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First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community

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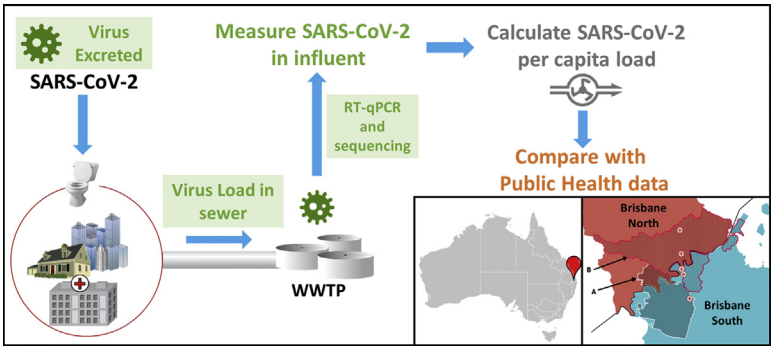
HIGHLIGHTS

- First study that reports the detection of SARS-CoV-2 in wastewater in Australia.
- The presence of SARS-CoV-2 was confirmed by sequencing.
- A median range of 171 to 1090 infected persons was identified in the catchment.
- Further methodological and molecular assay validation will be required.

GRAPHICAL ABSTRACT



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ABSTRACT

Infection with SARS-CoV-2, the etiologic agent of the ongoing COVID-19 pandemic, is accompanied by the shedding of the virus in stool. Therefore, the quantification of SARS-CoV-2 in wastewater affords the ability to monitor the prevalence of infections among the population via wastewater-based epidemiology (WBE). In the current work, SARS-CoV-2 RNA was concentrated from wastewater in a catchment in Australia and viral RNA copies were enumerated using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) resulting in two positive detections within a six day period from the same wastewater treatment plant (WWTP). The estimated viral RNA copy numbers observed in the wastewater were then used to estimate the number of infected individuals in the catchment via Monte Carlo simulation. Given the uncertainty and variation in the input parameters, the model estimated a median range of 171 to 1,090 infected persons in the catchment, which is in reasonable agreement with clinical observations. This work highlights the viability of WBE for monitoring infectious

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Wastewater
Human health risks
Enveloped viruses

diseases, such as COVID-19, in communities. The work also draws attention to the need for further methodological and molecular assay validation for enveloped viruses in wastewater.

1. Introduction

The ongoing global pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been a public health emergency of international concern (WHO, 2020a, 2020b). The reported symptoms of COVID-19 patients include cough, fever, difficulty in breathing and diarrhea, and SARS-CoV-2 ribonucleic acid (RNA) has been detected in feces of not only symptomatic but also asymptomatic patients (Gao et al., 2020; Holshue et al., 2020; Jiehao et al., 2020; Tang et al., 2020; Wölfel et al., 2020; Zhang et al., 2020a; Zhang et al., 2020b). These clinical observations imply that municipal wastewater of affected communities might contain the virus. Wastewater-based epidemiology (WBE) is a promising approach to understand the prevalence of viruses in a given wastewater treatment plant (WWTP) catchment population, because wastewater contains viruses excreted from symptomatic and asymptomatic individuals in a catchment (Sinclair et al., 2008; Xagoraraki and O'Brien, 2020). WBE is especially useful for early warning of disease outbreaks and informing the efficacy of public health interventions, as previously demonstrated for enteric viruses, such as norovirus, hepatitis A virus, and poliovirus (Hellmér et al., 2014; Asghar et al., 2014). In Australia, the first case of COVID-19 was recorded on 25/1/2020, and there have been >6,300 confirmed cases as of April 13, 2020 (Australian Government Department of Health, 2020). In the State of Queensland, there have been 998 cases of COVID-19 recorded on 14/4/2020. The first case of COVID-19 was reported in Brisbane on 21/2/2020 (two passengers from the Diamond Princess Cruise Ship) and increased to 541 confirmed cases on the 14th April 2020 in Queensland's Brisbane North and Brisbane South Primary Health Networks (PHNs). To date, there has been no detection of SARS-CoV-2 in wastewater in Australia, while we note a few recent studies conducted in other parts of the world (i.e., the Netherlands and USA) have reported molecular detection of SARS-CoV-2 in wastewater samples (Lodder and de Roda Husman, 2020; Medema et al., 2020; Wu et al., 2020a). Here, we report the first evidence for the presence of SARS-CoV-2 RNA in wastewater in Australia. Our preliminary findings demonstrate the applicability of WBE for COVID-19 surveillance as a potential tool for public health monitoring at the community level.

2. Materials and methods

2.1. Wastewater sampling

Untreated wastewater (sewage) samples were collected from one suburban pumping station (PS) and two WWTPs representing urban catchments in Southeast Queensland (SEQ). The two WWTP catchments represent approximately 21% (WWTP A) and 50% (WWTP B) of the combined populations of the two PHNs (Fig. 1). Fig. 2a and b showing sampling dates, the number of cases and the potential detection windows (28 days) of SARS-CoV-2 for wastewater samples in the two PHNs (Wu et al., 2020c). Sampling personnel wore face standard personal protective equipment (PPE) for wastewater sampling, such as long pants, steel capped boots, hard hats, safety glasses and gloves. Samples were collected using two types of automated sampling techniques – either a conventional refrigerated autosampler or a submersible in-situ high frequency autosampler (at WWTP A) as well as grab sampling techniques (pumping station and WWTP B). Samples were transported on ice to the laboratory and stored at 4 °C until further analysis.

2.2. Sample concentration and RNA extraction

Viruses were concentrated using two previously published methods. These methods are referred to as Method A (direct RNA extraction from electronegative membranes) (Ahmed et al., 2015) and Method B (ultrafiltration) (Ikner et al., 2011). Method A began with adjustment of the sample pH to ~3.5 to 4 using 2.0 N HCl. The samples (100–200 mL) were then passed through 0.45-μm-pore-size, 90-mm-diameter electronegative membranes (HAWP09000; Merck Millipore, Ltd., Sydney, Australia) via a glass funnel and base (Merck Millipore). For RNA extraction, a combination of two kits (RNeasy PowerWater Kit and RNeasy PowerMicrobiome Kit; Qiagen, Hilden, Germany) were used. A 5-mL bead tube from RNeasy PowerWater Kit was used to accommodate the electronegative membrane. A Precellys 24 tissue homogenizer (Bertin Technologies, France) was used to homogenize the samples at conditions ranging from 3 × 20 s at 8000 rpm at a 10 s interval. From here on RNA was extracted using RNeasy Power Microbiome kit as per manufacturer's instruction.

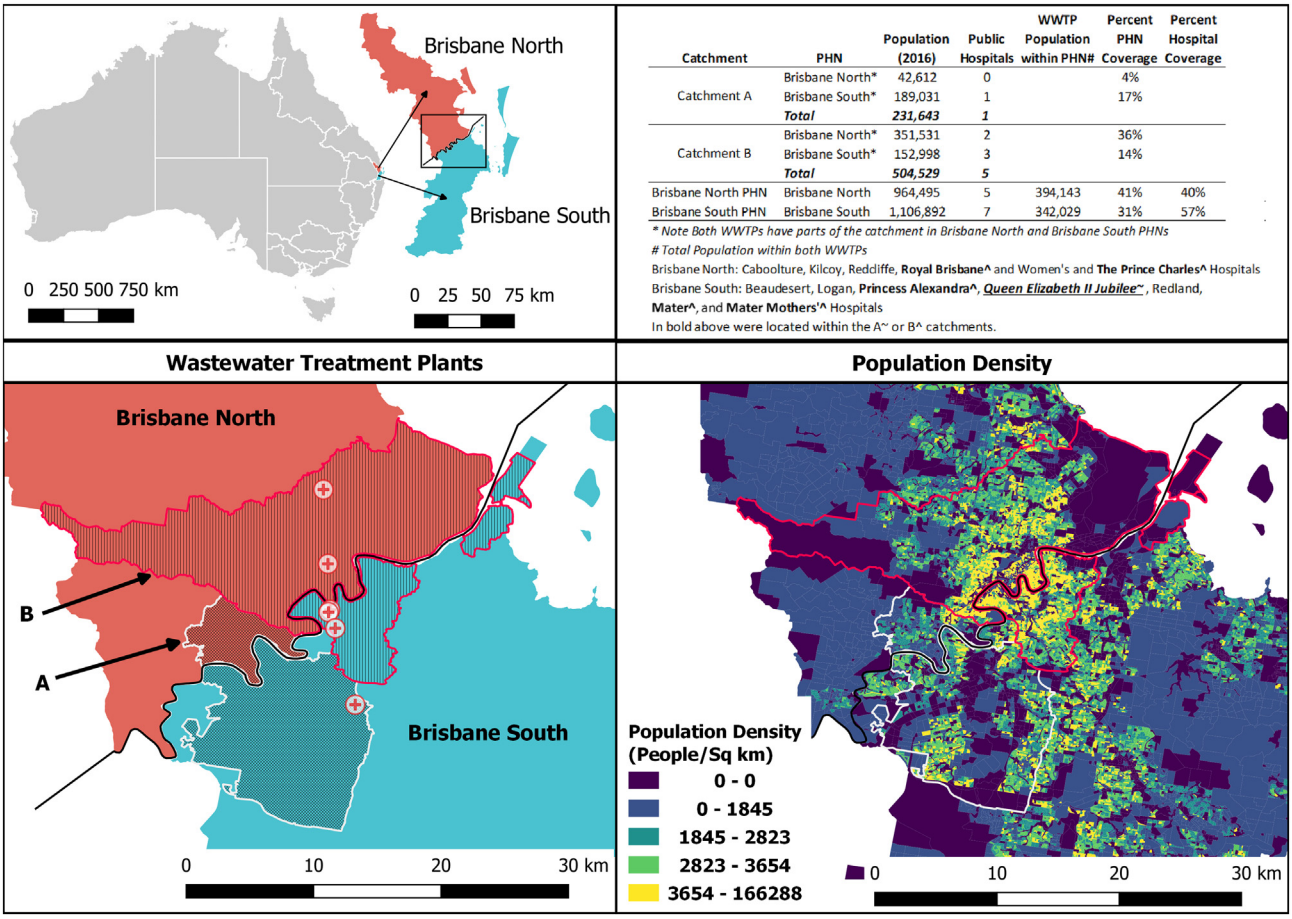
Method B began with centrifugation of wastewater samples (100–200 mL) at 4750*g* for 30 mins. Supernatant was then removed carefully without disturbing the pellet and centrifuged at 3500*g* for 15 min through Centricon® Plus-70 centrifugal filter with a cut-off of 10 kDa (Merck Millipore). The concentrate cup was inverted and placed on top of the sample filter cup. The device was centrifuged at 1000*g* for 2 min. The concentrated sample (~250 μL) was collected from the concentrate collection cup with a pipette. RNA was directly extracted from the concentrate using RNeasy PowerMicrobiome Kit (Qiagen). A QIAcube Connect platform was used to extract RNA to a final volume of 100 μL.

2.3. RT-qPCR analysis

Recently published RT-qPCR assays were used for the detection of SARS-CoV-2 RNA in wastewater samples (Corman et al., 2020; Shirato et al., 2020). The sequences for primers and probes are shown in Table 1 along with qPCR cycling parameters. For both RT-qPCR assays, gBlocks gene fragments were purchased from Integrated DNA Technologies (Coralville, IA, USA) and used as standards or positive controls. All RT-qPCR amplifications were performed in 40 μL reaction mixtures using iTaq™ Universal Probes One-Step Reaction Mix (Bio-Rad Laboratories, Richmond, CA). N_Sarbeco RT-qPCR mixtures contained 20 μL of Supermix, 600 nM of forward primer, 800 nM of reverse primer, 200 nM of probe, 0.50 μL of iScript advanced reverse transcriptase and 6 μL of template RNA. NIID_2019-nCOV_N RT-qPCR mixtures contained 20 μL of Supermix, 500 nM of forward primer, 700 nM of reverse primer R2, 700 nM of reverse primer R2Ver3, 200 nM of probe and 6 μL of template RNA. The RT-qPCR assays were performed using a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories). All RT-qPCR reactions were performed in triplicate. For each qPCR run, a series of three positive and negative controls were included.

2.4. RT-qPCR inhibition and quality control

An experiment was conducted to determine the presence of RT-qPCR inhibition in RNA extracted from wastewater samples using a



Catchment	PHN	Population (2016)	Public Hospitals	WWTP Population within PHN#	Percent PHN Coverage	Percent Hospital Coverage
Catchment A	Brisbane North*	42,612	0		4%	
	Brisbane South*	189,031	1		17%	
	Total	***231,643***	***1***			
Catchment B	Brisbane North*	351,531	2		36%	
	Brisbane South*	152,998	3		14%	
	Total	***504,529***	***5***			
Brisbane North PHN	Brisbane North	964,495	5	394,143	41%	40%
Brisbane South PHN	Brisbane South	1,106,892	7	342,029	31%	57%

** Note Both WWTPs have parts of the catchment in Brisbane North and Brisbane South PHNs*
 # Total Population within both WWTPs
Brisbane North: Caboolture, Kilcoy, Redcliffe, **Royal Brisbane^** and Women's and **The Prince Charles^** Hospitals
Brisbane South: Beaudesert, Logan, **Princess Alexandra^**, ***Queen Elizabeth II Jubilee~***, Redland, **Mater^**, and **Mater Mothers'^** Hospitals
In bold above were located within the A~ or B^ catchments.

Fig. 1. Maps of the WWTP catchments and Primary Health Networks (PHNs) showing overlap in area and population.

Sketa22 real-time PCR assay (Haugland et al., 2005). A known copy number (10^4/reaction) of *Oncorhynchus keta* (*O. keta*) was added in the DNase and RNase free water and the Cq (quantification cycle) value obtained acted as a reference point. If the Cq value of a wastewater sample increases compared to the reference Cq value, the sample is considered to have PCR inhibitors. Wastewater samples with a 2-Cq delay was considered to have RT-qPCR inhibition (Staley et al., 2012). All RNA samples were stored at −80 °C and subjected to RT-qPCR analysis within the same day after RNA extraction. A reagent blank and an extraction blank were included for each batch of RNA extraction to ensure no carryover contamination occurred during RNA extraction. No carryover contamination was observed in reagent blank samples. To minimize potential RT-qPCR contamination, RNA extraction and RT-qPCR setup were performed in separate laboratories.

2.5. Sequencing and bioinformatics

TaqMan RT-qPCR products (wastewater sample collected on 26/03/2020) were sequenced using both Sanger (Applied Biosystems, Foster City, CA, USA) and Illumina (MiSeq, Illumina, San Diego, CA, USA) sequencing platforms. For Sanger sequencing, 10 μL TaqMan qPCR products were run on a 2% agarose gel. Target bands of expected size were excised from the gel, then cleaned and concentrated with a Zymo Gel Recovery Kit (Zymo, Irvine, CA, USA). Final elution volume was 8 μL and 2 μL of the post-gel product was run on a Tapestation (Agilent, Santa Clara, CA, USA) to size and determine concentration of the product. 3 ng of the product was mixed with 10 pmol each of the N_Sarbeco forward or reverse primerl. These were then submitted to a service provider for Sanger sequencing (AGRF, Brisbane, Australia).

For MiSeq sequencing, the TaqMan PCR product was cleaned with 1× ratio of AMpure XP (BeckmanCoulter, Brea, CA, USA) and eluted in 15 μL. Illumina adapters were ligated on using the NEB UltraII Total RNA kit (New England Biolabs, Ipswich, MA, USA) with the modification of beginning the library prep process at the End Repair step. Libraries were amplified with 10 cycles of PCR. Samples were pooled in equimolar amounts for sequencing and sequenced as a 150 bp Paired end run using a 300 cycle v3 MiSeq kit (Illumina, USA).

Primer sequences were removed from de-multiplexed reads using cutadapt (ver. 2.9), with reads not containing primers discarded (–discard-untrimmed). Poor quality reads were identified and removed with trimmomatic (ver. 0.39) using a sliding window of 4 bases with an average quality of 15 (SLIDINGWINDOW:4:15). Reads were cropped to 120 bp (CROP:120), with any <120 bp in length discarded (MINLEN:120). Overlapping forward and reverse reads were merged using bbmerge from the BBMap suite (ver. 38.41, https://sourceforge.net/projects/bbmap/). Quality-controlled, merged reads were then mapped to the MT276598.1 reference genome using CoverM 'make' (ver 0.4.0, B. Woodcroft, unpublished, https://github.com/wwood/CoverM). Low quality read mappings were removed with CoverM 'filter' (minimum identity 90% and minimum aligned length of 75%). Read depth profiles for each sample were calculated using samtools (ver. 1.9).

2.6. Infection prevalence estimation

The prevalence of SARS-CoV-2 infection within the catchment was estimated using a mass balance on the total number of viral RNA copies in wastewater each day, as measured in wastewater by RT-qPCR, and the number of SARS-CoV-2 RNA copies shed in stool by an infected

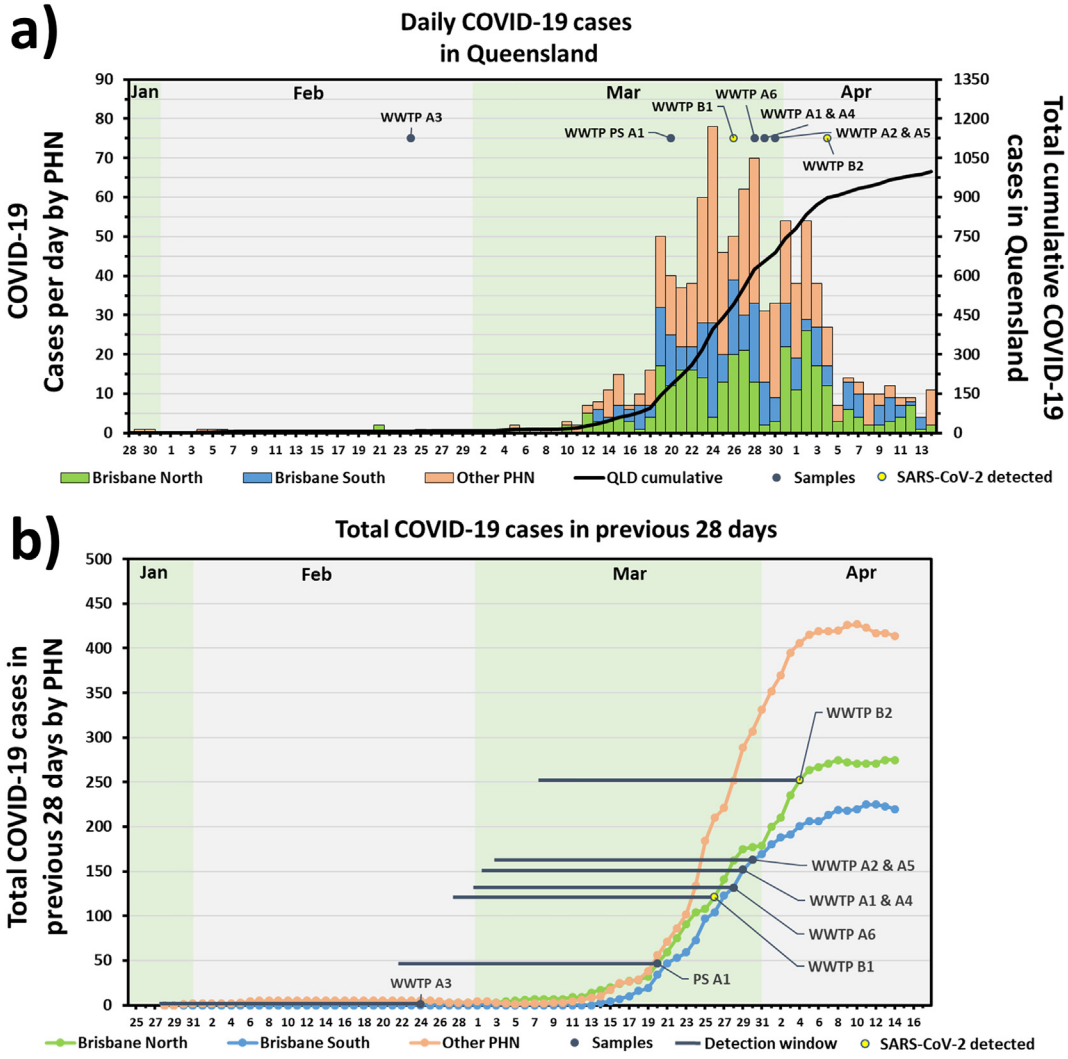


Fig. 2. a) and b) showing sampling dates, the number of cases and the potential detection windows (28 days) of SARS-CoV-2 for wastewater samples in the two PHNs (Brisbane North and South).

individual each day (Eq. (1)).

$$\text{Persons Infected} = \frac{\left(\frac{RNA\ copies}{liter\ wastewater}\right) * \left(\frac{liters\ wastewater}{day}\right)}{\left(\frac{g\ feces}{person-day}\right) * \left(\frac{RNA\ copies}{g\ feces}\right)} \quad (1)$$

Uncertainty and variability in the independent variables were incorporated using a Monte Carlo approach executed in Oracle Crystal Ball (Release 11.1.2.4.600, Redwood City, CA). SARS-CoV-2 RNA copies/L of wastewater were modelled as point estimates for each date of detection and as a uniform distribution between the minimum and maximum counts observed. The daily flow rate of wastewater was calculated as a point estimate using the product of the at-home population in the

Table 1
Primers and probes used in this study.

Organisms	Target gene	Assay name	Sequence (5′–3′)	Cycling parameters	Reference
Oncorhynchus keta	–	Sketa22	F-GGTTTCCGCAGCTGGG R-CCGAGCCGTCCTGGTCTA P-FAM-AGTCGCAGGCGGCCAC CGT-TAMRA	95 °C for 10 min; 40 cycles of 95 °C for 15 s, 63 °C for 45 s.	Haugland et al., 2005
SARS-CoV-2	N protein	N_Sarbeco	F-CACATTGGCACCCGCAATC R-GAGGAACGAGAAGAGGCTTG P-FAM-ACTTCCTCAAGGAACAACA TTGCCA-BHQ1	50 °C for 10 min for RT; 95 °C for 3 min and 45 cycles of 95 °C for 15 s, 58 °C for 30 s.	Corman et al., 2020
		NIID_2019-nCOV_N	F-AAATTTTGGGGACCAGGAAC R2-TGGCAGCTGTGTAGGTCAAC R2Ver3-TGGCACCTGTGTAGGTCAAC P-FAM-ATGTCGCGCATTGGCA TGGA-BHQ1	50 °C for 10 min for RT; 95 °C for 15 min; and 45 cycles of 95 °C for 15 s and 60 °C for 1 min	Shirato et al., 2020

catchment of approximately 600,000 persons (capita), and the observed average per capita wastewater rate of 250 L/person/day (Tscharke et al., 2019). The daily stool mass in $\log_{10} g$ per person was modelled as a normal distribution with a mean of 2.11 and standard deviation of 0.25 per data from high-income countries reported in Rose et al. (2015). Lastly, the shedding rate of SARS-CoV-2 RNA copies/g of feces was modelled as a log-uniform distribution from 2.56 to 7.67 as observed during the periods of heaviest shedding among mild cases of COVID-19 in Germany (Wölfel et al., 2020).

Summary statistics concerning the number of SARS-CoV-2 infections were generated by propagating a vector of each independent variable, drawn per the probability distribution describing it, through the model 10,000 times. For each estimate of infected persons, the corresponding prevalence was calculated by dividing the number of persons infected by the number of persons in the catchment. Sensitivity of the estimated number of cases to each model input was estimated by calculating the Spearman's correlation coefficient between each input and the estimated number of cases. For the purposes of the sensitivity analysis, the per capita daily wastewater flow was modelled as a triangle distribution with a minimum of 200 L, likeliest value of 250 L, and maximum of 300 L. Importantly, the model was conceptualized and executed blinded to any details regarding the geographic location of the catchment or clinical prevalence data.

3. Results

3.1. RT-qPCR inhibition and performance characteristics of RT-qPCR assays

None of the wastewater RNA samples had RT-qPCR inhibition, as confirmed by the Sketa22 RT-qPCR assay. The Cq values obtained for wastewater RNA samples were within 1 Cq of the reference Cq value. The slope of the standards for N_Sarbeco and NIID_2019-nCOV_N assays were −2.99 and −3.10, respectively. Y-intercept values were −36.85 (for N_Sarbeco) and −35.71 (for NIID_2019-nCOV_N). The amplification efficiencies for these two assays were 116 (for N_Sarbeco) and 108% (for NIID_2019-nCOV_N). The correlation coefficient (R^2) values for N_Sarbeco and NIID_2019-nCOV_N were 0.995 and 0.998%, respectively.

3.2. Detection of SARS-CoV-2 RNA in wastewater samples

Among the nine wastewater samples tested, two (22.2%) samples collected from WWTP B on two separate sampling events (27/03/20 and 01/04/20) were positive for SARS-CoV-2 using the N_Sarbeco assay. Wastewater samples from the PS and WWTP A were negative for SARS-CoV-2. The total number of reported cases of COVID-19 in the two PHNs that cover the greater Brisbane area were 297 and 404 on 27/03/20 and 01/04/20, respectively (Fig. 1). RT-qPCR assays produced inconsistent results. The N_Sarbeco assay returned both positive results, while the NIID_2019-nCOV_N assay failed to detect SARS-CoV-2 in any of the wastewater samples (Table 2). The concentration methods used also produced inconsistent results. Method A yielded positive signal for sample WWTP B1, yet the same sample was negative for SARS-CoV-2 when Method B was used. In contrast, concentration Method B generated a positive signal for sample WWTP B2; however, the same sample was negative for SARS-CoV-2 when Method A was used. Both RT-qPCR positive samples were below the RT-qPCR quantification level.

The Cq values of the WWTP B1 and B2 samples were 37.5 and 39, corresponding to 12 and 1.9 copies/100 mL of untreated wastewater, respectively. The aligned forward and reverse Sanger sequences confirmed a 100% identity match to the SARS-CoV-2 isolate and aligned to the N-protein (28,200–29,500) (Wu et al., 2020b). This result was further confirmed with the Illumina MiSeq sequencing. Specifically, the quality-controlled reads mapped between positions ~28,700 to 28,800 of the SARS-CoV-2/ISR_IT0320/human/2020/ISR genome.

Table 3
Number of SARS-CoV-2 infected persons and prevalence in the treatment catchment basin as estimated by viral RNA copies detection in wastewater and Monte Carlo simulation.

RNA copies/100 mL	Number of infections median (95% CI)	Prevalence of infection (%) median (95% CI)
12 copies/100 mL (27/3/2020)	1090 (748–1460)	0.181 (0.124–0.249)
1.9 copies/100 mL (1/4/2020)	171 (122−233)	0.028 (0.019–0.039)
Uniform distribution: 1.9 to 12 copies/100 mL	563 (391–764)	0.096 (0.064–0.142)

3.3. Prevalence estimate

Since the probability distributions for two input variables are right skewed in arithemetic space, the model summary statistic is reported as the median and 95% confidence interval (CI) determined by bootstrapping the model with 200 experiments of 1,000 draws each. The median is less sensitive to extreme values drawn from the input distributions, and given the large uncertainties and variability associated with relevant SARS-CoV-2 data across geographies the median represents a conservative estimator. As summarized in Table 3 below, the Monte Carlo simulation estimates a median number of infections ranging from 1,090 on 27/3/2020 to 171 on 1/4/2020 in the catchment basin. Using the observed counts in wastewater as maximum and minimum bounds (uniform distribution) results in a median prevalence of

Table 2
Detection of SARS-CoV-2 in wastewater samples at three WWTPs in Southeast Queensland, Australia.

Sources of wastewater and sample ID	Types of samples	Sampling date	Sample volume processed	Virus concentration methods and RT-qPCR assays			
				Method A		Method B	
				N_Sarbeco	NIID_2019-nCOV	N_Sarbeco	NIID_2019-nCOV
PS A1	Composite grab sample	20/03/2020	200 mL	ND	ND	ND	ND
WWTP A1	Composite autosampler[a]	29/03/2020	200 mL	ND	ND	ND	ND
WWTP A2	Composite autosampler[b]	30/03/2020	200 mL	ND	ND	ND	ND
WWTP A3	Composite autosampler[b]	24/02/2020	100 mL	ND	ND	ND	ND
WWTP A4	Composite autosampler[a]	29/03/2020	100 mL	ND	ND	ND	ND
WWTP A5	Composite autosampler[a]	30/03/2020	100 mL	ND	ND	ND	ND
WWTP A6	Composite autosampler[a]	28/03/2020	100 mL	ND	ND	ND	ND
WWTP B1	Composite grab sample	27/03/2020	100 mL	+ (~12.0)[c]	ND	ND	ND
WWTP B2	Composite grab sample	01/04/2020	100 mL	ND	ND	+ (~1.90)[c]	ND

PS: Pumping station; ND: Not detected
[a] A conventional refrigerated autosampler.
[b] A submersible in-situ high frequency autosampler.
[c] Copies/100 mL of untreated wastewater.

0.096% (95% CI: 0.064–0.142) in the catchment in the six day period covered by the surveillance. The sensitivity analysis indicates the estimated number of infections and prevalence are strongly correlated with the $\log_{10}$ SARS-CoV-2 RNA copies in stool (−0.977), followed by the RNA copies detected in wastewater (0.145), and $\log_{10}$/g of feces/person/day (−0.142). The model was least sensitive to the daily per capita flow rate of wastewater (0.042).

4. Discussion

In this proof of concept study, we investigated whether the presence of SARS-CoV-2 in untreated wastewater can be used as an early warning for COVID-19 infections in communities. For the detection of SARS-CoV-2, the N_Sarbeco and NIID_2019-nCOV_N assays were used based on results published in a recent study that reported the improved performance of these two assays against the synthesized control RNA template in the QuantiTect assay (Shirato et al., 2020). Subsequently, these two assays were used as diagnostic test systems in Japan. As far as we are aware, 16 RT-PCR/qPCR assays have been developed for the detection of SARS-CoV-2 in clinical samples. Some of these assays, especially CDC N1, N2 and N3, and E_Sarbeco have been used to detect SARS-CoV-2 in wastewater samples from the Netherlands (Medema et al., 2020) and USA (Wu et al., 2020a). To the best of our knowledge, this is the first study that reports the detection of SARS-CoV-2 in wastewater samples using the N_Sarbeco assay.

In the present study, the N_Sarbeco assay produced positive signals for two wastewater samples, while the same samples were negative when tested using NIID_2019-nCOV_N. It is possible that the N_Sarbeco assay is more sensitive than the NIID_2019-nCOV_N assay. The LOD of the N_Sarbeco assay was 8.3 copies/reaction (Corman et al., 2020). As far as we know, the LOD of the NIID_2019-nCOV_N assay is not known. Medema et al. (2020) also noted discrepancies between CDC N1 with CDC N2, CDC N3 and E_Sarbeco assays for several wastewater samples (Medema et al., 2020). Wu et al. (2020a) reported the concentration of SARS-CoV-2 RNA in wastewater samples in Massachusetts, USA using CDC N1, N2 and N3 assays. All three assays produced RT-qPCR quantifiable results with variable levels of SARS-CoV-2 RNA in wastewater samples (Wu et al., 2020a).

Another recent study determined the relative performance of the E_Sarbeco, CDC N1, N2, and N3 assays by testing ten nasopharyngeal or oropharyngeal SARS-CoV-2 positive samples. Among the five assays tested, E_Sarbeco and CDC N2 assays were the most sensitive (Nalla et al., 2020). Vogels et al. (2020) showed that most RT-qPCR assays can be used to detect SARS-CoV-2 by seeding SARS-CoV-2 RNA into RNA extracted from nasopharyngeal swabs, but there are apparent differences in the ability to differentiate between true negatives and positives when concentrations are at or below ten copies/μL of RNA for China CDC N, China CDC ORF1, USA CDC N2, and USA CDC N3 assays. In this study, the level of SARS-CoV-2 in two RT-qPCR positive samples were near the lower limit of detection (i.e, amplified between 37 and 40 cycles). This may have contributed to the inconsistent results between N_Sarbeco and NIID_2019-nCOV_N assays. When the concentration of the target gene is low, subsampling error may also occur (Taylor et al., 2019). Therefore, the performance of the currently used assays needs to be cross validated by seeding SARS-CoV-2 RNA into untreated wastewater samples followed by further inter-laboratory validation. However, we could confirm the specificity of the RT-qPCR by Sanger and MiSeq Illumina sequencing. Since wastewater is a complex matrix and some assays may produce false-positive results, we recommend sequencing RT-qPCR products for confirmation.

We used two virus concentration methods because limited information is available on the effectiveness of enveloped virus recovery from wastewater matrices using the existing virus concentration methods. The electronegative membrane used in this study is typically used for concentrating enteric viruses from wastewater and environmental waters with modest recovery (Rigotto et al., 2009; Ahmed et al., 2020). The rationale for using the electronegative membrane is that greater adsorption of enveloped viruses such as mouse hepatitis virus and *Pseudomonas* phage Φ6 to the solid fraction of wastewater compared to nonenveloped viruses (Ye et al., 2016). Furthermore, koi herpesvirus (i.e., an enveloped virus) showed high adsorption efficiency to the electronegative membrane (Haramoto et al., 2009). Besides the electronegative membrane, the Centricon® Plus-70 centrifugal filter has been successfully used to recover SARS-CoV-2 in wastewater samples in the Netherlands (Medema et al., 2020). A recent study successfully recovered SARS-CoV-2 from wastewater using a polyethylene glycol (PEG 8000) concentration method (Wu et al., 2020a). None of the reported studies to date have provided information on the percent recovery of SARS-CoV-2 from wastewater due to the risk associated with handling SARS-CoV-2 and the requirements for a BSL-3 facility. To the best of our knowledge, only one study has to date reported the percent recovery of SARS-CoV from wastewater which was estimated to be only 1% using an electropositive membrane (Wang et al., 2005). Since the characteristics of SARS-CoV-2 are different to enteric viruses, more research is needed for the effective recovery of SARS-CoV-2 from wastewater.

Little has been documented on the concentration and detection of SARS-CoV-2 in wastewater. Medema et al. (2020) reported binary RT-qPCR data, while another recent study reported ~250 copies/mL SARS-CoV-2 in wastewater in Massachusetts, USA (Wu et al., 2020a). The authors acknowledged that the estimated concentration was much greater (5% of all fecal samples in the catchment) than the confirmed cases (0.026%). The authors listed several factors and assumptions for this discrepancy and considered their results conservative. The estimated numbers of SARS-CoV-2 in our study were 3–4 orders of magnitude lower than Wu et al. (2020a). In our study, despite only two wastewater samples being RT-qPCR positive, we attempted to provide some quantitative estimation of SARS-CoV-2 in wastewater samples and relate these to the COVID-19 cases in the community.

The wastewater surveillance and Monte Carlo data suggest a median SARS-CoV-2 infection prevalence of 0.096% in the catchment basin during the six day period. The clinical prevalence would be equivalent to 450 cases in the catchment, but the upper bound of the 95% confidence interval around the median would suggest up to 764 total cases – 314 undiagnosed cases or roughly 7 undiagnosed infections for every 10 diagnosed infections. Unfortunately clinical prevalence data are only available for Queensland at the PHN level, and, therefore extrapolating these to the catchment population at this stage is not possible (Fig. 1) making direct comparisons difficult. Close collaboration with Health Departments will be essential in making these comparisons. Given the current variability and uncertainty in SARS-CoV-2 data, the median is a conservative measure of central tendency that demonstrates reasonable agreement with clinical observations. The model, as currently formulated, is parsimonious with opportunites for refinement as more data become available.

Sensitivity analysis indicates localized measures of viral RNA shedding in stool of infected individuals are an important consideration. However, the model does not yet include the proportion of infected patients shedding viral RNA in their stool, since this appears subject to substantial geographic variation – 27% in one cohort in China to 88% in a German cohort (Zhang et al., 2020a; Wölfel et al., 2020). The effect of this exclusion would be to increase our estimated prevalence by as much as 4-fold. The model also does not yet include any uncertainty or variation associated with the experimental workflow itself such as recovery of SARS-CoV-2 from wastewater. For example, if the observed mean recovery of virus concentration method was 10%, then the estimated number of infection would increase 10-fold. Given the uncertainty in the input parameters, additional research will be required.

In regards to the safety aspect of sampling wastewater, we consider that routine safe work practices and PPE have been and continue to be effective in protecting sampling personnel from exposure to pathogens including SARS-CoV-2 (CDC, 2020; USEPA, 2020). There is no evidence to date that SARS-CoV-2 or the related SARS-CoV has been transmitted

via wastewater (CDC, 2020; WHO, 2020a, 2020b). Furthermore, initial experiments indicated that fecal loads of SARS-CoV-2 are not infectious (Hennig and Drosten, 2020). We therefore recommend that future wastewater sampling efforts adhere to established safety procedures. Also, ethical guidelines should be consulted and approvals may be needed when developing monitoring programs involving community surveillance through WBE.

This is a proof of concept study and we have shown that SARS-CoV-2 RNA can be detected in untreated wastewater in Australia. One of the biggest challenges will be to establish quantitative predictions from the viral RNA concentrations measured in the sewage to the actual numbers of cases in the community. For application of WBE of SARS-CoV-2 infection, further systematic research is needed covering aspects from effective sampling and preservation through to data interpretation. Since application may be particularly useful in remote communities and confined populations, the availability of effective sampling techniques is of obvious importance. Furthermore, we need to identify or develop effective enveloped virus concentration methods and local data on the prevalence and concentration of SARS-CoV-2 in patients. We need robust RT-qPCR assay(s) that can measure SARS-CoV-2 in complex matrices such as wastewater with high sensitivity. It has been reported that digital RT-PCR (dRT-PCR) is more sensitive and suitable for the detection of SARS-CoV-2 in clinical specimens with low viral loads (Dong et al., 2020). Understanding the decay of SARS-CoV-2 in wastewater is also crucial in terms of their detection with RT-qPCR assays. Information on the composition of wastewater and environmental factors such as stormflow and its impact on wastewater may also be useful. These requirements are expected to be achievable and should provide accurate information on the disease burden in the community.

5. Conclusions

- This is the first study that reports the detection of SARS-CoV-2 in wastewater in Australia using RT-qPCR assay, confirmed by sequencing.
- The Monte Carlo simulation estimated a median number of infections ranging from 171 to 1,090 in the catchment basin (population 600,000). Although method refinement will be required, our initial data indicate that wastewater monitoring has great potential to provide early warning signs on how broadly SARS-CoV-2 is circulating in the community, especially in those individuals showing mild symptoms or no symptoms at all.
- Currently RT-qPCR assays developed for clinical specimen testing are being used for SARS-CoV-2 RNA detection in wastewater samples. Since different assays may produce conflicting results when the concentration is low in wastewater, these assays need to be evaluated head to head in intra- and inter-laboratory studies.
- The virus concentration method is another essential factor that requires attention for improving the sensitivity of detection of SARS-CoV-2 in wastewater.

CRediT authorship contribution statement

Warish Ahmed: Investigation, Resources, Writing - original draft. **Nicola Angel:** Resources. **Janette Edson:** Formal analysis, Writing - original draft, Investigation. **Kyle Bibby:** Formal analysis. **Aaron Bivins:** Formal analysis, Writing - original draft. **Jake W. O'Brien:** Resources. **Phil M. Choi:** Resources, Writing - original draft. **Masaaki Kitajima:** Formal analysis, Writing - original draft. **Stuart L. Simpson:** Resources, Writing - original draft. **Jiaying Li:** Resources. **Ben Tscharke:** Resources. **Rory Verhagen:** Resources. **Wendy J.M. Smith:** Resources. **Julian Zaugg:** Formal analysis, Writing - original draft. **Leanne Dierens:** Resources. **Philip Hugenholtz:** Resources, Writing - original draft, Writing - review & editing. **Kevin V. Thomas:** Conceptualization, Resources, Writing - original draft, Writing - review & editing. **Jochen F. Mueller:** Supervision, Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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References

Ahmed, W., Harwood, V.J., Gyawali, P., Sidhu, J.P.S., Toze, S., 2015. Comparison of concentration methods for quantitative detection of sewage-associated viral markers in environmental waters. Appl. Environ. Microbiol. 81 (6), 2042–2049.

Ahmed, W., Payyappat, S., Cassidy, M., Harrison, N., Besley, C., 2020. Sewage-associated marker genes illustrate the impact of wet weather overflows and dry weather leakage in urban estuarine waters of Sydney, Australia. Sci. Tot. Environ. 705, 135390.

Asghar, H., Diop, O.M., Weldegebriel, G., Malik, F., Shetty, S., Bassioni, L. El, Akande, A.O., Maamoun, E. Al, Zaidi, S., Adeniji, A.J., Burns, C.C., Deshpande, J., Oberste, M.S., Lowther, S.A., 2014. Environmental surveillance for polioviruses in the global polio eradication initiative. J. Infect. Dis. 210, S294–S303.

Australian Government Department of Health, 2020. Coronavirus (COVID-19) Current Situation and Case Numbers [WWW Document].

CDC. (2020, 10/03/2020). "Water Transmission and COVID-19." Retrieved 27/03/2020, 2020, from https://www.cdc.gov/coronavirus/2019-ncov/php/water.html.

Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K.W., Bleicker, T., Brunink, S., Scheider, J., Schmidt, M.L., Mulders, D.G.J.C., Haagmans, B.L., van der Veer, B., van den Brink, S., Wijsman, L., Goderski, G., Romette, J.-L., Ellis, J., Zambon, M., Peiris, M., Goossens, H., Reusken, C., Koopman, M.P.G., Drosten, C., 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 25 (3), 2000045.

Dong, L., Zhou, J., Niu, C., Wang, Q., Pan, Y., Sheng, S., Wang, X., Zhang, Y., Yang, J., Liu, M., Zhao, Y., Zhang, X., Zhu, T., Peng, T., Xie, J., Gao, Y., Wang, D., Zhao, Y., Dai, X., Fang, X., 2020. Highly accurate and sensitive diagnostic detection of SARS-CoV-2 by digital PCR. medRxiv, 20036129.

Gao, Q.Y., Chen, Y.X., Fang, J.Y., 2020. 2019 novel coronavirus infection and gastrointestinal tract. J. Dig. Dis. 1–2.

Haramoto, E., Kitajima, M., Katayama, H., Ito, T., Ohgaki, S., 2009. Development of virus concentration method for detection of koi herpesvirus in water. J Fish Disease 32 (3), 297–300.

Haugland, R.A., Siefring, S.C., Wymer, L.J., Brenner, K.P., Dufour, A.P, 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res. 39 (4), 559–568.

Hellmér, M., Paxéus, N., Magnius, L., Enache, L., Arnholm, B., Johansson, A., Bergström, T., Norder, H., 2014. Detection of pathogenic viruses in sewage provided early warnings of hepatitis A virus and norovirus outbreaks. Appl. Environ. Microbiol. 80, 6771–6781.

Hennig, K., Drosten, C., 2020. Coronavirus Update. Folge 21. K. Hennig.

Holshue, M., DeBolt, C., Lindquist, S., Lofy, K., Wiesman, J., Bruce, H., Spitters, C., Ericson, K., Wilkerson, S., Tural, A., Diaz, G., Cohn, A., Fox, L., Patel, A., Gerber, S., Kim, L., Tong, S., Lu, X., Lindstrom, S., Pallansch, M., Weldon, W., Biggs, H., Uyeki, T., Pillai, S., 2020. First case of 2019 novel coronavirus in the United States. N. Engl. J. Med. 382, 929–936.

Ikner, L.A., Soto-Beltran, M., Bright, K.R., 2011. New method using a positively charged microporous filter and ultrafiltration for concentration of viruses from tap water. Appl. Environ. Microbiol. 77 (10), 3500–3506.

Jiehao, C., Jing, X., Daojiong, L., Lei, X., Zhenghai, Q., Yuehua, Z., Hua, Z., Xiangshi, W., Yanling, G., Aimei, X., He, T., Hailing, C., Chuning, W., Jingjing, L., Jianshe, W., Mei, Z., Children, N., Women, H., Central, S., Zeng, M., 2020. A case series of children with 2019 novel coronavirus infection: clinical and epidemiological features. Clin. Infect. Dis. pii: ciaa198.

Lodder, W., de Roda Husman, A., 2020. SARS-CoV-2 in wastewater: potential health risk, but also data source. Lancet Gastroenterol. Hepatol. 1253, 30087.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., 2020. Presence of SARS-Coronavirus-2 in Sewage. medRxiv https://doi.org/10.1101/2020.03.29.20045880.

Nalla, A.K., Casto, A.M., Huang, M.W., Perchetti, G.A., Sampoleo, R., Shrestha, L., Wei, Y., Zhu, H., Jerome, K.R., Greninger, A.L., 2020. Comparative performance of SARS-CoV-

2 detection assays using seven different primer/probes sets and one assay kit. J. Clin. Microbiol. (pii: JCM.00557-20).

Rigotto, C., Kolesnikovas, C.K., Moresco, V., Simoes, C.M., Barardi, C.R., 2009. Evaluation of HA negatively charged membranes in the recovery of human adenoviruses and hepaptitis A virus in different water matrices. Mem. Inst. Oswaldo Cruz 104 (7), 970–974.

Rose, C., Parker, A., Jefferson, B., Cartmell, E., 2015. The characterization of feces and urine: a review of the literature to inform advanced treatment technology. Crit. Rev. Environ. Sci. Technol. 45 (17), 1827–1879.

Shirato, K., Nao, N., Katano, H., Takayama, I., Saito, S., Kato, F., Katoh, H., Nakatsu, Y., Mori, Y., Kageyama, T., Matsuyama, S., Takeda, M., 2020. Development of genetic diagnostic methods for novel coronavirus 2019 (nCoV-2019) in Japan. Jpn. J. Infect. Dis. https://doi.org/10.7883/yoken.JJID.2020.061.

Sinclair, R.G., Choi, C.Y., Riley, M.R., Gerba, C.P., 2008. Pathogen surveillance through monitoring of sewer systems. Adv. Appl. Microbiol. 65, 249–269.

Staley, C., Gordon, K.V., Schoen, M.E., Harwood, V.J, 2012. Performance of two quantitative PCR methods for microbial source tracking of human sewage and implications for microbial risk assessment in recreational waters. Appl. Environ. Microbiol. 78 (20), 7317–7326.

Tang, A., Tong, Z., Wang, H., Dai, Y., Li, K., Liu, J., Wu, W., Yuan, C., Yu, M., Li, P., Yan, J., 2020. Detection of novel coronavirus by RT-PCR in stool specimen from asymptomatic child. China. Emerg. Infect. Dis. J. 26.

Taylor, S.C., Nadeau, K., Abbasi, M., Lachance, C., Nguyen, M., Fenrich, J., 2019. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. Trends Biotechnol. 37 (7), 761–774.

Tscharke, B.J., O'Brien, J.W., Ort, C., Grant, S., Gerber, C., Bade, R., Thai, P.K., Thomas, K.V., Mueller, J.F., 2019. Harnessing the power of the census: characterizing wastewater treatment plant catchment populations for wastewater-based epidemiology. Environ. Sci. Technol. 53 (17), 10303–10311.

USEPA, 2020. Coronavirus and drinking water and wastewater. Coronavirus Retrieved 27/03/2020, 2020, from. https://www.epa.gov/coronavirus/coronavirus-and-drinking-water-and-wastewater.

Vogels, C.B.F., Brito, A.F., Wyllie, A.L., Fauver, J.R., Ott, I.M., Kalinich, C.C., Petrone, M.E., Casanovas-Massana, A., Muenker, M.C., Moore, A.J., Klein, J., Lu, P., LuCulligan, A., Jiang, X., Kim, D.J., Kudo, E., Mao, T., Moriyama, M., Oh, J.E., Park, A., Silva, J., Song, E., Takehashi, T., Taura, M., Tokuyama, M., Venkataraman, A., Weizman, O.-.E., Wong, P., Yang, Y., Cheemarla, N.R., White, E., Lapidus, S., Earnest, R., Geng, B., Vijaykumar, P., Odio, C., Fournier, J., Bermejo, S., Farhadian, S., Cruz, C.D., Iwasaki, A., Ko, A.I., Landry, M.-L., Foxman, E.F., Grubaugh, N.D., 2020. Analytical sensitivity and efficiency comparisons of SARS-CoV-2 qRT-PCR assays. https://doi.org/10.1101/2020.03.30.20048108.

Wang, X.W., Li, J.S., Jin, M., Zhen, B., Kong, Q.X., Song, N., Xiao, W.J., Yin, J., Wii, W., Wang, G.J., Si, B.Y., Guo, B.Z., Liu, C., Ou, G.R., Wang, M.N., Fang, T.Y., Chao, F.H., Li, J.W., 2005. Study on the resistenace of severe acute respiratory syndrome-associated coronavirus. J. Virol. Methods 126 (1–2), 171–177.

WHO, 2020a. Statement on the Second Meeting of the International Health Regulations (2005) Emergency Committee Regarding the Outbreak of Novel Coronavirus (2019-nCoV) (WWW Document).

WHO, 2020b. Water, sanitation, hygiene, and waste management for the COVID-19 virus. World Health Organisation 6.

Wölfel, R., Corman, V.M., Guggemos, W., Seilmaier, M., Zange, S., Mueller, M.A., Niemeyer, D., Vollmar, P., Rothe, C., Hoelscher, M., Bleicker, T., Bruenink, S., Schneider, J., Ehmann, R., Zwirglmaier, K., Drosten, C., Wendtner, C., 2020. Virological assessment of hospitalized cases of coronavirus disease 2019. Nature https://doi.org/10.1038/s41586-020-2196-x.

Wu, F., Xiao, A., Zhang, J., Gu, X., Lee, W., Kauffman, K., Hanage, W., Matus, M., Ghaeli, N., Endo, N., Duvallet, C., Moniz, K., Erickson, T., Chai, P., Thompson, J., Alm, E., 2020a. SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. medRxiv https://doi.org/10.1101/2020.04.05.20051540.

Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y., Yuan, M.L., Zhang, Y.L., Dai, F.H., Liu, Y., Wang, Q.M., Zheng, J.J., Xu, L., Holmes, E.C., Zhang, Y.Z., 2020b. A new coronavirus associated with human respiratory disease in China. Nature 579 (7798), 265–269.

Wu, Y., Guo, C., Tang, L., Hong, Z., Zhou, J., Dong, X., Yin, H., Xiao, Q., Qu, X., Kuang, L., Fang, X., Mishra, N., Lu, J., Shah, H., Jiang, G., Huang, X., 2020c. Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. Lancet Gastroenterol. Hepatol. 5 (5), 434–435.

Xagoraraki, I., O'Brien, E., 2020. Wastewater-based epidemiology for early detection of viral outbreaks. In: O'Bannon, D. (Ed.), Women in Water Quality. Springer Nature Switzerland, pp. 75–97. https://doi.org/10.1007/978-3-030-17819-2.

Ye, Y., Ellenberg, R.M., Graham, K.E., Wigginton, K.R, 2016. Survivability, partitioning, and recovery of enveloped viruses in untreated municipal wastewater. Environ. Sci. Technol. 50 (10), 5077–5085.

Zhang, W., Du, R.H., Li, B., Zheng, X.S., Yang, X. Lou, Hu, B., Wang, Y.Y., Xiao, G.F., Yan, B., Shi, Z.L., Zhou, P., 2020a. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. Emerg. Microbes Infect. 9, 386–389.

Zhang, J., Wang, S., Xue, Y., 2020b. Fecal specimen diagnosis 2019 novel coronavirus–infected pneumonia. J. Med. Virol., 0–2 https://doi.org/10.1002/jmv.25742.