Passive sampling of viruses for wastewater-based epidemiology: a case-study of SARS-CoV-2

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Abstract

The shedding of pathogens by infected humans enables the use of sewage monitoring to conduct wastewater-based epidemiology (WBE). Although most WBE studies use data from large sewage treatment plants, timely data from smaller catchments is needed for any targeted public health action. Traditional sampling methods, like autosamplers or grab sampling, are not conducive to quick

ad hoc deployments and high-resolution monitoring at these smaller scales. This study develops and validates a cheap and easily deployable passive sampler unit, made from readily available consumables, with relevance to the SARS-CoV-2 pandemic but with broader use for WBE. We provide the first evidence that passive samplers can be used to detect SARS-CoV-2 in wastewater, demonstrating their ability at three different scales (lot, suburb and city). A side by side evaluation of passive samplers and traditionally collected wastewater samples verified that the passive samplers were at least as sensitive at detecting SARS-CoV-2 in wastewaters. On all days where the average SARS-CoV-2 concentration in the wastewater equalled or exceeded the detection limit of 1 copy per mL, at least one of the passive samplers deployed at the same site on the same day was also positive. Moreover, on five occasions where wastewater SARS-CoV-2 concentrations were less than 1 copy per mL, one or more passive samplers were positive, suggesting a higher sensitivity than traditional wastewater sampling methods. Finally, there was a statistically significant ($p<0.05$) positive relationship between the concentrations of SARS-CoV-2 in wastewater and the levels found on the passive samplers, indicating that with some further testing these devices could be used semi-quantitatively in the future. Passive samplers have the potential for wide use in WBE with attractive feasibility attributes of cost, ease of deployment at small scale locations and continuous sampling of the wastewater. Further research will focus on the optimisation of laboratory methods including elution and extraction and continued parallel deployment and evaluations in a variety of settings to inform optimal use in wastewater surveillance.

1 Introduction

Viral pathogens or their fragments can be excreted in the faeces of infected individuals for weeks and sometimes years after the onset of infection (Alexander *et al.*, 1997; Dunn *et al.*, 2015). Viruses can also be shed by humans via respiratory and other bodily secretions, be in bathing, showering and hand-washing waters or in surface cleaning matrices (e.g. of household floors and sinks) (Sinclair *et al.*, 2008). As such, sewer systems collect pathogen inputs over a wide area, facilitating wastewater-based epidemiology (Kitajima *et al.*, 2020; Moore, 1951; Sattar and Westwood, 1977; Sikorski and Levine, 2020), the process of detecting pathogens of concern in wastewater streams and the subsequent inference about the health of the contributing population (Hart and Halden, 2020; Jones *et al.*, 2020; Orive *et al.*, 2020; Randazzo *et al.*, 2020).

SARS-CoV-2, the virus responsible for the current COVID-19 pandemic, is detectable in respiratory secretions as well as the faeces of infected humans. Viral fragments have been found in the stool of both asymptomatic and symptomatic persons infected with SARS-CoV-2 for 6 weeks or more from the time of infection with high intra and inter-individual variability spanning the early infectious and later non-infectious periods (Gupta *et al.*, 2020; Wu *et al.*, 2020). These characteristics make WBE a promising additional environmental surveillance tool to complement individual clinical testing and to inform the response to the current COVID-19 pandemic.

Most studies that use WBE for pathogens undertake sampling at the intakes to sewage treatment plants (STPs), providing very useful city-, town- or suburban scale information about infected populations both retrospectively (Ahmed *et al.*, 2020a) and as an early warning tool to identify infections and take action before clinical cases manifest (Hart and Halden, 2020). However, monitoring at large STPs cannot provide timely information at the scale needed for targeted public health actions. This is especially the case for STPs in large cities, such as Melbourne, Australia, where two STPs treat wastewater from ~3.7 million people.

WBE at smaller scales, such as lot or suburb, can be achieved by monitoring within the wastewater network, including at pumping stations and from sewer manholes. This allows for a disaggregation of the catchment into specific smaller geographically defined sub-catchments, appropriately sized for targeted action and traceback. Such monitoring may also be used at smaller upstream locations including at specific facilities considered "at-risk" for rapid transmission and/or high morbidity or mortality (e.g. aged care facilities). Further opportunities exist at correctional facilities, industry sites (abattoirs, distribution centres), schools, university campuses and hotel quarantine locations (Hassard *et al.*).

Although the application of WBE at smaller scales is appealing, collection of representative samples within the sewerage network presents several challenges. The collection of spot or grab samples is an option, but the high temporal variability of wastewater flows and pollutant concentrations at these smaller scales (Langergraber *et al.*, 2008; Metcalf *et al.*, 2003) suggests that single grab sampling may reduce sensitivity and miss important information, such as shedding events (Aymerich *et al.*, 2017). Ideally, multiple grab samples could be collected from each site, but this significantly increases costs and safety risks. To account for the dynamics of wastewater at these smaller scales, monitoring stations could be established with autosamplers and flow meters programmed to take frequent flow- or time- proportional samples (Aymerich *et al.*, 2017). Apart from requiring specialised skillsets, such installations are difficult at this scale because of: (1) installation and maintenance costs, (2) equipment availability, (3) limited space and access to the sampling site, (4) safety concerns, especially as traffic management is commonly required, (5) the absence of a reliable power supply for refrigerated samplers, and (6) excessive sampling depths, which may be more than 10 m and is beyond the capacity of most autosampler pumps. As such, the wide-spread application of WBE to smaller scales requires alternative sampling approaches.

Passive sampling presents a cheap, safe and easy alternative to traditional wastewater sampling within the sewage catchment for WBE. Passive sampling involves the deployment of a device in a

98 waterbody for a known time period, allowing for pollutants in the water to interact with the device
99 (Almeida *et al.*, 2016; Birch *et al.*, 2013; O'Connor Šraj *et al.*, 2018). This interaction could include the
100 association of a pollutant with a particular medium or substance (Birch *et al.*, 2013) or induces a
101 chemical reaction within the device (Almeida *et al.*, 2016). At the end of the deployment, the passive
102 sampler is analysed through visual inspection or via advanced laboratory analytical methods. A
103 notable advantage of passive sampling in water systems is that the deployment is easy (i.e. no
104 specialised skills required), rapid and usually does not require confined space entry permits.
105 Furthermore, the continuous exposure of the passive sampler to the water column reduces the
106 sampling errors that exist when taking discrete water samples. Consequently, passive sampling has
107 had a significant uptake in freshwater resource settings, especially in the field of water chemistry,
108 where both time- and flow-based passive sampling techniques have been validated (Birch *et al.*,
109 2013; O'Connor Šraj *et al.*, 2018).

110 The application of passive sampling in water and wastewater microbiology has not received much
111 research attention, with only seven peer reviewed studies identified (Cassemiro *et al.*, 2016; Moore,
112 1951; Organization, 2003; Sattar and Westwood, 1977; Sikorski and Levine, 2020; Vincent-Hubert *et*
113 *al.*, 2017; Voisin *et al.*, 2015). Two studies have used glass bead passive samplers, one to
114 characterise colonising biofilms in groundwater (Voisin *et al.*, 2015) and the other to monitor for
115 pathogens in wastewater (Organization, 2003). Vincent-Hubert *et al.* (Vincent-Hubert *et al.*, 2017)
116 trialled several passive samplers, including Zetapor membranes, nylon materials, low-density
117 polyethylene and polyvinylidene difluoride for the detection of herpesviruses and noroviruses in
118 seawater. The other four studies monitored pathogens in wastewater systems using the Moore's
119 swab, which is a piece of medical gauze that is placed in the wastewater for 1 to 7 days and is
120 attached to a string for retrieval (Moore, 1951). Slight modifications to the Moore's swab have been
121 adopted by Cassemiro *et al.* (Cassemiro *et al.*, 2016), who utilised Sattar and Westwood's (Sattar and
122 Westwood, 1977) method to monitor for polioviruses in wastewater. Sikorski and Levine (Sikorski

and Levine, 2020) recently revived the Moore's swab to monitor *Salmonella* bacteria in surface waters and wastewaters.

Although these studies provided proof of concept that passive samplers can be used for pathogen detection in wastewater, significant research questions remain prior to their use in WBE. Firstly, none of the above studies evaluated the sensitivity of the devices for detecting pathogens in wastewater, nor how this sensitivity compares to traditional auto-sampling or grab sampling techniques. Secondly, the above papers do not explore whether the accumulation of pathogens on the passive samplers is correlated with the concentration of pathogens in the water column, which is essential information if passive samplers are to help quantify the number or the level of infections in sub-catchments. Lastly, none of the above studies tested whether passive samplers can be used to detect SARS-CoV-2 in wastewater, which is critical in the context of the current COVID-19 pandemic.

The aim of this research study was to provide proof of concept for the use of simple passive samplers for the detection of SARS-CoV-2 in wastewaters from low case number settings which would be relevant for surveillance aiming at early detection use cases. This study validated the sensitivity of the passive samplers against traditional wastewater monitoring methods and assessed the potential of passive samplers at a variety of scales, ranging from single allotments to small- and large-scale sewage treatment plants.

2 Methodology

2.1 Selection of passive materials

Three commonly available and cheap materials for passive sampling of viruses in wastewater were used: 75 mm by 75 mm medical gauze swabs (i.e. as for the original Moore's swab (Cassemiro *et al.*, 2016; Moore, 1951; Sattar and Westwood, 1977; Sikorski and Levine, 2020; Vincent-Hubert *et al.*, 2017); Handy® 8ply, BSN Medical, Germany), typical laboratory grade electronegative filter

membranes (Cellulose Nitrate Filter, 11406-47-ACN, Sartorius, Germany, as per (Ahmed *et al.*, 2020b; Vincent-Hubert *et al.*, 2017)) and cotton buds (Swisspers, China), which were especially attractive due to their small footprint and ease of use in subsequent extraction steps.

2.2 Design of passive sampler units

We initially used the traditional Moore's swab design (medical gauze attached to a string), but the fouling rates were high. As such, we opted to place the passive samplers inside housings to minimise fouling. The design of the housing for the passive samplers varied depending on the scale of the site in question and our experience as the study progressed regarding clogging/fouling/ragging rates. In total, four designs were developed and trialled (Figure 1), with each sampler having an internal metal weight to ensure it was submerged. Each passive sampler unit was fixed to a secure anchor point with a 3 mm diameter rope.

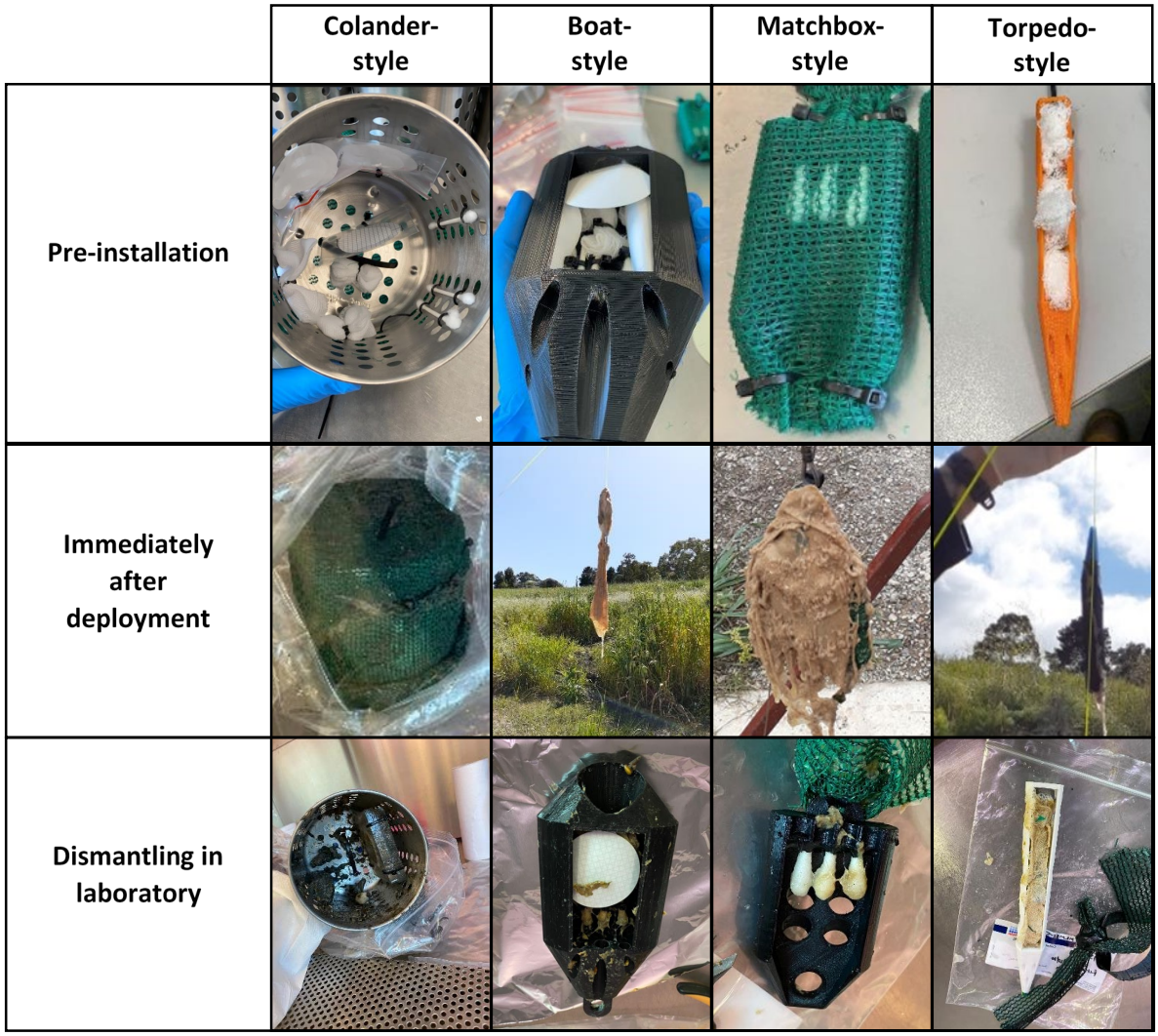

Figure 1. Four designs of passive sampler units, Colander (far left column), Boat (mid left column), Matchbox (mid right column) and Torpedo (far right column), before deployment (top row), directly after deployment (middle row) and during processing in the laboratory (bottom row).

Colander-style units. A larger passive sampling device (120 mm diameter, 135 mm high) and made from a readily available cutlery colander (ORDNING, IKEA, Sweden) was used for sampling at sewage treatment plants (Figure 1). Each colander contained triplicates of each passive sampling material, tied in place using cable ties (note that the electronegative membranes were first placed inside a hollow Perspex holder to protect them from damage and to ensure it remained in contact with the wastewater flow). The colander was wrapped in shade cloth (Rainforest 90% UV Shade Cloth, Coolaroo, Australia) to ensure mass-transfer efficiencies were maintained.

Boat-style units. Medium sized passive sampler housings (170 mm long, 80 mm wide, 37 mm high) were designed for sewer-line installations (i.e. pipes >500 mm in diameter) (Figure 1). These housings were created using a 3D printer (Creator Pro, FlashForge, China), files for which are available in the Supplementary Information section. As with the colander design, there were multiple entry points for the wastewater, including at the front, top and bottom. Each boat was wrapped in shade cloth and contained triplicates of each of the passive sampling materials.

Matchbox-style units. Small housings (70 mm long, 40 mm wide and 10 mm high) were designed for installation in sewer pipes less than or equal to 150 mm in diameter and 3D-printed (files available in in the Supplementary Information section). Each matchbox style sampler had multiple entry points for the wastewater at the front, top and on the bottom (Figure 1). They contained three cotton buds, hot-glued into location and were wrapped in shade cloth to prevent ragging (Figure 1).

Torpedo-style units. A new sampler was designed to resemble a torpedo (Figure 1), to allow for any rags caught on the anchor rope to skim off the housing. This sampler was again 3D printed and had multiple entry points for wastewater to interact with the passive samplers (front, top, sides and bottom; Figure 1). Each contained up to six passive sampling materials (sometimes daisy-chained to have three replicates of each material in two boats) and were again wrapped in shade cloth. To further reduce ragging, hot-glue and tape was used to attach the shade cloth instead of cable ties.

2.3 Study sites, passive sampler deployment and traditional wastewater sampling

Study sites. Eight study sites in Victoria, Australia (Figure 2) were used in this study to represent different scales, ranging from systems that collect the wastewaters of 260 residents and staff in an aged care facility, to Melbourne's largest sewage treatment plant that collects wastewater from over two million inhabitants (Table 1). These sites were chosen due to having known cases of COVID-19 upstream on the downward slope of the second wave of infections in Victoria (June 2020 to November 2020). This was purposive to provide field conditions which would simulate low viral shedding levels similar to those which might occur in an early detection scenario relevant for

Australia's extremely low prevalence setting with no or few cases of community transmission (noting Victoria's second wave reduced from a peak of 687 diagnoses/day on 4th of August 2020 to below five per day in early November 2020).

Seven of the eight sites were in metropolitan Melbourne while one was in Colac, a small town in regional Victoria (Figure 2). The aged care facility ("Aged care") had a known outbreak and was in lockdown, with the last case diagnosed 11 days prior to the initiation of sampling with a 21 day duration of sampling. Recent cases including other aged care outbreaks were known to be within the area of Melbourne and much of its wastewater is treated at the Western sewage Treatment Plant ("WTP"). The five trunk sewer sampling sites (sites Sewer 48K, Sewer 49K, Sewer 70K, Sewer 95K and Sewer 491K) are all on the same wastewater line that then connect to WTP (Figure 2) and therefore are expected to have recent cases in these sub-catchments within the expected shedding period. Furthermore, the Aged care facility drains into the same sewer line between Sewer 48K and Sewer 49K (Figure 2). The Colac STP (Figure 2) also had a known outbreak, with the last known case identified more than four weeks prior to initiation of sampling, with a sampling duration of 15 days.

Table 1. Site characteristics of the eight field trials conducted, noting the upstream population contributing to each sampling location, the style(s) of passive sampling units used, the deployment durations, the number of deployments trialled at each site and the type (if any) of paired traditional wastewater sampling methods.

Site Name	Sewer type, sewer diameter	Upstream population	Passive sampler		Paired traditional wastewater sampling during deployment period?
			Deployment duration	Number of deployments [*d*] (sampler unit/housing used)	
Aged care	At-site, 150 mm	260	3-7 hours	d=8 (6 Matchbox, 2 Torpedo)	Yes, flow-weighted composite from grab sampling every 10 to 30 minutes
Sewer 48K	Trunk sewer, 720 mm	48.9K	24 hours	d=5 (4 Boat, 1 Torpedo)	No
Sewer 49K	Trunk sewer, 720 mm	49.2K	24 hours	d=5 (4 Boat, 1 Torpedo)	No
Sewer 70K	Trunk sewer, 800 mm	70K	24 hours	d=5 (4 Boat, 1 Torpedo)	No
Sewer 95K	Trunk sewer, 1140 mm	95K	24 hours	d=5 (4 Boat, 1 Torpedo)	No
Sewer 491K	Trunk sewer 2950 mm	491K	24 hours	d=5 (4 Boat, 1 Torpedo)	No
Colac STP	STP*	13K	24 hours	d=5 (5 Colander)	Yes, time-averaged composite refrigerated autosampler
WTP**	STP*	2.2M	24 hours	d=7 (7 colander)	Yes, flow-weighted composite refrigerated autosampler

*STP – sewage treatment plant. **WTP - Western Treatment Plant, Melbourne's largest STP.

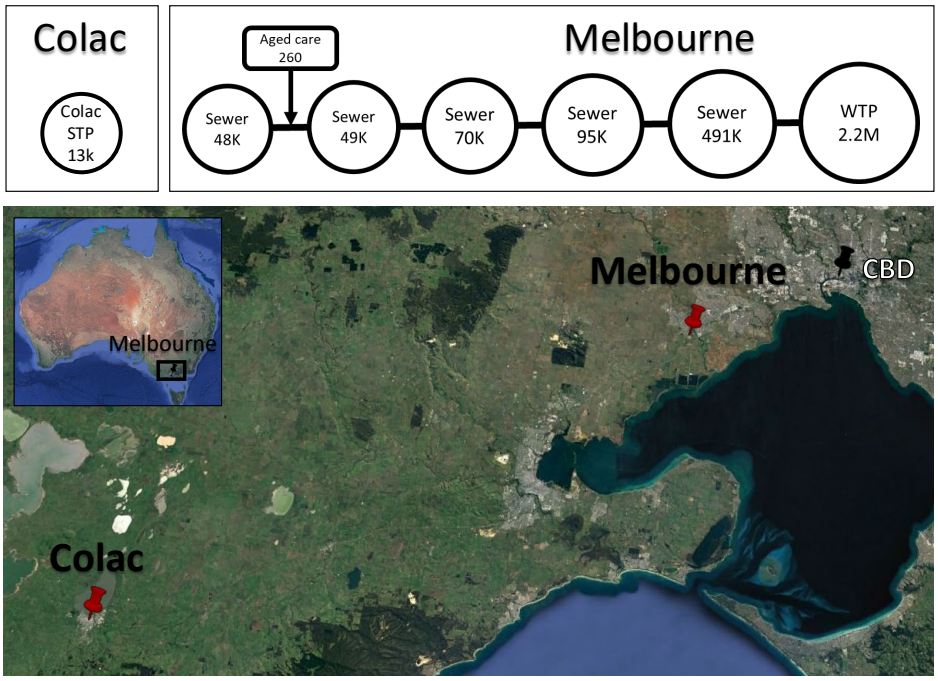

Figure 2. Location of the two study areas: (1) the aged care facility, the trunk sewer sites, and the Western Treatment Plant (red pin) located in Melbourne's metropolitan area within 40km of the Central Business District (CBD) (black pin), (2) Colac sewage treatment plant, located in regional Victoria (red pin), located 120km from Melbourne's CBD.

Passive sampler deployment and traditional wastewater sampling strategies. For the five trunk sewer sites (Sewer 48K, Sewer 49K, Sewer 70K, Sewer 95K and Sewer 491K), paired wastewater sampling using traditional approaches was not possible due to cost and logistical constraints. At these sites passive samplers were deployed and retrieved 24 hours later. In total, five 24-hour long deployments were performed, representing data acquired from both the boat-style (four deployments) and the torpedo-style units (one deployment).

Traditional grab or automatic wastewater sampling was conducted at the other three sites paired alongside the passive sampler deployments. At the STPs, refrigerated automatic samplers were available, and these were programmed to take samples across the entire passive sampler deployment period. For the WTP, 12 discrete samples were taken each day, where one bottle was filled every two hours, using four pulses of water, each 30 minutes apart. Using the measured flow at the inlet of the WTP, these samples were then combined to create a flow-weighted composite sample. At the Colac STP, a time-averaged composite sample was created *in-situ*, where an equal pulse of water was distributed into a single container every 15 minutes across the day.

The site serving the aged care facility was the smallest with a 150 mm diameter sewer. It was not possible to install permanent auto-sampling equipment at this site (manhole and pipe too small for equipment) and hence frequent grab samples were taken across the duration of the passive sampler deployment. To ensure representative wastewater samples were taken at this site, we opted for intensive and frequent sampling to occur during the period of passive sampler deployment. We also considered potential occupational health and safety risks of our samplers and limited the sampling and passive sampler deployment durations to be between three and seven hours in length (i.e. overnight sampling was not conducted). This also allowed us to maintain constant visual contact with the passive sampler during the deployments, to mitigate any possible creation of blockages or backflow issues (none were observed). For the three-hour deployment duration, we collected grab

243 samples every 10 minutes from the sewer which were then pooled using flow weightings to make
244 composite samples. For the seven-hour deployments, the first and last hours of sampling were
245 intensive (10 minutes intervals) because these were also at periods of high toilet use (i.e. after
246 breakfast, after lunch or after dinner) while the middle hours were less intense (every 30 minutes).

247 2.4 Laboratory analysis

248 In total, 38 wastewater samples collected using traditional techniques and 150 passive sampling
249 materials were pre-processed, extracted and analysed for SARS-CoV-2.

250 **Pre-processing and storage.** All samples were transported to the laboratory on ice and pre-
251 processed on the day of collection. Wastewater samples were processed similarly to others in the
252 literature (Ahmed *et al.*, 2020b), where 50 mL of wastewater was filtered through a 47 mm
253 diameter, 0.45 µm pore size, electronegative membrane (Satorius, Germany). RNA extraction from
254 these filters typically occurred directly after filtration, but some were stored at -80^{o}C until extraction
255 was possible.

256 Immediately after retrieval, passive sampling units were cleared of all obvious ragging materials.
257 Passive sampling units were dismantled on the day of retrieval, resulting in up to nine individually
258 stored passive samplers for each site, each day. Electronegative membranes and cotton buds were
259 either used immediately for RNA extraction or directly frozen at -80^{o}C until extraction was possible.
260 Gauzes were either directly frozen at -80^{o}C or immediately eluted by placing them in a sterile
261 stomacher bag with 10 mL of 1x sterile phosphate buffer solution mixed with 0.05% Tween 80
262 (Fisher, T164) and 0.001% Y-30 antifoam emulsion (Sigma catalog no. A-5758; (Hill *et al.*, 2005)).
263 After stomaching at 200 rpm for 2 minutes, the gauze was moved to one side of the bag which was
264 held on an angle. After squeezing the remaining liquid from the gauze, the elution buffer was then
265 filtered through a 47 mm diameter, 0.45 µm pore size, electronegative membrane. These were used
266 immediately for extraction.

RNA Extraction. The electronegative membranes and cotton buds were directly placed into 2 mL garnet-type bead-beating tubes and then processed using a Qiagen RNeasy PowerMicrobiome kit (Qiagen, Germany), with the following modifications: (1) use of phenol:chloroform:isoamyl, (2) beat-beating for 30sec at 4m/s (MP-Bio, USA), (3) DNase treatment was conducted for 15 minutes, and (4) final elution done using 50 µL of DEPC water (Sigma-Aldrich, Germany), passed through twice to ensure maximum yield. At least once on every day extraction was conducted, we also processed a method extraction blank. On some occasions, the Qiagen RNeasy PowerMicrobiome kits were not available so we used the Macherey-Nagel NucleoSpin RNA Stool kit (Macherey-Nagel, Germany) as per manufacturer's instructions. Our initial validation studies showed this produces a higher recovery rate (data not shown). Passive sampling materials and wastewater samples collected using traditional techniques on any given day were processed using the same kit.

Reverse Transcription and qPCR. The SARS-CoV-2 Real-time RT-PCR Assay (PerkinElmer, USA; hereafter referred to as the PE assay), which is a combined reverse transcription and TaqMan based qPCR, was used to detect both the nucleocapsid N and the ORF-1ab genes of the SARS-CoV-2 virus. After significant testing, our process included slight variations from that of the PE manufacturer's recommendation: 5µL of template was used in each reaction together with 10µL of the PE mastermix and 15µL of ultrapure DNase/RNAase free water (Invitrogen, USA). A minimum of two technical reactions for each sample were conducted, while on some occasions this was extended to between three and five technical replicates to help resolve any between-replicate variability. On nearly all occasions we also ran our replicates at 1:10 dilutions of the template to help ensure and determine that assays were not being inhibited. We always ran standard curves using five dilutions of the Twist synthetic SARS-CoV-2 RNA control 1 (GenBank ID: MT007544.1, Cat No: 102019), resulting in very high coefficients of determination, consistent intercepts (N gene: 39.65; ORF-1ab gene: 38.89) and slopes (N gene: -3.41; ORF-1ab gene: -3.32). The supplied MS2 phage internal control was added to samples prior to bead-beating, but this sometimes appeared to shear the RNA, limiting its use as a full extraction control as after shearing the RNA was not detectable. All assays

were run on a Bio-Rad Laboratories CFX-96 qPCR machine (Bio-Rad, USA). The qPCR protocol is available in the Supplementary material.

Detection limits. According to the above protocol (50 mL filter volume, 50 µL extraction volume, 5 µL template into each qPCR well) 5mL of equivalent volume of wastewater was placed into each qPCR well. A gamma irradiated preparation of a SARS-CoV-2 Australian isolate (kindly provided by the Victorian Infectious Diseases Reference Laboratory at the Doherty Institute) was used to determine that 95% of the time between 1 and 10 copies per reaction could be detected by our protocol. Using this dataset, we reached a detection limit of 5 copies per 5 mL; or rather 1 copy per mL of wastewater. Furthermore, we challenged our entire process (50 mL wastewater filtering, RNA extraction using the Qiagen PowerMicroBiome and 5 µL template into RT-qPCR using PE assays) by spiking gamma-irradiated SARS-CoV-2 into wastewater. From this we confirmed that our operational detection limit was 1 copy per mL.

2.5 Data analysis and comparisons

Each amplification curve was manually inspected by the same individual and cross-checked by another. In the absence of amplification, Ct values were recorded as >45, while reactions that had evidence of a late amplification were set to >43. As such, we recorded these values not to the limit of the cycle run (45), but instead to 43 to imply a detection, albeit low. All wells that had Ct values of <43 were recorded without adjustment.

The above recorded data was used for the calculation of both qualitative and quantitative results. We defined *a priori* four categories to assign each assay: (1) highly probable detection (where at least duplicates of N gene or ORF-1ab gene technical wells had Ct values of <43), (2) probable detection (where at least one replicate of either N gene or ORF-1ab gene had Ct values of <43), (3) possible detection (where at least one replicate of either N gene or ORF-1ab gene had Ct values of >43 and <45), and (4) no detection (where all replicates had Ct values of >45). In our analyses, samples that fell into the first two categories were deemed to have detectable SARS-CoV-2, while

318 those that fell into the second two categories were deemed to have non-detectable SARS-CoV-2. We
319 note that this is likely a conservative position as our amplicon sequencing (data not shown) suggests
320 that these late amplifications (of Ct>43 and Ct<45) were positive for SARS-CoV-2.

321 For the calculation of concentrations of SARS-CoV-2 in wastewater and passive samplers, we used
322 each recorded Ct value to estimate the number of copies per reaction using the stated intercepts
323 and slopes. For the wastewater samples, these values were then divided by the amount of
324 wastewater that was placed into each qPCR well to estimate the concentration of SARS-CoV-2 in the
325 wastewater (copies/mL). The average of these concentrations was calculated using all individual
326 estimates (from all replicates and dilutions) to finally estimate the number of copies per mL of
327 wastewater. For the passive samplers, the copies per reaction value was divided by the proportion
328 of RNA extract used in each qPCR well to obtain the number of copies of SARS-CoV-2 per passive
329 sampler (copies/sampler).

330 The average daily $\log_{10}$ concentration of SARS-CoV-2 measured in the wastewater was correlated
331 (Pearson *r*) to the average $\log_{10}$ copies of SARS-CoV-2 detected on the passive samplers deployed on
332 the same day at all sites using $\log_{10}$ transformed datasets and a Student's *t*-test was used to
333 determine the significance of this correlation ($p<0.05$)

334 3 Results and discussion

335 3.1 Fouling and clogging rates of passive sampler units

336 As expected, ragging and clogging of the passive sampler units occurred throughout the study
337 (Figure 1; middle row). The boat style unit experienced the most significant ragging and clogging,
338 likely because the rags that collected along the anchor rope slid down and were trapped on the wide
339 body of this unit (Figure 1). The matchbox style unit also experienced ragging too, again likely
340 because of the wide shape (relative to the anchor rope) and catching ability of the cable ties used to
341 fix the unit to the rope (Figure 1). The larger colander design was very rarely covered in rags (Figure

1), likely because they were always installed in the intake to sewage treatment plants where the water had often been through pumps that had macerated the wastewater's contents. Finally, the torpedo style unit experienced very little ragging, where 10% were retrieved with visible ragging materials and the front holes were blocked less than 5% of the time. While further optimisation of the design could be warranted to reduce ragging and clogging of openings, these 3D-printed devices are attractive as they are easily available, cheap and require very low expertise to print, assemble and deploy.

3.2 Detection of SARS-CoV-2 on passive samplers

SARS-CoV-2 was detected in 21 of the 38 traditionally collected wastewater samples (Table 2), which aligns well with the fact that all sites analysed for water samples were chosen because there were known outbreaks of COVID-19 upstream albeit at low levels with no recent new infections. There was a slight tendency for higher detection rates at the locations fed by larger populations. This is likely because it is easier to sample and capture this virus in larger systems, where there is less granularity of the output from the larger number of infected individuals and significant attenuation of sewage in the network. Smaller sites in close proximity to the infection source have a greater dependency on sampling the precise moment a toilet pulse occurs from a few individuals.

Of the 150 passive samplers analysed, 36% of them had detections of SARS-CoV-2 (Table 2), with a slightly higher number of electronegative membranes and gauzes (39%) having detections than cotton buds (32%). These results indicate that cotton buds, electronegative membranes and gauzes can be used as passive samplers of SARS-CoV-2 in human wastewaters and provides the first proof of concept that one or more of these passive samplers could be prime candidates for further optimisation for use in WBE of viruses more generally.

*Table 2. The number of samples processed (*n*) and the percentage of these that had detectable levels of SARS-CoV-2, ordered by sites and sample type. Empty cells indicate sample was not used at this site. Also noted is the number of days where sampling was conducted (d).*

		Aged care d=8	Sewer 48K d=5	Sewer 49K d=5	Sewer 70K d=5	Sewer 95K d=5	Sewer 491K d=5	Colac STP* d=5	WTP** d=7	Total detected / number sampled
Traditionally collected wastewater samples		50% n=24						50% n=6	75% n=8	55% n=38
Passive samplers	Cotton buds	32% n=28	20% n=5	40% n=5	40% n=5	20% n=5	40% n=5	20% n=5	43% n=7	32% n=65
	Electronegative membranes		40% n=5	60% n=5	60% n=5	20% n=5	40% n=5	33% n=6	20% n=5	39% n=36
	Gauze	33% n=3	57% n=7	38% n=8	57% n=7	29% n=7	29% n=7	33% n=6	25% n=4	39% n=49
	Total detected / number sampled	32% n=31	41% n=17	44% n=18	53% n=17	24% n=17	35% n=17	29% n=17	31% n=16	

*STP – sewage treatment plant. **WTP - Western Treatment Plant, Melbourne's largest STP.

The proportion of passive samples that were positive for SARS-CoV-2 at Sewer 48K (41 %) and Sewer 49K (44 %) were similar (Table 2) likely because they share very similar sub-populations, with just 300 extra people contributing to Sewer 49K than Sewer 48K, almost all of whom were residents and staff at our Aged Care facility. Sewer 70K contributes another 20,000 people, and the slightly higher detection rates compared to Sewer 49K could imply a higher rate of infection in that sub-catchment. Importantly, the detection rates at Sewer 95K decrease quite substantially, and this coincides with a large input to the sewer from industrial land-uses 1 km upstream of Sewer 95K. This industrial input could have two effects: (1) it could dilute the wastewater so that the concentration of SARS-CoV-2 is insufficient to allow for effective association with the passive samplers during deployment, or (2) the industrial effluent causes the association rate to decrease and/or the dissociation rate to increase due to chemical changes in the wastewater (e.g. change in ionic strength). Sewer 491K is 86 m downstream of Sewer 95K but combines wastewater effluent from another catchment of 380,000 people. The slight increase in passive sampler detection rates between Sewer 95K and Sewer 491K could imply the contributing population has a higher rate of infection, or simply that this water is less dilute.

While the above results and interpretations of the datasets seem to coincide with some catchment-level observations, it is hard to utilise such data effectively in WBE applications until these passive

sampler results are directly compared to what we are observing in the wastewater itself. As such, the remaining sections of the paper will focus on this aspect.

3.3 Do passive samplers detect SARS-CoV-2 when we detect it in the wastewater?

The concentrations of SARS-CoV-2 in the wastewater proximal to the Aged Care Facility, and those at the inlet of the WTP and Colac STPs were highly variable (Figure 3, black diamonds), ranging from below the detection (1 copy / mL; the lower limit of the secondary axes in Figure 3), to over 9,000 copies per mL detected at the Colac Sewage Treatment plant on the 8th October, 2020. It is important to note that while Table 2 shows the total number of samples taken over the entire period of time (and reports 21 positive samples), Figure 3 displays daily averages for the sites, resulting in fewer daily detections as multiple wastewater samples were processed on some days at some sites.

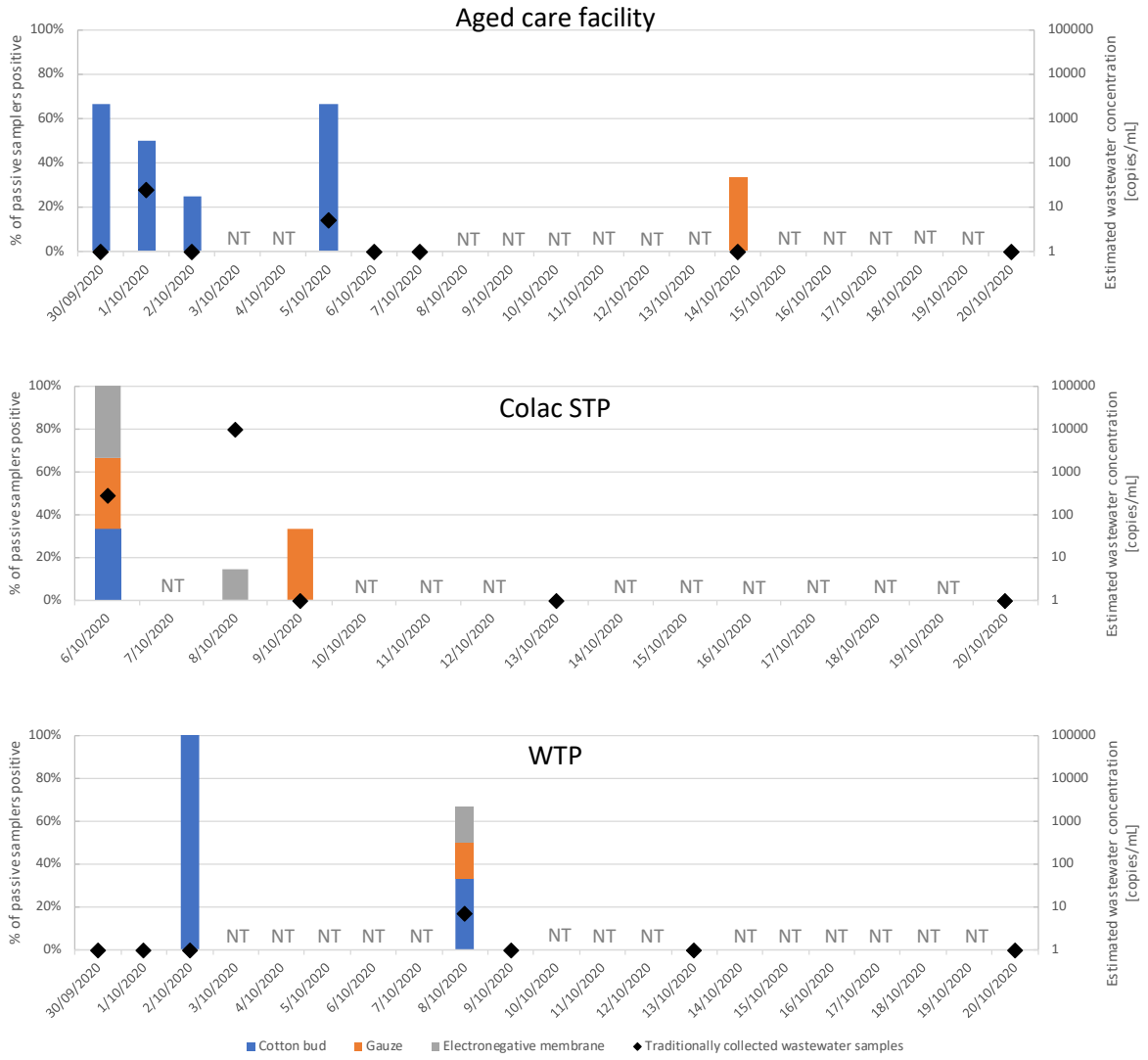

Figure 3. Detection frequency of SARS-CoV-2 in passive samplers (bar charts, left-hand axis) and estimated average concentration of SARS-CoV-2 in wastewater samples collected using traditional methods (black diamonds, right hand log-axis). Blue bars represent the contributions from cotton bud, orange bars represent the contributions from gauze and grey represent the contributions from electronegative membrane passive samplers. Passive samplers and traditional wastewater samples were always deployed/taken on the same dates and represent the same time period. Dates labelled with 'NT' indicates No Tests were conducted for either traditional wastewater samples or passive samplers. Dates where bar charts are not visible, yet black diamonds exist, indicate that, while they were analysed, no passive samplers were retrieved which yielded detectable SARS-CoV-2. The estimated limit of detection for the traditionally collected wastewater samples was 1 copy per mL, and hence black diamonds that sit here indicate the concentrations were equal to or less than this limit.

On each date where the average concentration of SARS-CoV-2 was greater than 1 copy per mL, at least one of the passive samplers deployed on that same day also had detectable levels of SARS-CoV-2 (Table 3). On the other hand, there were five days where the traditional wastewater sampling failed to detect SARS-CoV-2, while at least one passive sampler had detectable levels (Table 3). This might reflect the continuous contact that the passive samplers have with the sewage, which was not the case for the traditional wastewater sampling methods, even though they took samples very frequently (every 10, 15 to 30 minutes). This work demonstrates for the first time the potential for using passive samplers for WBE and suggests that the passive samplers are as, if not more, sensitive for detection of SARS-CoV-2 in wastewater than traditional water sampling processes.

Table 3. Frequency table reporting the number of days where SARS-CoV-2 was detected in at least one passive sampler as compared to the number of days where average wastewater concentrations were at or above 1 copy/mL.

		Passive samplers		
		Days with at least one detection	Days with no detection	Total
Wastewater samples collected using traditional methods	Days with avg. conc. ≥ 1 copy / mL	5	0	5
	Days with avg. conc. < 1 copy / mL	5	10	15
	Total	10	10	20

While these results demonstrate the sensitivity of the passive samplers to qualitatively detect SARS-CoV-2 in the wastewater when concentrations are at or above 1 copy per mL, the percentage of positive passive samplers did not always reflect the concentrations of SARS-CoV-2 in the wastewater when they were greater than 1 copy per mL. The most significant example of this was shown at the Colac STP on the 8th of October when the concentrations in the traditionally collected wastewater samples were around 10,000 copies per mL, yet only one of the nine tested passive samplers was positive (Figure 3). While it was originally proposed that the type of housing unit could help explain these observations because of differential ragging rates limiting mass transfer efficiencies, the data currently does not support that hypothesis. Indeed, a colander style unit was consistently used

throughout the study at the Colac site. Further work is required to understand whether wastewater quality variations or other environmental impacts could explain these observations.

3.4 Do the levels of SARS-CoV-2 captured on the passive samplers relate to the concentrations seen in the wastewater?

When pooling the three sites (Aged Care, Colac and Western STPs), a weak yet statistically significant correlation was observed between the average daily $\log_{10}$ concentration of SARS-CoV-2 measured in the wastewater and the average $\log_{10}$ copies of SARS-CoV-2 detected on the passive samplers deployed on the same day ($p<0.05$ $R = 0.46$). Looking further into individual sites revealed similarly moderate to strong correlations (R values ranging from between 0.55 and 0.96), but inferential statistics were not calculated as the number of samples per site was too low for separate reporting (n=5, n=7, n=8). Although these results provide necessary proof of concept that higher wastewater concentrations yield higher accumulation of SARS-CoV-2 on passive samplers, further work is required to optimise the laboratory methodologies for each passive sampler prior to any further quantitative inference.

3.5 General discussion

The presented work here demonstrates that passive samplers are an effective, easy to deploy and scalable option for monitoring SARS-CoV-2 in wastewater systems, providing evidence that passive samplers have the potential for wider adoption in WBE. In fact, the data directly demonstrate that when the daily average SARS-CoV-2 concentration in the wastewater equals or exceeds 1 copy per mL, at least one of the passive samplers deployed at the same site on the same day was also positive. Furthermore, the statistically significant correlation between the concentrations of SARS-CoV-2 in the wastewater and the concentrations found on the passive samplers further demonstrates that these samplers have the potential to provide meaningful quantitative data.

However, there are several aspects of this research which should be further strengthened as researchers begin to unlock the potential of passive samplers for SARS-CoV-2 WBE applications. For

instance, our work did not fully uncover the strengths and weaknesses of each individual passive sampling material, and instead we combined the datasets to answer our research questions. Further work should be conducted on each passive sampling material, answering questions such as: (1) what is the association rate of SARS-CoV-2 with each passive sampling material?, (2) what are their maximum association capacities?, and (3) what are the most optimal elution, extraction and assay methods for each passive sampling material?

Furthermore, this study did not calculate the concentration of SARS-CoV-2 per mass, per volume or per area of each passive sampling material and instead estimated the total amount of virus captured by each passive sampler. Together with flow and dilution rates, concentrations of SARS-CoV-2 on passive sampling materials would be required to estimate the number of infected individuals within a specific sub-catchment. We also did not determine the detection limits for each material, which is also important for moving to quantitative results. Further work should explore whether different positioning of the passive sampling materials in the housings, or the method used to deploy and fix the housings, change the SARS-CoV-2 association rates. More work should also be conducted on optimising the duration of deployment, not only with respect to what is best suited for supporting health responses, but also the ability of the unit to handle ragging, reduced mass transfer efficiencies and resilience to variations in wastewater quality. For longer deployment periods, the question of RNA degradation and disassociation will also need to be addressed.

4 Conclusions

Evidence that easily available and cheap materials (cotton bud, medical gauze and electronegative membrane) can be used as passive samplers of SARS-CoV-2 in wastewater was demonstrated in this study . Furthermore, a suitable 3D-printed housing unit was developed that protected against ragging and clogging, maintaining mass transfer efficiencies between the wastewater and the passive sampling materials. The freely available housing unit design can be made with commonly available 3D printers, is quick to assemble and easy to deploy. The passive samplers were deployed

in wastewater sewers at eight locations in Victoria, Australia, representing lot, suburb and city scales. This validation is a critical first step in the process of applying passive sampling for wastewater-based epidemiology. Furthermore, the simultaneous collection of wastewater using traditional sampling methods highlighted the sensitivity of the passive samplers and their potential to reflect SARS-CoV-2 concentrations in the wastewater. Further work is suggested, including laboratory testing of the passive sampling materials for their association rates and maximum capacities, and optimising the laboratory processing methods for each passive sampling material.

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References

[1]Ahmed, W., N. Angel, J. Edson, K. Bibby, A. Bivins, J.W. O'Brien, P.M. Choi, M. Kitajima, S.L. Simpson, J. Li, B. Tscharke, R. Verhagen, W.J.M. Smith, J. Zaugg, L. Dierens, P. Hugenholtz, K.V. Thomas and J.F. Mueller, 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, Science of The Total Environment, 2020a. **728**: p. 138764.

[2]Ahmed, W., P.M. Bertsch, A. Bivins, K. Bibby, K. Farkas, A. Gathercole, E. Haramoto, P. Gyawali, A. Korajkic, B.R. McMinn, J.F. Mueller, S.L. Simpson, W.J.M. Smith, E.M. Symonds, K.V. Thomas, R. Verhagen and M. Kitajima, Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater, Science of The Total Environment, 2020b. **739**: p. 139960.

[3]Alexander, J.P., Jr., H.E. Gary, Jr. and M.A. Pallansch, Duration of poliovirus excretion and its implications for acute flaccid paralysis surveillance: a review of the literature, J Infect Dis, 1997. **175 Suppl 1**: p. S176-82.

[4]Almeida, M.I., A.M. Silva, R.A. Coleman, V.J. Pettigrove, R.W. Cattrall and S.D. Kolev, Development of a passive sampler based on a polymer inclusion membrane for total ammonia monitoring in freshwaters, Anal Bioanal Chem, 2016. **408**(12): p. 3213-22.

[5]Aymerich, I., V. Acuña, C. Ort, I. Rodríguez-Roda and L. Corominas, Fate of organic microcontaminants in wastewater treatment and river systems: An uncertainty assessment in view of sampling strategy, and compound consumption rate and degradability, Water Research, 2017. **125**: p. 152-161.

[6]Birch, H., A.K. Sharma, L. Vezzaro, H.-C.H. Lützhøft and P.S. Mikkelsen, Velocity dependent passive sampling for monitoring of micropollutants in dynamic stormwater discharges, Environmental Science and Technology, 2013. **47**(22): p. 12958-12965.

[7]Cassemiro, K.M.S.d.M., F.M. Burlandy, M.R.F. Barbosa, Q. Chen, J. Jorba, E.M. Hachich, M.I.Z. Sato, C.C. Burns and E.E. da Silva, Molecular and Phenotypic Characterization of a Highly Evolved Type 2 Vaccine-Derived Poliovirus Isolated from Seawater in Brazil, 2014, PLOS ONE, 2016. **11**(3): p. e0152251.

[8]Dunn, G., D. Klapsa, T. Wilton, L. Stone, P.D. Minor and J. Martin, Twenty-Eight Years of Poliovirus Replication in an Immunodeficient Individual: Impact on the Global Polio Eradication Initiative, PLOS Pathogens, 2015. **11**(8): p. e1005114.

[9]Gupta, S., J. Parker, S. Smits, J. Underwood and S. Dolwani, Persistent viral shedding of SARS-CoV-2 in faeces - a rapid review, medRxiv, 2020: p. 2020.04.17.20069526.

[10]Hart, O.E. and R.U. Halden, Computational analysis of SARS-CoV-2/COVID-19 surveillance by wastewater-based epidemiology locally and globally: Feasibility, economy, opportunities and challenges, Science of The Total Environment, 2020. **730**: p. 138875.

[11]Hassard, F., L. Lundy, A.C. Singer, J. Grimsley and M. Di Cesare, Innovation in wastewater near-source tracking for rapid identification of COVID-19 in schools, The Lancet Microbe.

[12]Hill, V.R., A.L. Polaczyk, D. Hahn, J. Narayanan, T.L. Cromeans, J.M. Roberts and J.E. Amburgey, Development of a rapid method for simultaneous recovery of diverse microbes in drinking water by ultrafiltration with sodium polyphosphate and surfactants, Applied and environmental microbiology, 2005. **71**(11): p. 6878-6884.

[13]Jones, D.L., M.Q. Baluja, D.W. Graham, A. Corbishley, J.E. McDonald, S.K. Malham, L.S. Hillary, T.R. Connor, W.H. Gaze, I.B. Moura, M.H. Wilcox and K. Farkas, Shedding of SARS-CoV-2 in feces and urine and its potential role in person-to-person transmission and the environment-based spread of COVID-19, Science of The Total Environment, 2020. **749**: p. 141364.

[14]Kitajima, M., W. Ahmed, K. Bibby, A. Carducci, C.P. Gerba, K.A. Hamilton, E. Haramoto and J.B. Rose, SARS-CoV-2 in wastewater: State of the knowledge and research needs, The Science of the total environment, 2020. **739**: p. 139076-139076.

[15]Langergraber, G., J. Alex, N. Weissenbacher, D. Woerner, M. Ahnert, T. Frehmann, N. Halft, I. Hobus, M. Plattes, V. Spering and S. Winkler, Generation of diurnal variation for influent data for dynamic simulation, Water Sci Technol, 2008. **57**(9): p. 1483-6.
[16]Metcalf, amp and I. Eddy, *Wastewater engineering : treatment and reuse*. 2003: Fourth edition / revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel. Boston : McGraw-Hill, [2003] ©2003.
[17]Moore, B., The detection of enteric carriers in towns by means of sewage examination, Journal of the Royal Sanitary Institute, 1951. **71**(1): p. 57-60.
[18]O'Connor Šraj, L., M.I.G.S. Almeida, C. Bassett, I.D. McKelvie and S.D. Kolev, Gas-diffusion-based passive sampler for ammonia monitoring in marine waters, Talanta, 2018. **181**: p. 52-56.
[19]*World Health Organization, Guidelines for environmental surveillance of poliovirus circulation*. 2003, World Health Organization: Geneva.
[20]Orive, G., U. Lertxundi and D. Barcelo, Early SARS-CoV-2 outbreak detection by sewage-based epidemiology, Science of The Total Environment, 2020. **732**: p. 139298.
[21]Randazzo, W., P. Truchado, E. Cuevas-Ferrando, P. Simón, A. Allende and G. Sánchez, SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area, Water research, 2020. **181**: p. 115942-115942.
[22]Sattar, S.A. and J.C. Westwood, Isolation of apparently wild strains of poliovirus type 1 from sewage in the Ottawa area, Canadian Medical Association journal, 1977. **116**(1): p. 25-27.
[23]Sikorski, M.J. and M.M. Levine, Reviving the "Moore Swab": a Classic Environmental Surveillance Tool Involving Filtration of Flowing Surface Water and Sewage Water To Recover Typhoidal Salmonella Bacteria, Applied and Environmental Microbiology, 2020. **86**(13): p. e00060-20.
[24]Sinclair, R.G., C.Y. Choi, M.R. Riley and C.P. Gerba, Pathogen surveillance through monitoring of sewer systems, Advances in applied microbiology, 2008. **65**: p. 249-269.
[25]Vincent-Hubert, F., B. Morga, T. Renault and F.S. Le Guyader, Adsorption of norovirus and ostreid herpesvirus type 1 to polymer membranes for the development of passive samplers, Journal of Applied Microbiology, 2017. **122**(4): p. 1039-1047.
[26]Voisin, J., B. Cournoyer and F. Mermillod-Blondin, Utilisation de billes de verre comme substrats artificiels pour la caractérisation des communautés microbiennes dans les nappes phréatiques : mise au point méthodologique, La Houille Blanche, 2015(4): p. 52-57.
[27]Wu, Y., C. Guo, L. Tang, Z. Hong, J. Zhou, X. Dong, H. Yin, Q. Xiao, Y. Tang, X. Qu, L. Kuang, X. Fang, N. Mishra, J. Lu, H. Shan, G. Jiang and X. Huang, Prolonged presence of SARS-CoV-2 viral RNA in faecal samples, The Lancet Gastroenterology & Hepatology, 2020. **5**(5): p. 434-435.

Supplementary material – RT-qPCR mix and protocol

Table 4. RT-qPCR mastermix used in this study

Reagent name	Volume per reaction
nCoV Reagent A	7.5 µL
nCoV Reagent B	1.5 µL
nCoV Enzyme Mix	1 µL
UltraPure DNase/RNase free water	15 µL
RNA template	5 µL

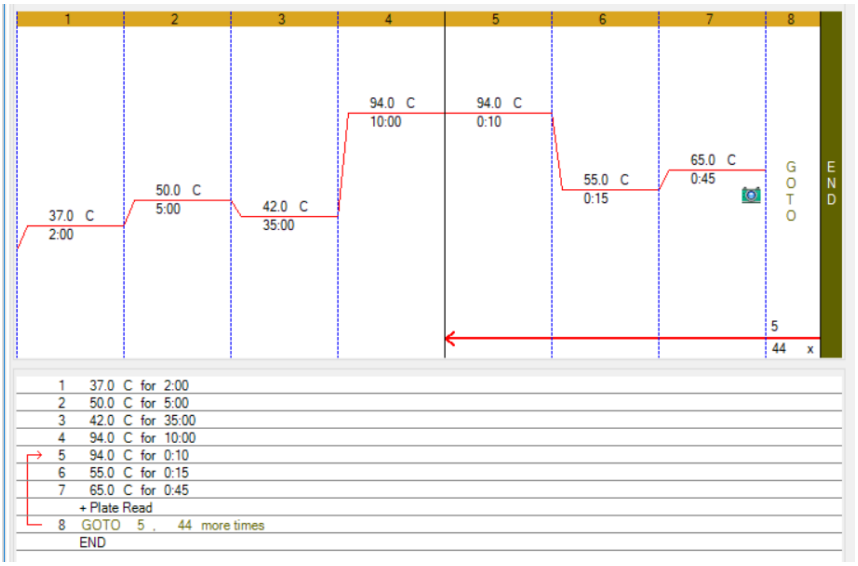
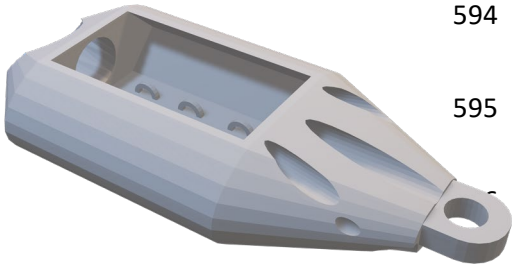


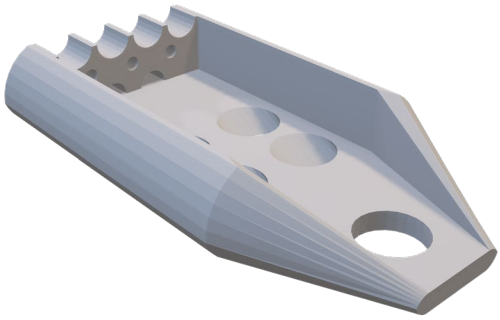
Figure 4. RT qPCR Run for PCr amplification and fluorescence detection on BIORAD CFX 96 (based on Instructions for PerkingElmer®SARS-CoV-2 Real-time RT-PCR Assay. Reaction volume 30 µL

Supplementary material – Passive Sampling Unit Designs

Boat style unit



Matchbox style unit



Torpedo style unit