference or loss through viral concentration and RNA extraction. Therefore, a direct comparison of results from Ni et al. (2021) and this study is not possible. Targeting multiple loci along the SARS-CoV-2 genome is expected to enhance sensitivity in a matrix such as wastewater where m~~M~~ultiple species of viral RNA are likely present (i.e., fragmented, genomic, sub-genomic) in non-stoichiometric amounts. On the other hand, this may increase error in wastewater surveillance where some of these primers may amplify off-target sequences from other microorganisms also present in wastewater. Therefore, a cut-off of 5 amplicon tile mapping sites was used to designate a sample positive or negative with ATOPlex sequencing. We acknowledge that in this study for ATOPlex, multiple replicates were not used which could have reduced the detection sensitivity.

While the ATOPlex sequencing assay was not as sensitive ~~for detection~~ as the US CDC N1 nor the combined RT-qPCR assay results, it nonetheless had similar sensitivity to three commonly utilized RT-qPCR assays (US CDC N2, CCDC N1, and CCDC ORF1ab) when analyzed independently at the lowest seed concentration (10^{-3} dilution). This demonstrates that ATOPlex could be utilized not only for monitoring for VOC~~genomic variants~~, but also for positive/negative detection of SARS-CoV-2 in wastewater samples. However, based on the observed trends in decreasing mapping rates and breadth of coverage with decreasing SARS-CoV-2 concentrations, the ATOPlex method seems more suited for wastewaters with medium to high viral concentrations (e.g., at or above the 10^{-2} dilution level). Previous studies utilizing genomic sequencing of SARS-CoV-2 in wastewater have observed mixed trends in genome coverage versus viral concentration, based on the different methods employed. Crits-Christoph et al. (2021) used hybridization-based probe capture followed by metatranscriptomic sequencing and observed no correlation between the relative abundance of SARS-CoV-2 and ~~genome copies~~GC quantified by RT-qPCR. Using multiplex tiling PCR with 500 bp amplicons for viral enrichment followed by sequencing with Nanopore, Izquierdo-Lara et al. (2021) found an inverse sigmoidal correlation between the breadth of genome coverage and the Cq values of both the N2 and the E primers/probe sets within a range of Cq values from 27 to 36. However, using multiplex tiling PCR with shorter amplicons (Swift Normalase® Amplicon SARS CoV-2 Panel), Fontenele et al. (2021) did not observe any correlation between breadth of coverage and RT-qPCR Cq values in the range of 27 and 36, even though there was an apparent decreasing trend in depth of genome coverage with increasing Cq. Thus, the sensitivity of multiplex tiling PCR assays may be dependent on aspects of the primer panel design, such as amplicon length. Such effects were systematically explored by Lin et al. (2021) by conducting a comparison of three different multiplex tiling PCR primer sets of different amplicon length (150 bp, 400 bp, 1200 bp) with the same wastewater samples with Cq values ranging from 29 to 36, which revealed that shorter amplicons (e.g. 150 bp Swift Biosciences) were less susceptible to decreasing detection rates at lower viral concentrations. However, it is also apparent that trade-offs exist in the design of multiplex tiling PCR assays, as Lin et al. (2021) found lower mapping rates and depth of genome coverage at high viral concentrations using the 150 bp amplicon panel versus 400 bp and 1200 bp, which was attributed to more primer-primer interactions and higher off-target amplification rates. As the ATOPlex assay used here relied on shorter amplicons (159 to 199 bp), it is thus possible that observed detection sensitivity was impacted by higher rates of off-target amplification and primer-primer interactions. It has also been shown that the sensitivity of multiplex tiling PCR is dependent on the wastewater matrix and/or extraction method (Lin et al., 2021). Therefore, it may be possible to optimize the sensitivity of multiplex tiling PCR for the application of SARS-CoV-2 detection based on a combined selection of wastewater matrix type, extraction method, and multiplex primer assay design, beyond what is presented in this current study.

Here, we show that the PLOD of RT-qPCR for the detection of SARS-CoV-2 in wastewater was lower than that of ATOPlex. Therefore, for applications where positive/negative detection is critical, and wastewater viral concentrations are low, RT-qPCR could beis the preferred method. There are additional benefits of RT-qPCR, such as its shorter turnaround time (within 4.5 h from concentration to results for RT-qPCR versus 48–60 h for ATOPlex), as well as its lower cost per sample. However, the application of multiplex tiling PCR based sequencing offers several critical advantages for wastewater surveillance, such as the ability to detect and monitor VOC. genomic variants (e.g., variants of concern/interest and novel/emerging variants) of concern. It could also be argued that sequencing provides more convincing detection of SARS-CoV-2 genomic fragments at low viral concentrations with verification via read mapping at multiple sites across the genome, as high Cq values in RT-qPCR can be difficult to discern between true detection from non-target amplification for non-specific RT-qPCR assays. This is especially an issue for the complex in the wastewater matrix which has greater chemical and nucleic acid complexity compared to human clinical specimens and thus more opportunity for off-target amplification or spurious probe hydrolysis. Therefore, a strategy of frequent RT-qPCR testing (e.g., daily) complemented by periodic multiplex tiling PCR sequencing (e.g., weekly or fortnightly) may represent a powerful combination for monitoring SARS-CoV-2 concentrations and evolutionary dynamics throughout the COVID-19 pandemic.

5 Conclusions

- Among the four RT-qPCR assays tested (US CDC N1, US CDC N2, CCDC N, CCDC ORF1ab), the US CDC N1 assay outperformed all other assays, as well as ATOPlex sequencing, especially in instances when SARS-CoV-2 levels wereare close to nearing detection thresholds.
- Combining multiple RT-qPCR assays can increase SARS-CoV-2 detection sensitivity, over any individual assay. This approach may be especially useful when SARS-CoV-2 levels in wastewater are low, while minimizing false negative results arising from mismatches in primer design due to viral genome mutations.
- The ATOPlex sequencing displayed similar or relatively lesser sensitivity to RT-qPCR assays in instances of low SARS-CoV-2 concentrations, but performance may be dependent on primer panel design features such as amplicon length, or other variables such as wastewater matrix, virus concentration and nucleic acid extraction. method used.
- The PLOD for RT-qPCR assays for SARS-CoV-2 detection in wastewater was lower than ATOPlex sequencing, however combination of both approachestechnologies could boost detections sensitivity while enabling identification of genomic variantsVOC as they emergearise.

Uncited references

China CDC (2020), Ahmed et al. (2020b), Ahmed et al. (2020c), Ahmed et al. (2021b), Ahmed et al. (2021c), Besselsen et al. (2002)

Disclaimers

The views expressed in this article are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. The U.S. Environmental Protection Agency through the Office of Research and Development provided technical direction but did not collect, generate, evaluate, or use the environmental data described herein.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118621.

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The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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Graphical abstract

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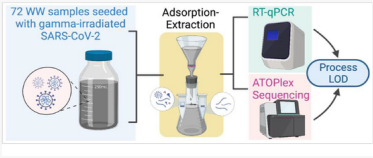

Highlights

- Wastewater PLOD values were determined for four SARS-CoV-2 assays and ATOPlex sequencing.
- The US CDC N1 RT-qPCR assays were the most sensitive assay.
- Combining multiple RT-qPCR assays can increase SARS-CoV-2 detection sensitivity.
- The PLOD for RT-qPCR assays for SARS-CoV-2 detection in wastewater was lower than sequencing.
- Combination of both RT-qPCR and ATOPlex sequencing could boost detections sensitivity.

Appendix Supplementary materials

Multimedia Component 1

alt-text: Image, application 1

Queries and Answers

Q1

Query: Please confirm that given names and surnames have been identified correctly.

Answer: Yes

Q2

Query: This section comprises references that occur in the reference list but not in the body of the text. Please position each reference in the text or, alternatively delete it.

Answer: has been fixed

Q3

Query: Please provide volume no. in Ref. "Ahmed et al. (2021a)".

Answer: I have reqested to delete this reference