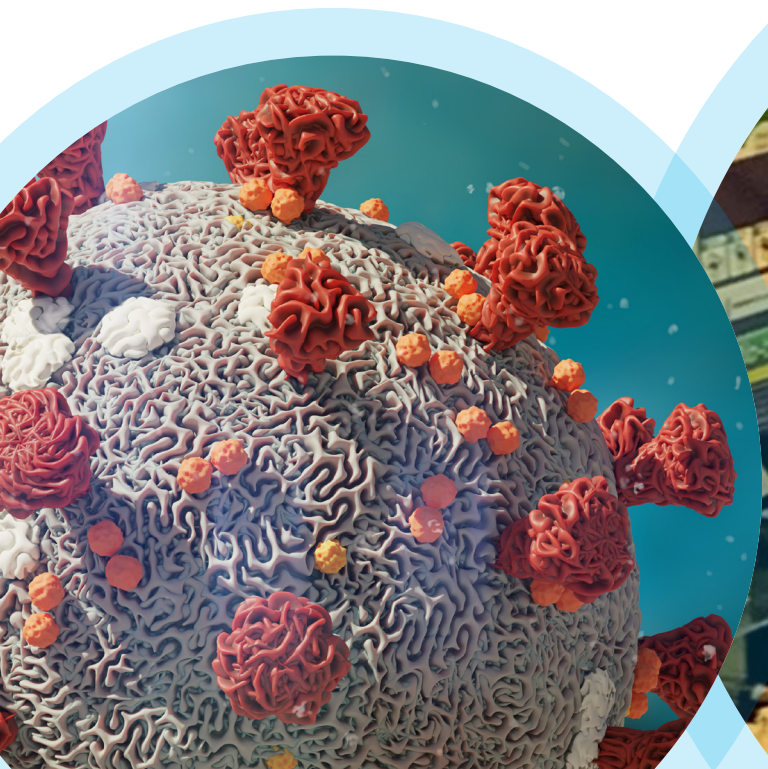
CREW CENTRE OF EXPERTISE FOR WATERS

Tracking SARS-CoV-2 via Municipal Wastewater



CREW CENTRE OF EXPERTISE FOR WATERS

Tracking SARS-CoV-2 via Municipal Wastewater

Full Report

Alexander Corbishley, David Gally, Stephen Fitzgerald, Alison Tidswell, Sean Mcateer

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CREW CENTRE OF EXPERTISE FOR WATERS

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Contents

Glossary

Influent – wastewater flowing into a wastewater treatment works

Effluent – treated water flowing out of a wastewater treatment works

Primary Sludge – the solids that settle out of wastewater shortly after arrival at a wastewater treatment works

Sludge Cake – sludge that has undergone further processing at a wastewater treatment works

SARS-CoV-2 RNA – the genetic material of the virus that caused COVID19

Faecal Shedding – presence of viral particles in the faeces of infected people

RT-qPCR assay – Reverse Transcriptase Quantitative Polymerase Chain Reaction assay. A technique that is used to convert viral RNA into cDNA that then measures the amount of cDNA (and hence viral RNA) in a sample

Cq Value – cycle quantification value. A measure of the amount of cDNA detected in an RT-qPCR assay. Smaller Cq values represent higher levels of cDNA.

Spike – a virus that is grown in the laboratory and added to samples intentionally

Surrogate virus – a virus that behaves in a similar way to the virus of interest

Infectious SARS-CoV-2 – the virus that causes COVID19 in a form that is able to infect cells in the laboratory

Heat killed SARS-CoV-2 – the virus that causes COVID19 that has been heat treated so that it is no longer infectious

PRRSv – Porcine Reproductive and Respiratory Syndrome virus. An enveloped pig virus in the same family as the virus that causes COVID19

Executive Summary

Objectives

This project set out to determine whether SARS-CoV-2 viral RNA could be detected in wastewater during the disease outbreak in Scotland and to explore whether this approach offered a potential tool for monitoring for the presence of SARS-CoV-2 within the community. The scientific objectives were to:

1) Complete a rapid literature review of human coronaviruses in treated and untreated municipal wastewater

2) Design and implement an appropriate sampling strategy to obtain a contemporaneous series of wastewater samples from wastewater treatment plants and specific locations, e.g. hospital effluent, draining into these works

3) Archive and extract RNA from these samples

4) Develop reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assays to quantify the presence of SARS-CoV-2 RNA relative to an internal sample control

5) Quantify SARS-CoV-2 RNA within wastewater samples

6) Use the results of 1-5 to propose changes to industry and public health policy, develop guidance for the wider implementation of the findings and to develop more substantial follow-on projects

Whilst examining whether SARS-CoV-2 RNA in wastewater represents the presence of infectious virus was not within the remit of this project, the fate of viral RNA within the wastewater treatment plant and hence whether viral particles are present in plant effluent was studied for one large treatment plant. A literature review was also undertaken to explore the utility of SARS-CoV-2 wastewater epidemiology and any potential hazard from SARS-CoV-2 viral RNA in wastewater to the community, plant staff or environment.

Finally, the project set out to identify a suitable surrogate virus for the fate of SARS-CoV-2 in wastewater, both to act as a process control during sample analysis and to optimise viral RNA extraction efficiency, whilst minimising the use of the SARS-CoV-2 virus itself.

Background

SARS-CoV-2 is a novel coronavirus of animal origin that causes severe respiratory disease in humans (COVID19). During the early stages of the COVID19 pandemic, studies of infected patients have shown that SARS-CoV-2 RNA can be detected in the faeces of patients for weeks after the onset of clinical signs. There is no data with respect to faecal shedding prior to the onset of clinical signs, however modelling data suggests that faecal shedding at the start of an outbreak may be extremely high. Testing at wastewater treatment plants in a number of countries has identified evidence of SARS-CoV-2 RNA in municipal wastewater, but not plant effluent. Given the significant logistical limitations to testing people individually, testing of wastewater in the sewage network could provide valuable information as to the level of infection within communities.

Determining the fate of viral RNA during wastewater processing is important to informing appropriate risk assessments with respect to any potential hazard from SARS-CoV-2 in wastewater.

Research undertaken

A series of experiments were undertaken to determine the extraction efficiency of SARS-CoV-2 RNA from wastewater. A suitable surrogate virus was validated to assist in these experiments and to act as a process control when analysing samples for the presence of SARS-CoV-2 RNA. Wastewater influent samples were received from six different treatment plants in Scotland shortly before the peak of the pandemic, whilst a time series of wastewater treatment plant influent, primary sludge, treated dewatered sludge (cake) and effluent samples were provided by Scottish Water from a large wastewater treatment plant during March, April and May 2020. Wastewater samples were also taken from two hospital sites. Published evidence on the utility of wastewater epidemiology for SARS-CoV-2 and the potential hazards from SARS-CoV-2 in wastewater was also reviewed.

Key Findings

- This project identified SARS-CoV-2 viral RNA in wastewater treatment plant influent during the COVID19 pandemic in Scotland.

- The fate of SARS-CoV-2 RNA within a single wastewater treatment plant was studied, with no evidence of viral RNA in plant effluent. Viral RNA was concentrated in primary sludge during the wastewater treatment process at this plant.

Key Findings (cont'd)

- Both live and heat inactivated Porcine Reproductive and Respiratory Syndrome virus (PRRSv) grown in tissue culture was shown to have a similar extraction efficiency to heat inactivated SARS-CoV-2 virus when spiked into a range of wastewater samples. The extraction efficiency was however highly variable and most likely affected by the physicochemical properties of the sample, which are different between treatment plants and also over time at the same plant.

- Three different approaches to viral RNA concentration were attempted:

    - The use of milk powder adsorption, whilst successful, was not scalable and so was not explored further.

    - The use of concentration columns was explored in detail. Viral RNA was lost at all stages of processing using concentration columns, with significant loss at the 50 kDa spin column stage.

    - PEG precipitation was compared to the use of concentration columns. When using the same volume of wastewater, PEG precipitation did not improve the efficiency of virus recovery compared to the use of concentration columns, however it has the potential to facilitate the processing of larger volumes of influent, which could improve the sensitivity of viral RNA detection.

- SARS-CoV-2 RNA was detected at higher levels in primary sludge than influent and so sampling primary sludge or larger volumes of plant influent may improve the sensitivity of viral RNA detection, which will become increasingly important as disease levels within the population decline. There are however technical, logistical and operational difficulties in sampling primary sludge, including, but not limited to, mixing of sludge streams within treatment works, different residence times within treatment plants and the lack of automatic samplers for primary sludge.

- No evidence was identified in the literature of transmission of COVID19 via wastewater, whilst reports as to whether infectious virus is present in faeces are conflicting.

Policy implications

1) The work in this project should be repeated across a range of wastewater treatment plants before generalised conclusions can be drawn regarding the fate of SARS-CoV-2 viral RNA during the wastewater treatment process.

2) Detection of SARS-CoV-2 RNA in wastewater treatment plant influent can be used to detect the presence of COVID19 within a population, but detecting SARS-CoV-2 RNA in primary sludge may offer a more sensitive approach than plant influent.

3) At present, it is not possible to infer what proportion of the population are infected from measurements made at a wastewater treatment plant.

4) There is no evidence that SARS-CoV-2 RNA is present in wastewater treatment plant effluent.

5) There is no evidence of transmission of COVID19 via wastewater.

Recommendations

1) Further work should be carried out to determine the potential of monitoring SARS-CoV-2 in wastewater streams as part of the wider surveillance of community spread of COVID.

2) Experiments should be undertaken at a number of wastewater treatment plants of different designs to determine whether viral RNA is concentrated in primary sludge and absent from effluent in all plant types.

3) Further work is required to explore how the levels of SARS-CoV-2 RNA in wastewater can be related to the proportion of the population that is infected with SARS-CoV-2r.

Introduction

Background

SARS-CoV-2 is a novel coronavirus of animal origin that causes severe respiratory disease in humans (COVID19). During the early stages of the COVID19 pandemic, studies of infected patients have shown that SARS-CoV-2 RNA can be detected in the faeces of patients for weeks after the onset of clinical signs. There is no data with respect to faecal shedding prior to the onset of clinical signs, however modelling data suggests that faecal shedding at the start of an outbreak may be extremely high. Testing at wastewater treatment plants in a number of countries has identified evidence of SARS-CoV-2 RNA in municipal wastewater, but not plant effluent. Given the significant logistical limitations to testing people individually, testing of wastewater in the sewage network could provide valuable information as to the level of infection within communities.

Determining the fate of viral RNA during wastewater processing is important to informing appropriate risk assessments with respect to any potential hazard from SARS-CoV-2 in wastewater.

The overarching aim of this project was to develop the sampling protocols and assays to detect SARS-CoV-2 RNA in municipal wastewater collected in Scotland during the COVID19 pandemic. The scientific objectives are set out below:

1) Complete a rapid literature review of human coronaviruses in treated and untreated municipal wastewater.*

2) Design and implement an appropriate sampling strategy to obtain a contemporaneous series of wastewater samples from wastewater treatment plants and specific locations, e.g. hospital effluent, draining into these works.

3) Archive and extract RNA from these samples.

4) Develop reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assays to quantify the presence of SARS-CoV-2 RNA relative to an internal sample control.

5) Quantify SARS-CoV-2 RNA within wastewater samples.

6) Use the results of 1-5 to propose changes to industry and public health policy, develop guidance for the wider implementation of the findings and to develop more substantial follow-on projects.

* *Through UK wide collaborations established by this project, the principle investigator contributed to two comprehensive literature reviews authored by David Polo and David Jones, documenting current knowledge with respect to both SARS-CoV-2 wastewater epidemiology and the potential for infectivity in water. These were provided to the project steering group during drafting and preprints/submitted copies are attached to this report.*

Methods

Wastewater treatment plant influent, primary sludge, cake and effluent samples were provided by Scottish Water from a large wastewater treatment plant during March, April and May 2020. Single influent samples were taken on 27th March 2020 from five other wastewater treatment plants in Scotland. Outflows from two hospital sites and the influent to their serving wastewater treatment plants were also taken. Samples were transported at 4°C and stored at -20°C prior to analysis.

Detailed laboratory methods are provided in Appendix 1. Samples were defrosted at 4°C prior to extraction of viral RNA. Liquid samples were clarified by centrifugation at 4669 x g followed by passage through a 0.45 µm filter prior to concentration and viral RNA extraction. The amount of liquid sample processed varied from 20 to 400 ml depending on the sample type. For primary sludge and cake, 2 ml or 2 g was processed.

Following viral RNA extraction, samples were subjected to a one-step RT-qPCR assay to detect the SARS-CoV-2 E gene. Where appropriate, a standard curve using a plasmid of known molecular weight containing the SARS-CoV-2 E gene was used to calculate the number of gene copies per litre of processed sample. Remaining RNA was archived at -80°C.

To assess the efficiency of viral RNA extraction, samples were spiked with tissue culture supernatants of Porcine Reproductive and Respiratory Syndrome virus (PRRSv) + tissue culture supernatants of SARS-CoV-2. All SARS-CoV-2 tissue culture supernatants were heat inactivated at 70°C for 10 minutes prior to use. PRRSv tissue culture supernatants were used either fresh or after heat inactivation as per the SARS-CoV-2 tissue supernatants. Where appropriate, a standard curve using a plasmid of known molecular weight containing the PRRSv qPCR amplicon was used to calculate the number of gene copies of PRRS virus.

RT-qPCR data were analysed using the software provided by the instrument manufacturer to calculate Cq values and where relevant, genome equivalents per litre of sample. Data were processed in Microsoft Excel, before plotting using Prism 8 (GraphPad).

Results and discussion

Methodological considerations

A series of experients were undertaken to determine whether SARS-CoV-2 viral RNA could be detected in wastewater treatment plant influent, primary sludge, cake and effluent samples taken in Scotland during the COVID19 pandemic. In an attempt to understand the sensitivity of the approach proposed, samples were also spiked using heat inactivated SARS-CoV-2 and/or PRRS virus. PRRSv is an enveloped pig virus in the same Nidovirus order as coronaviruses. Although an animal coronavirus would have been preferable as a spike control for this work, it was not possible to source one during the nationwide lockdown that was in place for the duration of this project.

At the outset of the project, it was proposed to try to detect three different SARS-CoV-2 targets using RT-qPCR, namely the E-gene, N1-gene and Orf1. Primers and probes were ordered for these three targets from three separate suppliers. Unfortunately, it was only possible to establish the E-gene assay during the project. The Orf1 assay had poorer sensitivity than the E-gene, whilst the N1-gene assay suffered from contamination (data not shown). The latter is a national problem relating to the manufacture of the primers and probes that other labs have also struggled with, as it results in high background signal levels, which make the assay unusable.

The team at the Roslin Institute has previous experience of using concentration columns for the extraction of viral RNA. This approach has also been adopted by other groups pursuing wastewater epidemiology e.g. University of Bangor and in the Netherlands. The team attempted viral RNA concentration using adsorption to milk powder, and whilst this approach was successful (data not shown), it was cumbersome and did not lend itself well to application at a large scale, due to the requirement to manipulate the pH of samples and to stir them continuously overnight prior to extraction. During the project, a number of groups released preprint papers using either concentration columns or polyethylene glycol (PEG) precipitation[1–3]a significant proportion of cases shed SARS-Coronavirus-2 (SARS-CoV-2) for the detection of SARS-CoV-2 RNA in wastewater. An additional preprint paper from The Commonwealth Scientific and Industrial Research Organisation (CSIRO, Australia) demonstrated that PEG precipitation was inferior to concentration column processing[4] for the concentration of viral RNA. For these reasons, the study focused on the use of concentration columns.

SARS-CoV-2 spike virus recovery is not affected by virus concentration

The first experiment set out to determine whether the extraction efficiency of SARS-CoV-2 RNA was affected by the concentration of virus in the sample. This is an essential requirement to determining whether the concentration of viral RNA in wastewater can be reliably quantified. The data from this experiment are shown in Figure 1, where it is demonstrated that across five orders of magnitude of virus concentration, there is no association between viral concentration and the efficiency of viral recovery. In this sample, approximately 5% of the spiked virus was recovered and it is evident that there is some variation between technical replicates. Clearly the sample that had been spiked with the second point of the SARS-CoV-2 dilution series resulted in a higher recovery of 10%, however this does not appear to be related to viral concentration and demonstrates the variability in extraction efficiency. It was decided that this finding required further investigation.

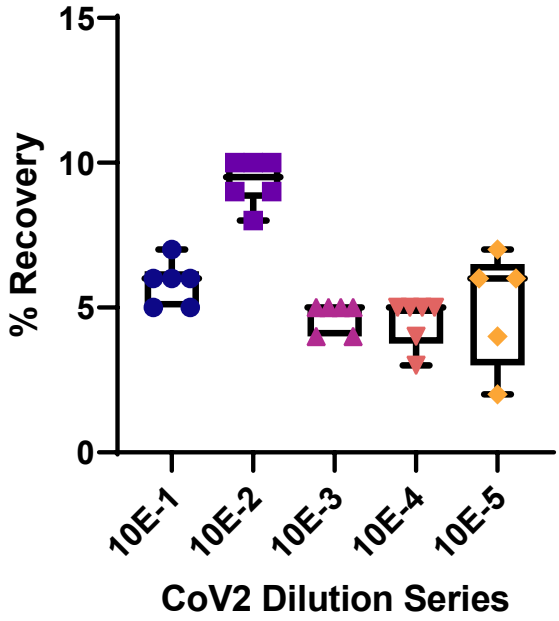

Figure 1: Percent recovery of heat inactivated SARS-CoV-2 from a single wastewater treatment plant influent sample. 3x20 ml technical replicates were spiked and subsequently analysed by RT-qPCR in duplicate.

Spike virus recovery is similar between heat inactivated SARS-CoV-2, live PRRSv and heat inactivated PRRSv

It was necessary to determine whether a PRRS virus spike represented a suitable surrogate for SARS-CoV-2 for two reasons. Firstly, SARS-CoV-2 is difficult to produce, as it must be cultured in a containment level 3 facility and hence supplies of the virus are limited. In addition, it is necessary to use a different virus to SARS-CoV-2 as a process control spike when quantifying SARS-CoV-2 in wastewater samples, as the signal from the process control must not interfere with the signal for SARS-CoV-2.

The results from this experiment are shown in Figure 2: Percent recovery of heat inactivated (killed) SARS-CoV-2, live PRRSv and heat inactivated (killed) PRRSv from a single wastewater treatment plant influent sample. 3x20 ml technical replicates were spiked either with heat inactivated SARS-CoV-2, heat inactivated SARS-CoV-2 or live PRRSv. Samples were analysed by RT-qPCR in duplicate, and the recovery of all three spike viruses was around 1%. Again, there is variation between the technical replicates, with up to 3% recovery for SARS-CoV-2. As the efficiency of recovery was similar for SARS-CoV-2 and PRRSv and there was no obvious impact of heat inactivation on the recovery efficiency of PRRSv, it was therefore agreed to undertake all subsequent experiments using heat inactivated PRRSv. This is because the PRRSv strain is an animal pathogen (SAPO2 designation) that must be handled in a containment level 2 laboratory. Heat inactivating the virus means it can be used by laboratories at a lower level of containment.

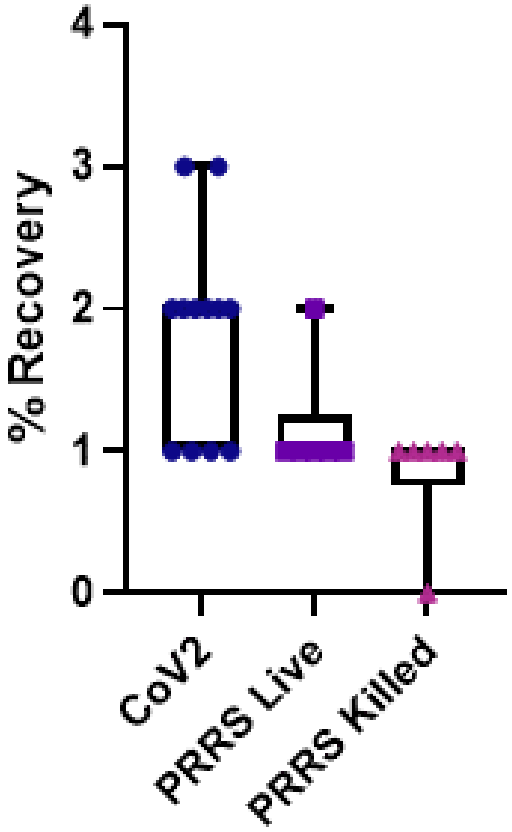

Figure 2: Percent recovery of heat inactivated (killed) SARS-CoV-2, live PRRSv and heat inactivated (killed) PRRSv from a single wastewater treatment plant influent sample. 3x20 ml technical replicates were spiked either with heat inactivated SARS-CoV-2, heat inactivated SARS-CoV-2 or live PRRSv. Samples were analysed by RT-qPCR in duplicate.

Spike virus recovery is not improved by removing the initial filtration or final concentration steps

Due to the low recovery efficiency observed in these experiments, it was important to determine whether modifications to the viral RNA concentration protocol would improve recovery. In consultation with Bangor University (Kata Farkas, personal communication) this viral RNA concentration protocol was modified to adopt a 10,000 x g initial spin to clarify samples and to use a single concentration column. This contrasts to the existing protocol, where samples are clarified at a lower speed (4669 x *g*) and passed through a 0.45 µm filter, followed by concentration through two separate concentration filter columns. To determine the impact of the second concentration filter column, a third protocol was attempted where the second concentration filter column was omitted.

No improvement was observed in the recovery of either SAR-CoV-2 or PRRSv spikes using these two modifications to the original protocol (Figure 3). If anything, the higher initial spin without the 0.45 µm filter stage had a slightly poorer recovery (Bangor (10,000g spin) in Figure 3). Removing the second concentration filter column from the protocol seemed to reduce variability between technical replicates, however if anything, it resulted in poorer viral RNA recovery (Roslin (1-filter) in Figure 3).

As modifications to the original protocol did not result in an improvement in spike recovery, it was decided to continue with the original protocol. A comparison between the Roslin (1-filter) and Roslin (2-filter) protocols using two different wastewater treatment plant samples found that using the Roslin (2-filter) technique resulted in increased variability, but improved spike virus recovery (data not shown).

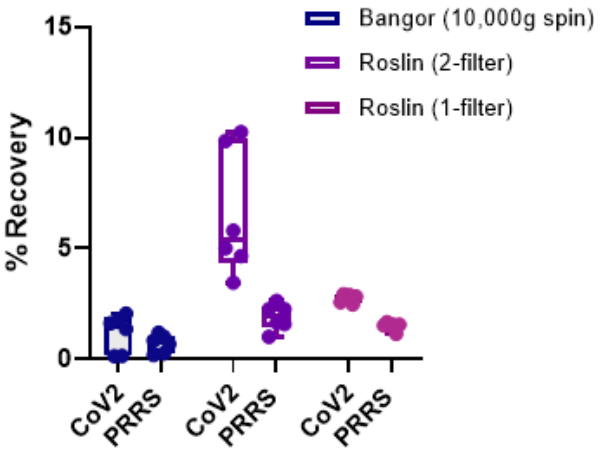

Figure 3: Percent recovery of heat inactivated SARS-CoV-2 and PRRSv from a single wastewater treatment plant influent sample processed using three different protocols. For each protocol, 3x20 ml technical replicates were spiked and subsequently analysed by RT-qPCR in duplicate.

SARS-CoV-2 and PRRSv spike recoveries are similar, however they vary considerably between samples from different wastewater treatment plants

All previous experiments had been conducted with samples from a single wastewater treatment plant. As both SARS-CoV-2 and PRRSv spike recovery was poor, i.e. under 10%, the next step was to determine wether this was consistently observed across samples from different wastewater treatment plants. There was also some indication that recovery of the SARS-CoV-2 spike may have been more efficient than the PRRSv spike recovery (Figure 2 and Figure 3), however these samples may have had an existing SAR-Cov-2 signal that would result in a higher apparent recovery of SARS-CoV-2 compared to PRRSv. To this end, influent samples from six wastewater treatment plants taken on 27th March 2020 were spiked with either PRRSv only or PRRSv and SARS-CoV-2.

In this experiment, a significant variation in spike virus recovery between samples from different wastewater treatment plants was observed, ranging from around 1 % to nearly 60 % (Figure 4). With the exception of wastewater treatment plant 5 (WW5), the recovery of SARS-CoV-2 and PRRSv from each sample was similar, hence confirming that the PRRSv spike is a suitable surrogate for a SARS-CoV-2 spike. Subsequent analysis showed that the sample from WW5 had a strong pre-existing SARS-CoV-2 signal prior to being spiked (Figure 7), hence the higher apparent recovery of the SARS-CoV-2 spike versus the PRRSv spike observed in this experiment.

It was beyond the project specification to investogate the physical properties e.g. pH, total suspended solids, conductivity etc of these samples responsible for the variability in spike virus recovery. However, it was found that spike virus recovery deteriorated the longer influent samples were stored at 4°C (data not shown).

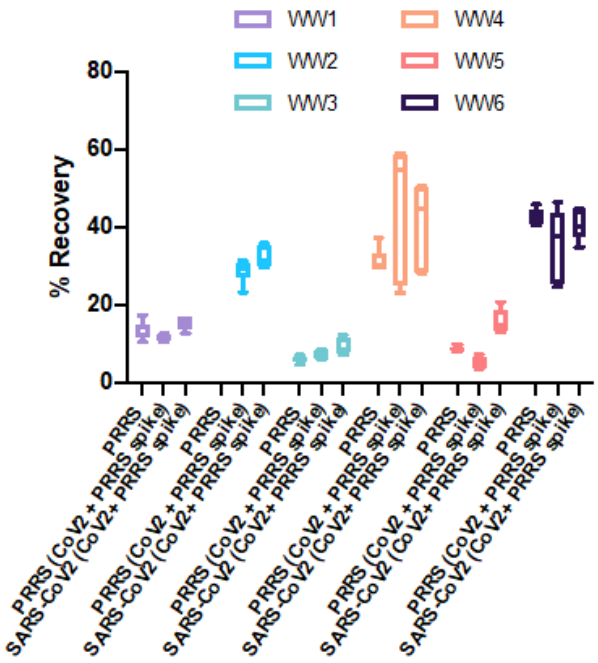

Figure 4: Percent recovery of heat inactivated SARS-CoV-2 and PRRSv from six wastewater treatment plant influent samples. For each sample, 3x20 ml technical replicates were spiked and subsequently analysed by RT-qPCR in duplicate.

Spike virus is lost at all stages of processing, but particularly at the stage of the first (50 kDa) concentration column

To determine where the spike virus was bring lost, initial experiments demonstrated that very little spike virus was detectable in the pellet after the first centrifugation to clarify the samples (data not shown), therefore an experiment was undertaken where PRRSv was spiked at various points during sample processing.

As can be seen in Figure 5, there is large jump in virus recovery when the spike is introduced after the first (50 kDa) concentration column stage. It is however notable that virus is lost at all stages of sample processing and particularly interesting that approximately 50% of the virus is lost during RNA extraction. This would suggest that residues from the wastewater sample are reducing the efficiency of viral RNA extraction in the kits that were used when compared to virus extracted directly from tissue culture supernatants. There wasn't scope to explore the performance of different RNA extraction kits in this project, however it is worth noting that even if the concentration of viral RNA in wastewater samples was completely optimised, around half of the virus would likely still be lost at the stage of extraction.

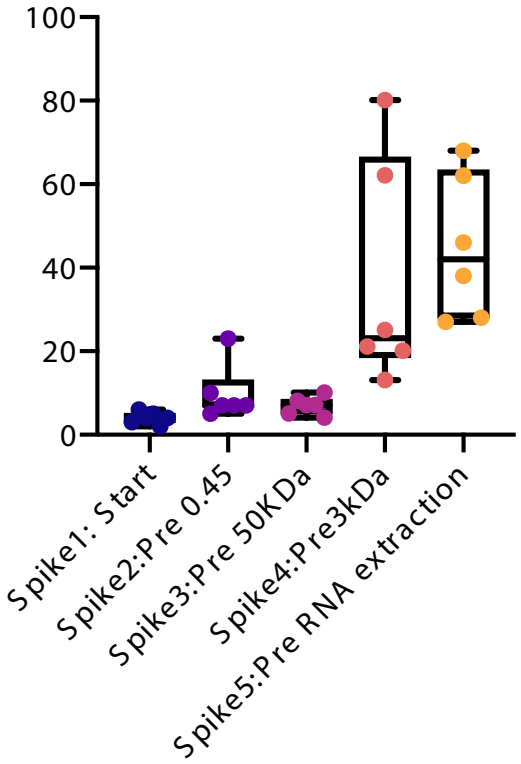

Figure 5: Percent recovery of heat inactivated PRRSv inoculated at different stages of processing of a single wastewater sample. For each spike, 3x20 ml technical replicates were processed and subsequently analysed by RT-qPCR in duplicate.

Spike virus recovery in distilled water is improved following pre-incubation with protein

As mentioned above, the spike virus extraction efficiency deteriorated the longer that samples were stored at 4°C, hence suggesting that the physicochemical properties of the samples have a significant impact on virus recovery. To investigate this further, the efficiency of recovery from distilled water was determined. As is evident in Figure 6, spike virus recovery from distilled water is poor, at around 2.5%. Note the poorer efficiency of recovery of the wastewater samples from WW2 and WW5 after storage at 4°C compared to that seen when processed immediately after defrosting (Figure 4).

The efficiency of recovery of the spike virus was considerably improved in distilled water after pre-incubation for 30 minutes in 1% skimmed milk powder or 1% bovine serum albumin (BSA). Proteins like skimmed milk powder and BSA are often used to block binding to laboratory plasticware and these results may suggest that a significant proportion of this cell culture spike virus is sticking to the plasticware used during sample concentration and processing. As might be expected, treatment with detergent (0.1% Triton-X100) reduced the extraction efficiency of the spike virus from distilled water.

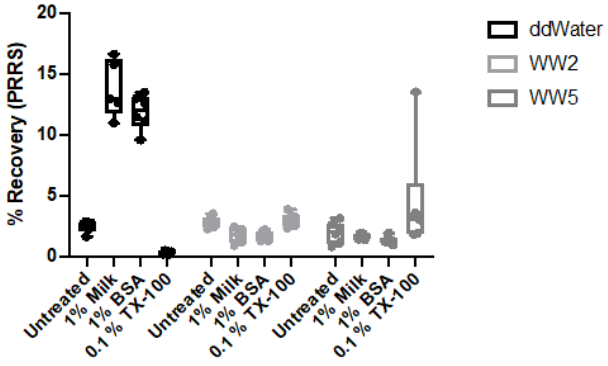

Figure 6: Percent recovery of heat inactivated PRRSv inoculated into distilled water or wastewater after pre-incubation with 1% skimmed milk powder, 1% bovine serum albumin (BSA) or 0.1% Triton-X100. Untreated = no preincubation of spike virus. For each spike, 3x20 ml technical replicates were processed and subsequently analysed by RT-qPCR in duplicate.

Somewhat unexpectedly, pre-treatment with protein did not improve recovery of the spike virus from two wastewater samples tested, whilst treatment with detergent did not reduce extraction efficiency. The failure of incubation with protein to improve spike virus recovery in wastewater samples is currently unexplained and indicates that further work is required to understand the physicochemical properties of wastewater samples that affect the efficiency of virus recovery.

It is important to note that these experiments involved the use of spike viruses (SARS-CoV-2 or PRRSv) that were produced in tissue culture, which is a significantly different matrix to faeces. Whether laboratory grown spike viruses behave the same way in these samples compared to virus shed by infected patients is unknown.

SARS-CoV-2 RNA can be detected in wastewater treatment plant influent collected in Scotland during the COVID19 pandemic

Wastewater treatment plant influent samples taken on 27th March 2020 (i.e. shortly before the COVID19 disease peak) were available from six plants. Preliminary experiments demonstrated that, as expected, processing of 40 vs 20 ml wastewater reduced the Cq value of the RT-qPCR experiments by a value of 1 (data not shown). Attempts to process larger volumes using the spin columns resulted in extended sample processing times as the filters became blocked. Therefore, to improve sensitivity of detection, 40 ml of wastewater was processed compared to 20 ml in previous experiments. One of these plants, WW5 had a strong positive signal that could be quantified (Figure 7). The levels in the other five plants generally sat between the limit of detection and limit of quantification, with all five samples having some technical replicates that were below the limit of detection.

The original plan in this project was to adjust the calculated value of genome equivalents of SARS-CoV-2 per litre of wastewater and limit of detection to reflect

the efficiency of spike virus recovery. As only one sample could be convincingly quantified, no adjustment was made to the figures to account for the efficiency of the spike virus recovery. (Figure 8). It is notable that despite following the same protocol and using the same samples as previous experiments, the spike virus recovery efficiency was lower (see Figure 4), however the relative efficiency of recovery between the samples was similar.

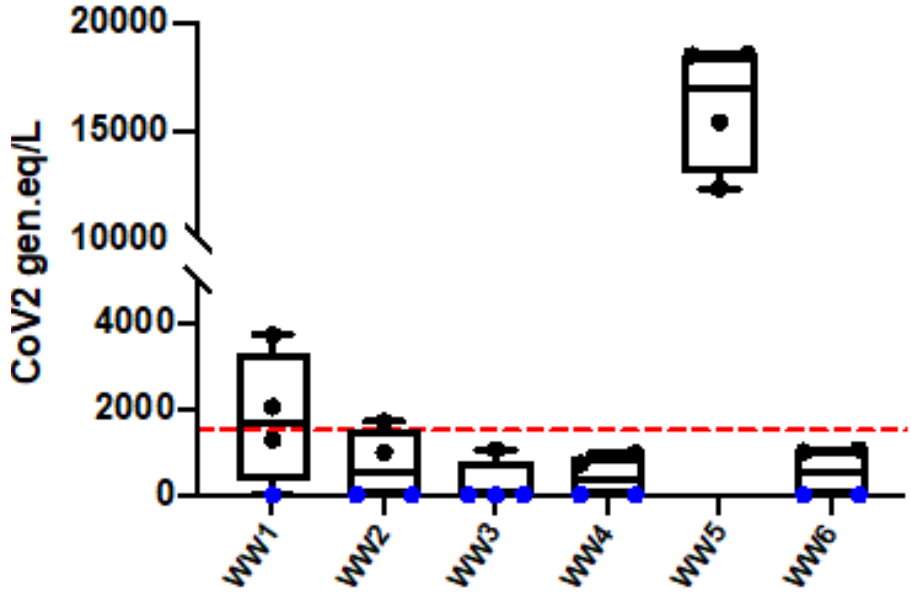

Figure 7: Quantification of SARS-CoV-2 RNA in wastewater treatment plant influent from six Scottish plants sampled on 27th March 2020. For each sample, 2x40 ml technical replicates were processed and subsequently analysed by RT-qPCR in duplicate. Gen.eq/L = SARS-CoV-2 genome equivalents per litre. Red dotted line = limit of quantification. Blue dots = below limit of detection.

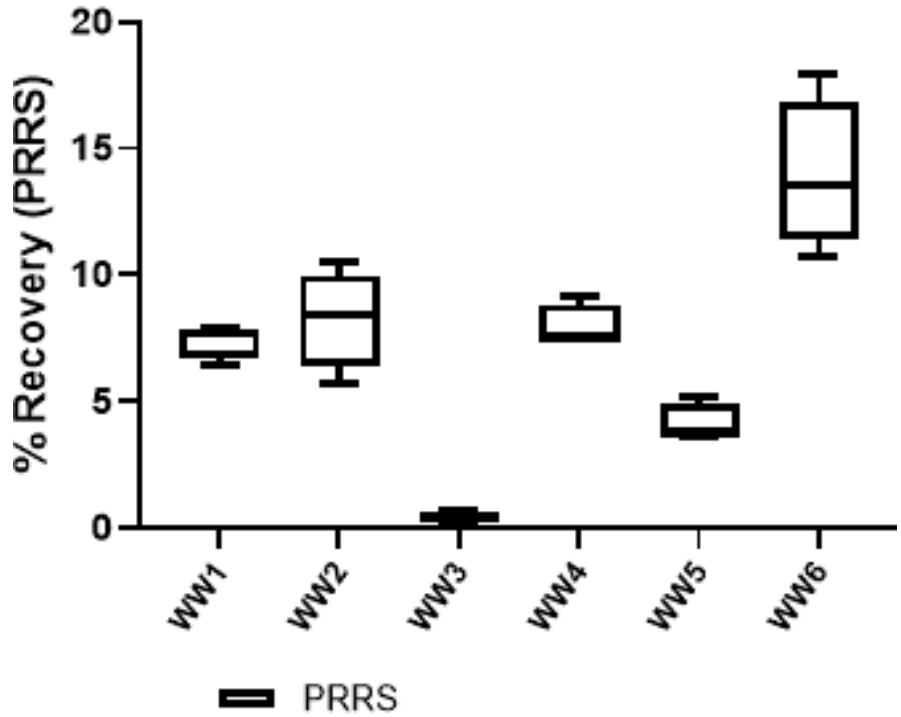

Figure 8: Percent recovery of heat inactivated PRRSv inoculated into wastewater samples prior to processing for SARS-CoV-2 detection in Figure 7.

The inclusion of a spike virus is an important process control to demonstrate that the process of viral concentration, RNA extraction and RT-qPCR has been successful. This is particularly important for wastewater samples where SARS-CoV-2 RNA cannot be detected. Whether adjusting SARS-CoV-2 genome equivalent calculations in line with the recovery of a spike virus grown in tissue culture is approximate and remains uncertain. Faeces are a significantly different matrix to tissue culture supernatants and virus shed in the faeces of infected patients may behave differently to that grown in tissue culture and spiked into wastewater.

For plant WW2, influent samples were received throughout April and May. Throughout this period, the SARS-CoV-2 RNA levels in the influent samples sat between the limit of detection and quantification and so drawing clear inferences regarding the level of infection within the population is not possible (Figure 9A). It is also of note that despite coming from the same catchment, spike virus recovery from these influent samples varied considerably over time (Figure 9B).

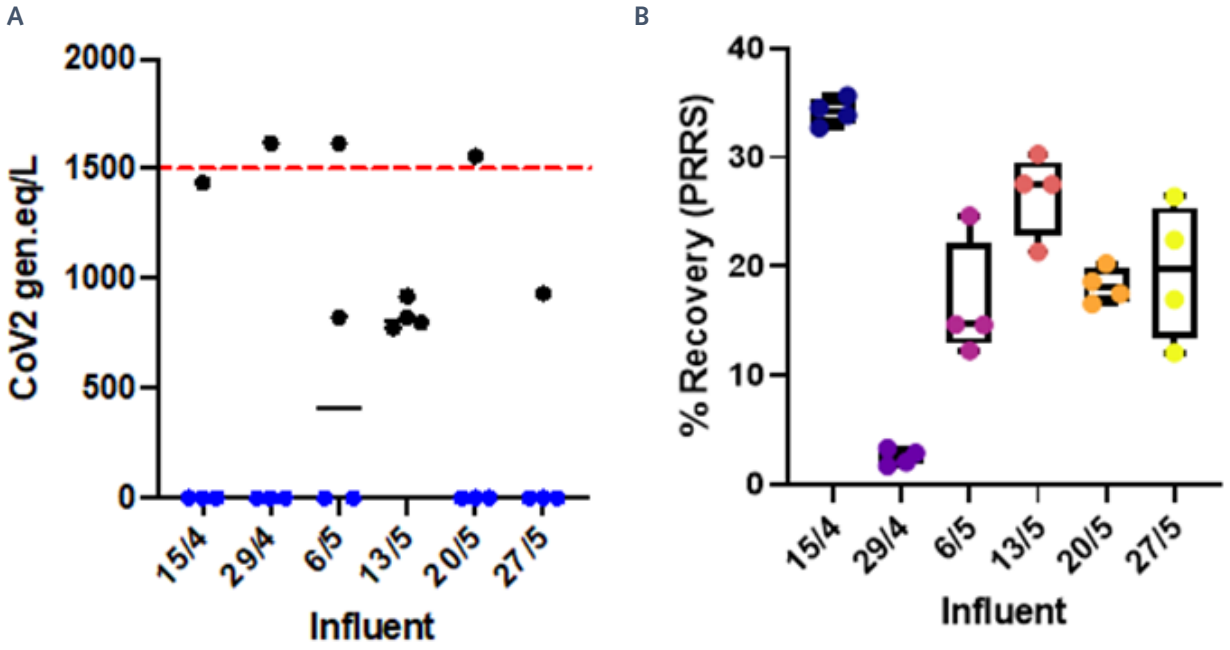

Figure 9: A) Quantification of SARS-CoV-2 RNA in wastewater treatment plant influent from plant WW2 during April and May. For each sample, 2x40 ml technical replicates were processed and subsequently analysed by RT-qPCR in duplicate. Gen.eq/L = SARS-CoV-2 genome equivalents per litre. Red dotted line = limit of quantification. Blue dots = below limit of detection. B) Percent recovery of heat inactivated PRRSv inoculated into wastewater samples prior to processing.

Primary sludge provides a more sensitive indicator of SARS-CoV-2 RNA presence than influent

Primary sludge, cake and effluent were also collected from plant WW2 during May. Despite the recovery of the spike virus being very low from sludge, cake and effluent (Figure 10B), levels of SARS-CoV-2 RNA were higher in primary sludge than influent (Figure 10A), hence suggesting that viral RNA is concentrated within primary sludge.

In addition, no viral RNA was detected in the effluent from this plant, indicating that the majority of SARS-CoV-2 RNA is retained within the solid phase of the works. This was further confirmed by the positive results for two of the three cake samples that were tested. The negative cake sample from 20th May remains unexplained.

Taken together, these results indicate that SARS-CoV-2 RNA is most consistently detected in primary sludge, even when levels in the influent are between the limit of detection and limit of quantification, whilst the failure to identify viral RNA in the effluent adds further weight to the current risk assessment that wastewater treatment work effluent represents a negligible risk with respect to SARS-CoV-2 virus transmission. The main caveat to the conclusions from this section are that these results have been obtained from a single wastewater treatment plant and therefore this experiment needs to be repeated at a variety of works to determine whether these findings can be generalised or not.

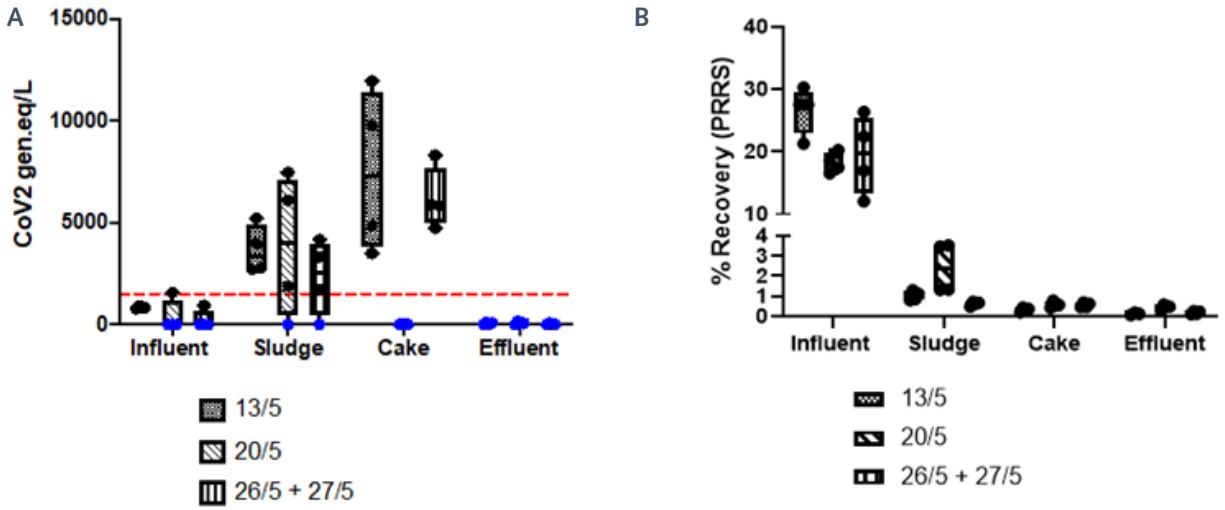

Figure 10: A) Quantification of SARS-CoV-2 RNA in wastewater treatment plant influent, primary sludge, cake and effluent from plant WW2 during May. For each sample, 2x40 ml (influent), 2x2 ml (slude/cake) and 2x400 ml (effluent) technical replicates were processed and subsequently analysed by RT-qPCR in duplicate. Gen.eq/L = SARS-CoV-2 genome equivalents per litre. Red dotted line = limit of quantification. Blue dots = below limit of detection. B) Percent recovery of heat inactivated PRRSv inoculated into samples prior to processing for SARS-CoV-2 detection.

SARS-CoV-2 RNA was not consistently detected in hospital sewage outflows, nor in the wastewater treatment plants serving these hospitals

To explore whether SARS-CoV-2 RNA could be detected from hospital sites, two hospitals were sampled at the end of April 2020. The research team was able to obtain samples from two hospitals beyond thier existing links. It therefore was not possible to comment on the relationship between the wastewater test results and case numbers within the hospital.

Whilst one replicate from one of the hospitals tested positive for SARS-CoV-2 RNA, viral RNA could not be consistently detected in these two samples, whilst the serving wastewater treatment plants were also negative (Figure 11). Whether the hospital sites were negative due to low numbers of cases in the hospital or the presence of detergents and disinfectants within hospital outflows cannot be determined from the data.

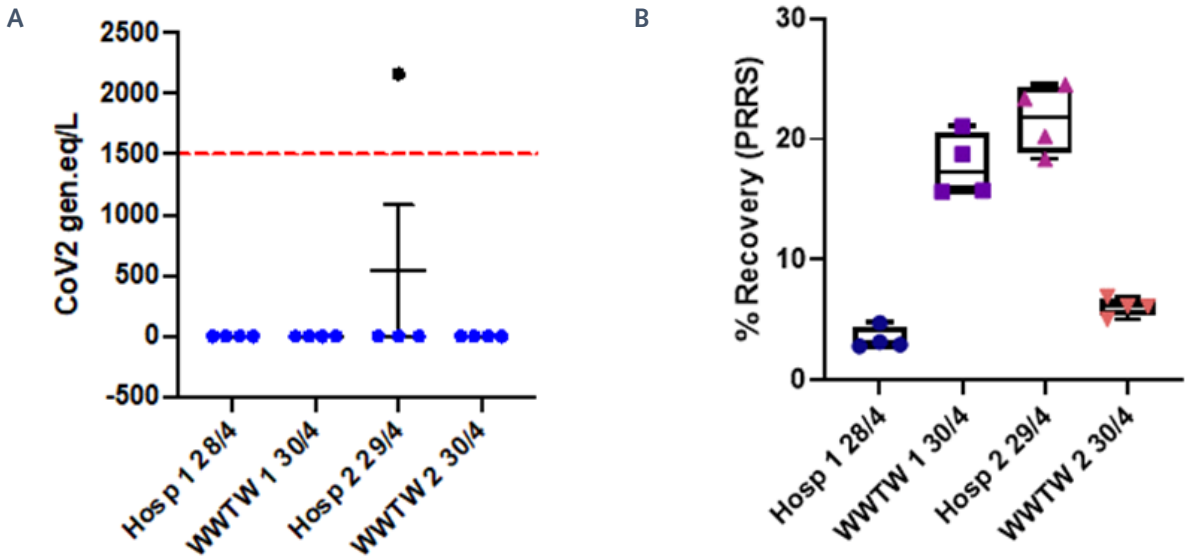

Figure 11: A) Quantification of SARS-CoV-2 RNA in hospital sewage outflows and wastewater treatment plant influent. For each sample, 2x40 ml technical replicates were processed and subsequently analysed by RT-qPCR in duplicate. Gen.eq/L = SARS-CoV-2 genome equivalents per litre. Red dotted line = limit of quantification. Blue dots = below limit of detection. B) Percent recovery of heat inactivated PRRSv inoculated into samples prior to processing for SARS-CoV-2 detection.PEG precipitation does not improve detection of SARS-CoV-2 or recovery of spike virus from wastewater treatment plant influent

Towards the end of this project, Bangor University reported improved sensitivity of SARS-CoV-2 RNA from wastewater samples using polyethylene glycol (PEG) precipitation. This was in contrast to previous reports in the literature that PEG precipitation was inferior to the use of concentration columns for viral RNA detection. This improvement in PEG precipitation sensitivity was attributed to a longer precipitation phase at 4°C compared to that published by CSIRO.

Consequently, the Bangor PEG precipitaion protocol was attempted with two samples from this study. WW5 which has a known strong positive SARS-CoV-2 RNA signal and WW2 which has a marginal signal, however testing of primary sludge confirmed that the plant was in fact positive for SARS-CoV-2 RNA. In general, with the exception of one replicate, PEG precipitation did not improve the sensitivity of viral detection (Figure 12A), whilst recovery of spike virus was also not improved (Figure 12B).

This experiment was conducted using the same volume of wastewater for both protocols. One potential advantage of the PEG precipitation technique is that it allows for larger volumes of wastewater to be processed e.g. detection could be improved tenfold if 400 ml of wastewater were processed. Furthermore, supply problems with the concentration columns mean that protocols that use these columns may be disrupted due to supply shortages. The disadvantage of the PEG precipitation technique is that laboratories must have access to centrifuges that can process large volume samples at 10,000 x *g*.

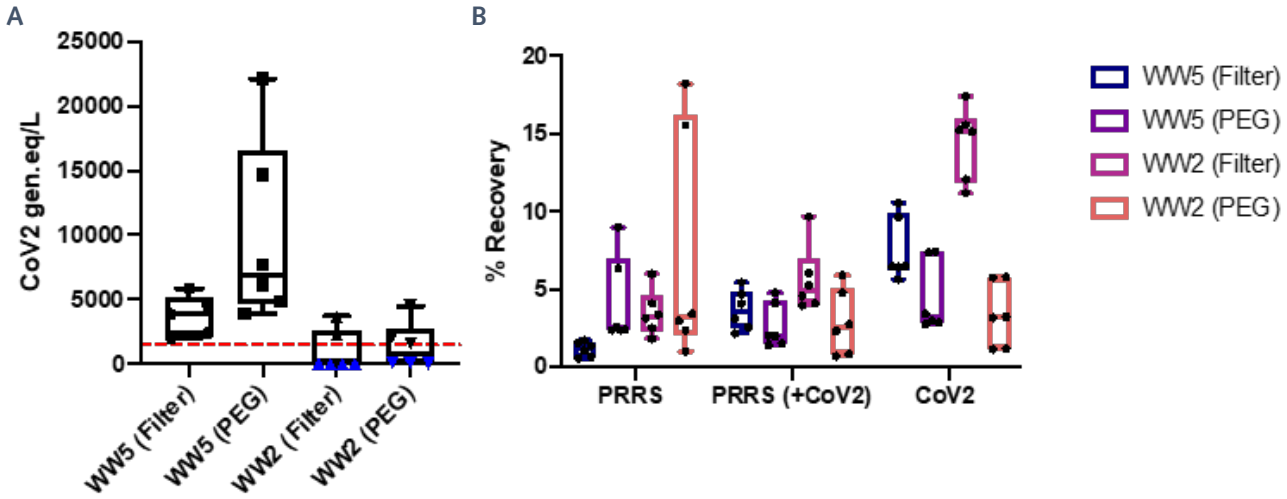

Figure 12: A) Quantification of SARS-CoV-2 RNA in wastewater treatment plant influent using either PEG precipitation or the Roslin (2-filter) protocols. For each sample, 2x40 ml technical replicates were processed and subsequently analysed by RT-qPCR in duplicate. Samples were spiked with either heat inactivated PRRSv alone or both heat inactivated PRRSv and SARS-CoV-2. Gen.eq/L = SARS-CoV-2 genome equivalents per litre. Red dotted line = limit of quantification. Blue dots = below limit of detection. B) Percent recovery of heat inactivated PRRSv and SARS-CoV-2 inoculated into samples prior to processing for SARS-CoV-2 detection.

Key findings

This project identified SARS-CoV-2 viral RNA in wastewater treatment plant influent during the COVID19 pandemic in Scotland.

The fate of SARS-CoV-2 RNA within a single wastewater treatment plant was studied, with no evidence of viral RNA in plant effluent. Viral RNA was concentrated in primary untreated sludge during the wastewater treatment process at this plant.

Both live and heat inactivated Porcine Reproductive and Respiratory Syndrome virus (PRRSv) grown in tissue culture was shown to have a similar extraction efficiency to heat inactivated SARS-CoV-2 when spiked into a range of wastewater samples. The extraction efficiency was however highly variable and most likely affected by the physicochemical properties of the sample, which are different between treatment plants and also over time at the same plant.

PEG precipitation does not improve the efficiency of virus recovery compared to the use of concentration columns, however it may permit the processing of larger volumes of influent, which could improve the sensitivity of viral RNA detection.

Sampling primary sludge or processing larger volumes of plant influent may improve the sensitivity of viral RNA detection, which will become increasingly important as disease levels within the population decline. Sampling of primary sludge however presents a number of challenges. Firstly, residence times in settlement tanks vary by plant type, whilst there may also be mixing of sludge streams, which introduces additional uncertainty with respect to the temporal relationship with disease levels in the population. Secondly, sludge samples are harder to obtain and normalise, whilst composite autosamplers are readily available for influent. Finally, the methodologies for processing sludge are not comparable to those used for sampling upstream and downstream of the plant.

The work in this project should be repeated across a range of wastewater treatment plants before generalised conclusions can be drawn regarding the fate of SARS-CoV-2 viral RNA during the wastewater treatment process.

Policy implications

1) Detection of SARS-CoV-2 RNA in wastewater treatment plant influent can be used to detect the presence of COVID19 within a population.

2) Viral RNA is concentrated in primary sludge, which may offer a more sensitive approach than plant influent for the detection of SARS-CoV-2 RNA in wastewater.

3) At present, it is not possible to infer what proportion of the population are infected from measurements made at a wastewater treatment plant.

4) There is no evidence that SARS-CoV-2 RNA is present in wastewater treatment plant effluent.

5) There is no evidence of transmission of COVID19 via wastewater.

Recommendations

1) Further work should be carried out to determine the potential of monitoring SARS-CoV-2 in wastewater streams as part of the wider surveillance of community spread of COVID.

2) Experiments should be undertaken at a number of wastewater treatment plants of different designs to determine whether viral RNA is concentrated in primary sludge and absent from effluent in all plant types.

3) Further work is required to explore how the levels of SARS-CoV-2 RNA in wastewater can be related to the proportion of the population that is infected with SARS-CoV-2.

The assistance of Scottish Water in providing all of the samples used in this project is gratefully acknowledged.

Future research

This project has helped to inform two substantial follow-on projects.

The first is a Scottish national wastewater epidemiology programme, involving the Scottish Environment Protection Agency (SEPA) and Scottish Water. Methodology and reagents from this project have been provided to SEPA who have started testing wastewater from 28 wastewater treatment plants representing the health boards in Scotland. This data will generate a national timeseries of SARS-CoV-2 levels in wastewater across Scotland, which can potentially be used as an early warning system and will inform the development of epidemiological models for disease control.

The second project is a UK wide collaboration funded by the Natural Environment Research Council (NERC): *National COVID-19 Wastewater Epidemiology Surveillance Programme (N-WESP)*. N-WESP is led by the Centre for Ecology and Hydrology and brings together investigators from the Universities of Bangor, Edinburgh, Oxford, Newcastle, Lancaster, Sheffield, Bath, Cranfield and the London School of Hygiene and Tropical Medicine. It addresses a number of outstanding research questions aligned to this project, specifically:

- How can the sensitivity and consistency of SARS-CoV-2 RNA detection be improved? Specifically, can existing protocols be optimised, does primary sludge represent a more appropriate sample than influent, what are the detection limits of different techniques and how can assays be standardised and normalised?

- How many people need to be infected with SARS-CoV-2 in a wastewater treatment catchment to be able to detect and quantify COVID-19 cases and how do viral RNA levels in wastewater relate to the proportion of the population that is infected?

- Is SARS-CoV-2 recovered from wastewater and sludge infectious?

- Is (infectious) SARS-CoV-2 environmentally persistent? How do different environmental factors impact on the survival of SARS-CoV-2?

- How precise and accurate are wastewater estimates of the population shedding COVID-19?

- Can wastewater epidemiology reveal temporal and spatial variability at the catchment and sub catchment scale?

Whilst these two follow on projects will answer a number of important questions with respect to the use of wastewater monitoring for the presence of SARS-CoV-2 in the population, a number of key questions remain unanswered. These include, but are not limited to:

- The range of SARS-CoV-2 shedding levels in the faeces of infected patients, including variation across age groups, correlations with the severity of clinical signs, chronic shedding, shedding prior to the onset of clinical signs and shedding in re-infected individuals.

- The factors that affect the decay rates of SARS-CoV-2 RNA in the wastewater network.

- Whether SARS-CoV-2 RNA can be detected in sewage impacted rivers and coastal waters and whether SARS-CoV-2 in untreated wastewater and sewage impacted rivers represents an environmental hazard.

Publications

Investigators from this project have contributed to two reviews authored by David Jones and David Polo. The paper by David Jones was published and is included in Appendix 2. At the time of publishing the CREW report the paper by David Polo was in the final stages of review and will be availabile on the CREW website shortly.

Making waves: Wastewater-based epidemiology for SARS-CoV-2 - Developing robust approaches for surveillance and prediction is harder than it looks. David Polo, Marcos Quintela-Baluja, Alexander Corbishley, Davey L. Jones, Andrew C. Singer, David W. Graham, Jesús L. Romalde. Submitted to Water Research.

Fecal shedding of SARS-CoV-2 and its potential role in person-to-person transmission and the environment-based spread of COVID-19. David. L. Jones, Marcos Quintela Baluja, David W. Graham, Alexander Corbishley, James E. McDonald, Shelagh K. Malham, Luke S. Hillary, Thomas R. Connor, William H. Gaze, Ines B. Moura, Mark H. Wilcox, Kata Farkas. Submitted to Science of the Total Environment.

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Appendix 1 – Detailed methods

Roslin Institute SARS-CoV-2 Detection in wastewater

1. Equipment/Reagents/Materials

1.1 0.45 µm syringe filters (Millipore)

1.2 Sartorius Vivaspin 20 Centrifugal Filter Unit (50 kDa) (Fisher)

1.3 Amicon Ultra-0.5 Centrifugal Filter Unit (10kDa) (Millipore)

1.4 QiAmp Viral RNA extraction kit (Qiagen)

1.5 Reliance One-Step Multiplex Supermix (Bio-Rad)

1.6 MBSC Class II for sample processing

1.7 Benchtop bucket centrifuge

1.8 Benchtop microfuge

2. Procedure

2.1 All sample processing is to be carried out within a Class II microbiological safety cabinet. For all centrifugation steps, samples must be placed in centrifuge buckets or directly into a rotor and sealed prior to removal from the cabinet. All spin steps to be carried out at 4 °C unless otherwise stated

2.2 Control: Samples are spiked with PRRS1 virus particles as a processing control

2.3 Remove solid matter from wastewater samples by centrifugation at 4669 x g for 10 min

2.4 Pass supernatants through a 0.45 µm syringe filter to remove larger microorganisms

2.5 Concentrate samples ~ x20 by centrifugation (1500g, 10 min) using a Centriprep Centrifugal Filter Unit (50 kDa)

2.6 Further concentrate ~ x10 by centrifugation (14,000g, 30 min) using a Millipore Amicon Ultra-0.5 Centrifugal Filter Unit

2.7 Samples should now be concentrated to a volume ~100 µl and ready for viral RNA extraction

2.8 Extract viral RNA using QIAmp viral RNA kit according to manufacturer guidelines. Final elution in 60 µl AE buffer

3. RT-qPCR

3.1 For each RNA extraction duplicate technical replicates are to be tested

3.2 For E-Sarbeco and N genes standard curves were made using the nCoV_all control plasmid (Eurofins). For PRRS the ORf7 gene was cloned in house onto plasmid pJET1.2 (Fisher). Eight point standard curves were generated for each plasmid by 10-fold serial dilution beginning at 10^7 copies per microliter

3.2 Prepare reaction master mix as follows: Per 20 µl reaction

5 µl Bio-Rad Reliance One-Step Multiplex Supermix

1 µl Primers (Final concentration 500 nM)

1 µl Probe (Final concentration 200 nM)

7 µl H_2O to final volume of 20 µl

Note: Adjust volumes/concentrations accordingly for multiplex qPCR

3.3 Add 14 µl of mastermix to each required well of a white 96 well PCR plate

3.4 Carefully pipette 6 µl of extracted template RNA into each well containing mastermix

3.5. Seal plate with clear adhesive PCR plate seal

3.6. Briefly centrifuge plate @ 2000 rpm for 30 s

3.7. Perform one-step qPCR on Bio-Rad CFX96 machine with the following cycling conditions:

50°C 10 min 1 cycle

95°C 10 min 1 cycle

95°C 10 sec 40 cycles

60°C 30 sec 40 cycles

Roslin Institute SARS-CoV-2 Detection in primary sludge and cake

1. Equipment/Reagents/Materials

1.1 Laboratory weigh scales

1.2 Benchtop Vortex

1.3 Qiagen RNeasy PowerSoil Total RNA kit

1.4 Reliance One-Step Multiplex Supermix (Bio-Rad)

1.5 MBSC Class II for sample processing

1.6 Benchtop bucket centrifuge

1.7 Benchtop microfuge

2. Procedure

2.1 All sample processing is to be carried out within a Class II microbiological safety cabinet. For all centrifugation steps, samples must be placed in centrifuge buckets or directly into a rotor and sealed prior to removal from the cabinet. All spin steps to be carried out at 4 °C unless otherwise stated

2.2 2 g of cake or sludge sample was weighed out directly into a 15 ml PowerBead tube supplied with the RNeasy PowerSoil Total RNA kit

2.3 Control: Samples are spiked with PRRS1 virus particles as a processing control

2.4 Total RNA was extracted from samples using an RNeasy PowerSoil Total RNA kit according to manufacturer guidelines. Final elution in 60 µl AE buffer

2.5 RT-qPCR was performed as outlined above

PEG-precipitation protocol

This protocol is for the processing of 40 ml samples.

1. Equipment/Reagents/Materials

1.1 Polyethylene Glycol 8000

1.2 $NaCl_2$

1.3 Trizol reagent

1.4 Reliance One-Step Multiplex Supermix (Bio-Rad)

1.5 MBSC Class II for sample processing

1.6 Temperature controlled centrifuge with swinging bucket rotor, sealed buckets and 50 ml tube inserts and speed up to 10,000 x g.

1.7 Benchtop microfuge

1.8 pH meter

1.9 0.45 µm syringe filters (Millipore)

2. Procedure

2.1 All sample processing is to be carried out within a Class II microbiological safety cabinet. For all centrifugation steps, samples must be placed in centrifuge buckets or directly into a rotor and sealed prior to removal from the cabinet. All spin steps to be carried out at 4 °C unless otherwise stated

2.2 Control: Samples are spiked with PRRS1 virus particles as a processing control

2.3 Samples are passed through a 0.45 µm syringe filter to remove larger microorganisms

2.4 Adjust sample pH to 7 – 7.5 if necessary

2.5 Add 3.2 g PEG8000 and 0.72 g $NaCl_2$ to each sample and mix until PEG is no longer visible

2.6 Incubate samples overnight at 4 °C

2.7 Centrifuge samples at 10,000 x g for 30 min

2.8 Discard supernatant

2.9 Dissolve PEG pellet in 1 ml Trizol reagent. Extra RNA according to Trizol manufacturer guidelines

2.10 RT-qPCR performed as above.

Primers/probes used for SARS-CoV-2 detection in wastewater

	Forward primer	Reverse primer	Probe
E-Sarbeco	ACAGGTACGTTAATAGTTAATAGCGT	ATATTGCAGCAGTACGCACACA	YAKYE-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1 (HEX alternative to YAKYE)
2019-nCoV-N1	GACCCCAAAATCAGCGAAAT	TCTGGTTACTGCCAGTTGAATCTG	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
HKU-Orf1	TGGGGYTTTACRGGTAACCT	AACRCGCTTAACAAAGCACTC	RED-TAGTTGTGATGCW ATCATGACTAG-BHQ2
Control (PRRSV1)	CAGGACTTCGGAGCCTCGT	AGCAACTGGCACAGTTGATTGA	Cy5-ACGAGCTGTTAAACGAGGA-3IAbRQSp

YAKYE = Yakima Yellow RED = Texas Red BHQ = Black hole quencher 3IAbRQSp=Iowa Black

Appendix 2 – Publication

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 20 July 2020 doi:10.20944/preprints202007.0471.v1

Fecal Shedding of SARS-CoV-2 and its Potential Role in Person-To-Person Transmission and the Environment-Based Spread of COVID-19

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Abstract

The recent detection of SARS-CoV-2 RNA in feces has led to speculation that it can be transmitted via the fecal-oral/ocular route. This review aims to critically evaluate the incidence of gastrointestinal (GI) symptoms, the quantity and infectivity of SARS-CoV-2 in feces and urine, and whether these pose an infection risk in sanitary settings, sewage networks, wastewater treatment plants, and the wider environment (e.g. rivers, lakes and marine waters). Overall, severe GI dysfunction is only evident in a small number of COVID-19 cases, with 11 ± 2% exhibiting diarrhea and 12 ± 3% exhibiting vomiting and nausea. In addition to these cases, SARS-CoV-2 RNA can be detected in feces from some asymptomatic, mildly- and pre-symptomatic individuals. Fecal shedding of the virus peaks in the symptomatic period and can persist for several weeks, but with declining abundances in the post-symptomatic phase. SARS-CoV-2 RNA is occasionally detected in urine, but reports in fecal samples are more frequent. The abundance of the virus genetic material in both urine (ca. 10^2-10^5 gc/ml) and feces (ca. 10^2-10^7 gc/ml) is much lower than in nasopharyngeal fluids (ca. 10^5-10^{11} gc/ml). There is strong evidence of multiplication of SARS-CoV-2 in the GI tract and infectious virus has occasionally been recovered from both urine and stool samples. The level and infectious capability of SARS-CoV-2 in vomit remain unknown. In comparison to enteric viruses transmitted via the fecal-oral route (e.g. norovirus, adenovirus), the likelihood of SARS-CoV-2 being transmitted via feces or urine appears lower due to the lower relative amounts of virus present in feces/urine. The biggest risk of transmission will occur in clinical and care home settings where secondary handling of people and urine/fecal matter occurs. In addition, while SARS-CoV-2 RNA genetic material can be detected by in wastewater, this signal is greatly reduced by conventional treatment. Our analysis also suggests the likelihood of

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infection due to contact with sewage-contaminated water (e.g. swimming, surfing, angling) or food (e.g. salads, shellfish) is extremely low or negligible based on very low predicted abundances and limited environmental survival of SARS-CoV-2. These conclusions are corroborated by the fact that over eight million global cases of COVID-19 have occurred, but exposure to feces or wastewater has never been implicated as a transmission vector.

Keywords: bathing waters, coronavirus, environmental transmission, faecal-oral route, infection risk, waterborne illness

1. Introduction

In recent years, several viral epidemics have impacted human populations, resulting in substantial morbidity, mortality and a negative impact on the global economy [e.g. Zika virus (ZIKV), Ebola virus (EBOV), severe acute respiratory syndrome coronavirus (SARS-CoV), and Middle East respiratory syndrome coronavirus (MERS-CoV)](Peckham, 2013)(Watkins, 2018). Of these, respiratory viruses such as coronaviruses (CoV) have proven particularly problematic to control due to their ease of human-to-human transmission and wide range of primary and secondary animal reservoirs(Assiri et al., 2013)(Damas et al., 2020). They were also recently highlighted by the World Health Organization in 2018 as priority areas for research given their potential to cause a public health emergency and the absence of efficacious drugs and/or vaccines(WHO, 2018). To date, seven human coronaviruses (HCoV) have been identified that can induce a range of respiratory symptoms with variable case fatality rates. These include the circulating seasonal HCoVs that are generally considered to cause mild respiratory symptoms (αCoVs; HCoV-229E and HCoV-NL63, β-CoVs; HCoV-HKU1 and HCoV-OC43), through to novel CoVs that lead to severe and potentially fatal respiratory tract infections (β-CoVs; SARS-CoV-1, MERS-CoV and SARS-CoV-2)(Y. R. Guo et al., 2020)(Pfefferle et al., 2011). The novel Coronavirus Disease 2019 (COVID-19), caused by SARS-CoV-2, presents with a range of respiratory symptoms which, in an estimated 14-17% of cases, leads to severe or critical disease such as severe pneumonia or acute respiratory distress syndrome (ARDS)(Petrosillo et al., 2020)(Wu and McGoogan, 2020)(Docherty et al., 2020). Although SARS-CoV-2 belongs to the same β-CoV genus as the CoVs responsible for the severe acute respiratory syndrome (SARS; caused by SARS-CoV) and Middle East respiratory syndrome (MERS; caused by MERS-CoV), this newly emerged virus tends to be associated with milder infections. For example, depending on the country, case fatality rates from COVID-19 have been estimated to be ca. 1-5%, significantly lower than the death rates for SARS (9.5%) and MERS (35%)(Wu and McGoogan, 2020)(De Wit et al., 2016)(Rajgor et al., 2020)(CDC, 2020). In addition, SARS and MERS are predominantly associated with nosocomial spread, whereas SARS-CoV-2 is much more widely transmitted in the community, particularly in care homes and prisons(Petrosillo et al., 2020).

Coronaviruses are enveloped, positively charged (at neutral pH), single-stranded viruses that possess the largest genomes of all known RNA viruses (26.4 to 31.7 kb), giving them considerable plasticity to accommodate, acquire and modify genes, enabling jumps between animal hosts(Woo et al., 2010)(Perlman and Netland, 2009). This is mainly evidenced by the by the observed spillover of SARS, MERS and now SARS-CoV-2 and the emergence of new variants of SARS-CoV-2 and thus the possibility for antigenic drift(Koyama et al., 2020). The genome size of SARS-CoV-2 lies at the upper end of the coronavirus range (29.9 kB) encoding a total of 11 genes with 11 open reading frames(Yoshimoto, 2020). The direct ancestor of SARS-CoV-2 appears to bats in which it has been circulating unnoticed for decades in bats and then transmitted to pangolins and then humans(Boni et al., 2020). SARS-CoV-2 is 96.2% identical to the bat CoV RaTG13, and is far more distantly related to both SARS-CoV-1 (79.5% identity) and MERS-CoV (50% identity) (Y. R. Guo et al., 2020)(Paraskevis et al., 2020)(Rabaan et al., 2020)(Andersen et

al., 2020). The genetic differences between SARS-CoV-1 and SARS-CoV-2 (380 amino acid substitutions) are largely clustered in non-structural protein genes; however, 27 mutations also are present in genes encoding the viral spike protein S responsible for receptor binding and cell entry. These differences have resulted in contrasting patterns of human infection (e.g. antigen detection) and replication compared with both SARS-CoV-1 and MERS-CoV. Although SARS-CoV-2 is thought to be largely spread by the inhalation of contaminated respiratory droplets or via contact with fomites, the fecal-oral route also has been suggested in its spread due to the fact that infected persons can shed SARS-CoV-2 RNA in bodily fluids (e.g. urine, feces)(L Peng et al., 2020)(T. Zhang et al., 2020). However, considerable debate exists about the relative important of this pathway, partially because a comprehensive review does not yet exist.

Here we critically assess current and previous available evidence on (i) gastrointestinal (GI) symptoms associated with COVID-19, (ii) the behavior of SARS-CoV-2 in the GI tract, (iii) the abundance of SARS-CoV-2 in feces and urine, (iv) the evidence that SARS-CoV-2 remains infectious after release from the body, and (v) whether feces and urine in sanitary environments, sewage systems and wastewater consequently pose a risk to human health.

2. Proportion of COVID-19 cases showing gastrointestinal symptoms

Patients infected with SARS-CoV-2 typically exhibit a wide range of symptoms including fever, coughing, dyspnea, sore throat and headaches. In addition, GI symptoms including nausea, vomiting, loss of appetite, diarrhea, and abdominal pain have been reported(Lo et al., 2020)(Adhikari et al., 2020). GI problems are also observed in other acute respiratory infections (e.g. influenza viruses, human rhinoviruses) and have been reported as a very common symptom of severe influenza in children(Poole et al., 2020). In some cases, this is due to co-infections with other organisms, but is frequently due to simultaneous viral replication in multiple organs, including the GI tract(Minodier et al., 2017) (Rovida et al., 2013).

Most reports on the symptoms of COVID-19 are derived from clinical cases. From these, however, the number, range and severity of symptoms associated with COVID-19 can vary largely from person to person. Overall, our analysis of the symptoms from 48 independently published studies has shown that a small, but significant number of patients experience gastrointestinal problems. Incidence of GI complaints, vomiting and diarrhea is similar to SARS-CoV-1 and MERS-CoV(Rabaan et al., 2020)(Kanwar et al., 2017). Current evidence also suggests that rates of GI symptoms from SARS-CoV-2 are comparable in both children and adults in symptomatic cases. However, it should be noted that there is a greater proportion of asymptomatic carriage and mild infections in children in comparison to adults(Dong et al., 2020)(Y. Wang et al., 2020). Further, other studies suggest the incidence of diarrhea is greatest in severely ill patients, while abdominal pain and vomiting are not(Yang et al., 2020)(Tian et al., 2020). Our analysis suggests that, on average, the number of hospitalized cases experiencing diarrhea is 11% ± 2% while those exhibiting vomiting and nausea is 12% ± 3% (mean ± SEM, n = 48 independent studies). It is unknown from the reported data to what extent these symptoms co-occur. In a rare number of cases, diarrhea has been shown to be the only COVID-19 symptom, making these cases very difficult to diagnose(R.-L. Li et al., 2020)(Taxonera et al., 2020). Although there are reports of renal organ failure from SARS-CoV-2, there are very few reports of urinary dysfunction as a result of infection(Prabhu et al., 2020). It should be noted that the data presented in Fig. 1 does not account for SARS-CoV-2 infections that are either asymptomatic or very mild, and do not require hospitalization. Asymptomatic cases may account for ca. 40-45% of SARS-CoV-2 infections, with the potential to transmit the virus for extended periods, possibly longer than 14 d(Oran and Topol, 2020). It is therefore likely the incidence of these symptoms is greater than shown in Fig. 1. This underreporting is common for gastrointestinal infections(Fletcher et al., 2013; Gleizes et al., 2006). The variability in the data may also be associated with different reporting criteria for each condition used in the different studies(Kwan et al., 2005). Further, data may also be slightly

confounded due to the administration of anti-viral drugs, antibiotics and traditional and alternative medicines to patients that also induce diarrhea and vomiting(Tian et al., 2020). While self-reporting of SARS-CoV-2 infection and symptoms has been used in some countries to capture mild cases of COVID-19, these data have large uncertainties due to 'hypochondriacal suspicion' and the inclusion of symptoms from other diseases also circulating in the population(Gong et al., 2020). For this reason, this type of data was considered unreliable.

As evidenced from Figure 1, abdominal pain is a common symptom of COVID-19. The extent to which this is directly due to viral infection of the GI tract or from general anxiety, however, remains unknown. A range of studies have shown that the threat of contracting COVID-19 can induce a range of somatic symptoms (e.g. sleep dysfunction, GI pain, headaches)(S. Liu et al., 2020; Yuan et al., 2020)(Shevlin et al., 2020). Somatic symptoms of nausea, vomiting, abdominal pain and diarrhea are also known to be common in society. In some cases, the levels of these GI-related symptoms in society are consistent with reports for symptom frequency in COVID-19 cases(T. T. Haug et al., 2002)(T. Tangen. Haug et al., 2002).

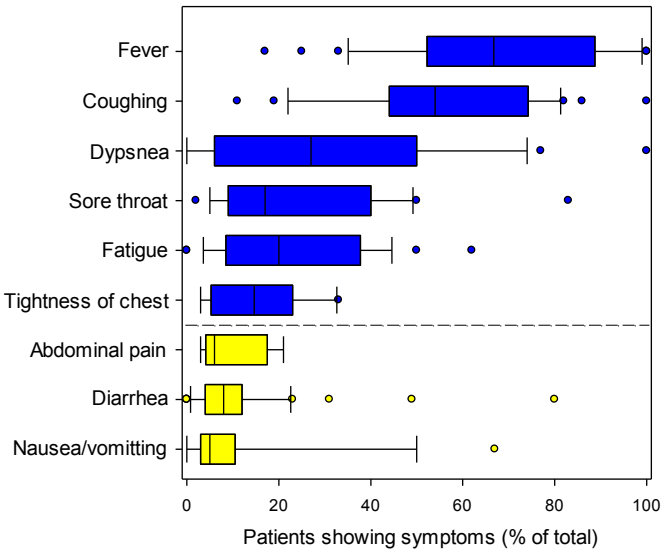

Figure 1. Summary of symptoms experienced in clinically reported SARS-CoV-2 infections. The data is the summary of 48 independent reports involving a total of 3706 patients. The yellow bars are those associated with gastrointestinal problems. In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a black line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Points above and below the whiskers indicate outliers outside the 10th and 90th percentiles. The average size of the cohort studies was 79 ± 21 (n = 48).

We conclude from our analysis that SARS-CoV-2 clearly causes gastrointestinal dysfunction in a small, but substantial proportion of COVID-19 cases (ca. 5-20%). However, the likelihood of prevalence could be much greater due to underreporting of mild infections. In addition, due to the prevalence of somatic symptoms, these symptoms should not be used as direct evidence for actual GI infection.

3. Fecal shedding patterns of SARS-CoV-2

Consistent with the symptoms presented in Fig. 1, SARS-CoV-2 RNA has been routinely detected in upper and lower respiratory tract fluids, sputum, saliva, stool, blood, and urine of infected persons(Yan et al., 2020)(Lu et al., 2020). The presence of the virus in feces appears to be similar in patients both with and without GI symptoms(Lin et al., 2020). Overall, however, SARS-CoV-2 is mostly detected in respiratory tract samples (typical range 70-100%), to a lesser extent in stool (typical range 30-60%), and rarely in urine (<5%)(Lo et al., 2020)(Y. Huang et al., 2020)(Kashi et al., 2020). In a few cases, even though it cannot be detected in the upper respiratory tract, the virus can be found in stools(W. Zhang et al., 2020)(Ling et al., 2020). However, in these cases the potential for false-negatives cannot be discounted(Piras et al., 2020). This range of symptoms has led to speculation that there are two different subtypes of COVID-19 manifestations referred to as "gut-tropism" and "lung-tropism", depending on where the virus enters the body (i.e. inhaled or ingested) and becomes established, and thus where symptoms develop(Lo et al., 2020). There is no evidence, however, to support this or that some strains of SARS-CoV-2 preferentially target the GI tract in comparison to the respiratory tract(Iwasaki and Grubaugh, 2020).

Shedding of the virus in feces and in respiratory droplets may occur ca. 3-5 days before other classic symptoms, such as fever or diarrhea manifest (i.e. pre-symptomatic)(Buscarini et al., 2020)(D. Wang et al., 2020)(He et al., 2020). Current evidence suggests that despite showing no symptoms, asymptomatic, pre-symptomatic or post-symptomatic people may still be shedding the virus at appreciable levels, although asymptomatic individuals may not shed it for as long or in as high amounts as in severely infected individuals that require hospitalization(Lu et al., 2020)(Su et al., 2020)(Shen et al., 2020)(Chau et al., 2020)(Byrne et al., 2020). Critically, however, it is not well established whether viral loads are similar between asymptomatic, and mild, moderate, or severe symptomatic cases, with conflicting reports present in the literature(Y. Wang et al., 2020)(Lu et al., 2020)(He et al., 2020)(Y. Liu et al., 2020)(Li et al., 2010)(Schwierzeck et al., 2020)(Zou et al., 2020). However, we note that if the viral loads are similar, the lack of coughing and diarrhea in asymptomatic cases should lower the risk of disease transmission.

The information available so far from COVID-19 cases suggests the temporal dynamics of viral shedding in feces follows a classic infection cycle pattern (i.e. rapid build-up phase followed by a slow decline)(Sethuraman et al., 2020) (Fig. 2). This is somewhat similar to that seen for SARS-CoV-1 where the rate of viral shedding in feces is low in the first five days of illness, but rises gradually to peak at days 9-14 with very high titres, often exceeding those of nasopharyngeal aspirates(Cheng et al., 2004). However, unlike SARS-CoV-1, it is known that shedding and transmission occurs with SARS-CoV-2 prior to symptom onset(Wei et al., 2020). In the case of SARS-CoV-2, initial reports provide good evidence of the rapid accumulation of viral loads in feces(W. Zhang et al., 2020) and that it can be detected in stools of fecal-positive patients for at least two weeks after the decline of symptoms(Y. Pan et al., 2020b). Since these early reports, the amount of fecal-positive cases in cohort-studies has been shown to be up to 75% of the total(Yan et al., 2020). Critically, however, it suggests that not all COVID-19 infections result in pronounced fecal shedding, consistent with the incidence of symptoms presented in Fig. 1. In addition, diarrhea is not always associated with viral shedding(Young et al., 2020). Taking all the available evidence on the temporal dynamics of viral shedding in feces suggests that shedding may occur for ca. five days prior to symptoms developing, ca. one week prior to hospitalization, and then for two weeks after symptoms have subsided(Lo et al., 2020)(Byrne et al., 2020)(Hosoda et al., 2020). Another diagnostic feature of COVID-19 cases is that SARS-CoV-2 can often be found in stool samples even after throat swabs appear negative in the post-symptomatic phase(T. Zhang et al., 2020)(Gupta et al., 2020)(Xu et al., 2020)(Jiang et al., 2020). For example, the median (IQR) time of detectable viral RNA was 18.5 (13-22) days for throat swabs, 22 (18-27) days for sputum, and 17 (11-32) days for stools (Fig. 2). In addition, viral loads in sputum and stool appear to decline slower than in throat swabs, with the longest shedding period recorded at 59 days(J. Huang et al.,

2020)(Xiao et al., 2020b)(Xu et al., 2020)(Y. Wu et al., 2020). This has led to the suggestion that detection of SARS-CoV-2 in stool samples should be used alongside testing of viral presence in sputum and saliva samples(Ahamed Mim et al., 2020)(J. Liu et al., 2020)(Ma et al., 2020). However, in the late stages of infection it is possible that SARS-CoV-2 in feces may not infectious and that RNA-based testing may result in unnecessary hospital bed-occupancy.

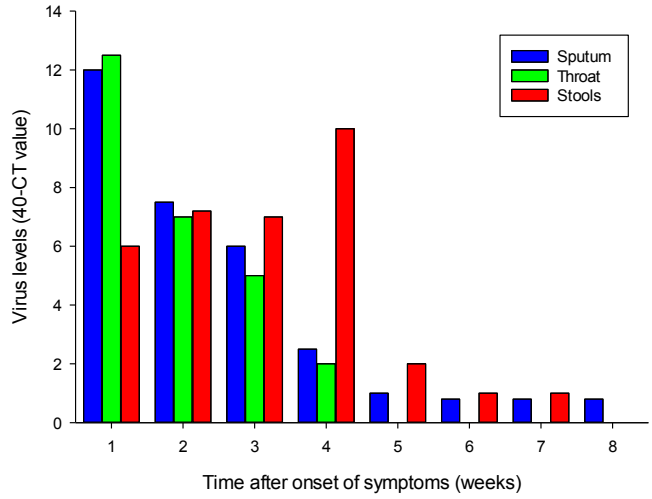

Fig. 2: Temporal dynamics of SARS-CoV-2 in the sputum, throat and stools. Data are from a cohort (n = 32) of COVID-19 patients in China. Adapted from [65].

The evidence presented above has also led to the supposition that the fecal-oral route may be an opportunity for transmission of SARS-CoV-2(Xu et al., 2020), as suggested previously also for SARS-CoV-1 and MERS-CoV(Yan et al., 2020). It is well established that stool samples contain an abundance of viruses in the human body and are an integral part of the transmission pathway for many pathogenic viruses (e.g. bocavirus, norovirus, rotavirus, astrovirus, sapovirus, adenovirus)(Rovida et al., 2013)(Drosten et al., 2013). Of the estimated 1.4 billion cases of diarrhea worldwide each year, viruses make up a considerable portion(Xie et al., 2013)(Kotloff et al., 2019). Although seasonal HCoVs only make up a small proportion of these cases in comparison to viruses such as norovirus (NoV), rotavirus (RoV), rhinovirus (RhV) and adenovirus (AdV), it does imply that SARS-CoV-2 is not unusual in inducing GI problems and this symptom may represent a part of its infection cycle (Fig. 3) (Rovida et al., 2013)(Drosten et al., 2013)(Kheyami et al., 2010)(Esper et al., 2010)(Risku et al., 2010).

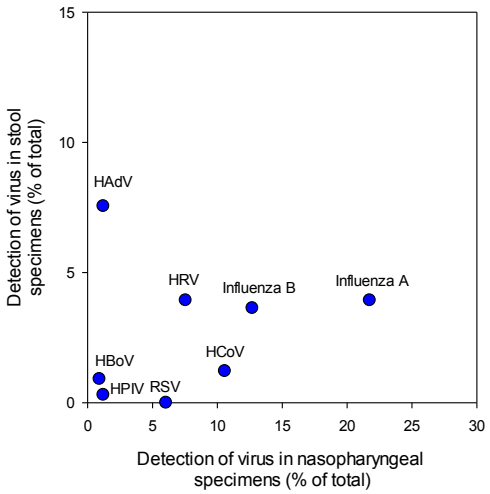

Fig. 3: Prevalence of human pathogenic viruses in nasopharyngeal and stool samples from individuals (n = 331). The points represent individual viruses including Human Coronavirus (HCoV), Influenza A, Influenza B, Human Rhinovirus (HRV), Respiratory syncytial virus (RSV), Human Adenovirus (HAdV), Human Bocavirus (HBoV) and Human Parainfluenzavirus (HPIV). Data calculated from(Minodier et al., 2017).

4. Multiplication of SARS-CoV-2 in the gut

If sputum is swallowed, viral particles enveloped in mucus may pass down the GI tract in a semi-protected state, and avoid degradation by gastric acid and bile/pancreatic juices(Hirose et al., 2017). This is likely to provide a primary route for infection of the GI tract, post-establishment of the virus in the upper respiratory tract. In addition, SARS-CoV-2 contained in sputum and saliva may also ultimately contribute to the viral load in feces, especially given the high viral load in these fluids and the large amounts (ca. 1.5 l person^{-1}) swallowed per day. Although SARS-CoV-2 has been detected in blood, the prevalence rates are extremely low (ca. 1% of infections exhibit viremia)(Lam et al., 2020), suggesting that this is not a primary route of infection of GI tract tissues and is a secondary manifestation of COVID-19. It is also possible that SARS-CoV-2 may reach the GI tract via contaminated food, however, there are no documented cases of food-borne transmission of SARS-CoV-2(Li et al., 2021). A rare exception to this would be the handling and consumption of products from animals which have contracted the virus. The widespread risk of this, however, is likely to be extremely low based on evidence from previous SARS-CoV-1 and MERS-CoV outbreaks(M. Wang et al., 2005)(Todd, 2017)(Rahman and Sarkar, 2019).

There is reasonable evidence to suggest that SARS-CoV-2 can replicate in the GI tract. Firstly, the GI tract contains an abundance of the metallopeptidase, angiotensin-converting enzyme 2 (ACE-2) which is the cell surface functional receptor (attachment site) for SARS-CoV-2(Bertram et al., 2012)(M. Y. Li et al., 2020). Secondly, it has been shown *in vitro* that HCoVs and SARS-CoV-2 can infect cells from the respiratory, gastrointestinal, hepatic and central nervous systems. Studies have indicated that SARS-CoV-2 has a 10–20 times greater affinity to ACE-2 receptors compared to SARS-CoV-1, with a potentially lower infectious dose(Galbadage et al., 2020). It has been shown that the ACE-2 receptor protein is highly expressed not only in lung cells but also in esophageal epithelial cells and absorptive enterocytes (epithelial cells) present in the stomach, duodenum, ileum, colon and rectum(Xiao et al., 2020b)(M. Y. Li et al., 2020)(H. Zhang et al., 2020)(A.-X. Guo et al., 2020)(Zang et al., 2020). Further, ACE-2 mRNA

transcripts have been reported to be more abundant in intestinal cells than in lung tissues(Du et al., 2020). The ACE-2 receptor is also present in renal tubes and the bladder, suggesting the potential for viral replication in the urinary system(Du et al., 2020)(M. Y. Li et al., 2020) and potentially explaining the subsequent recovery of SARS-CoV-2 in urine(Ling et al., 2020). Gastrointestinal tissue samples obtained from esophageal, esophageal non-lesion, gastric, duodenum and rectum mucosa have also tested positive for the presence of SARS-CoV-2 in clinical cases(Xiao et al., 2020b).

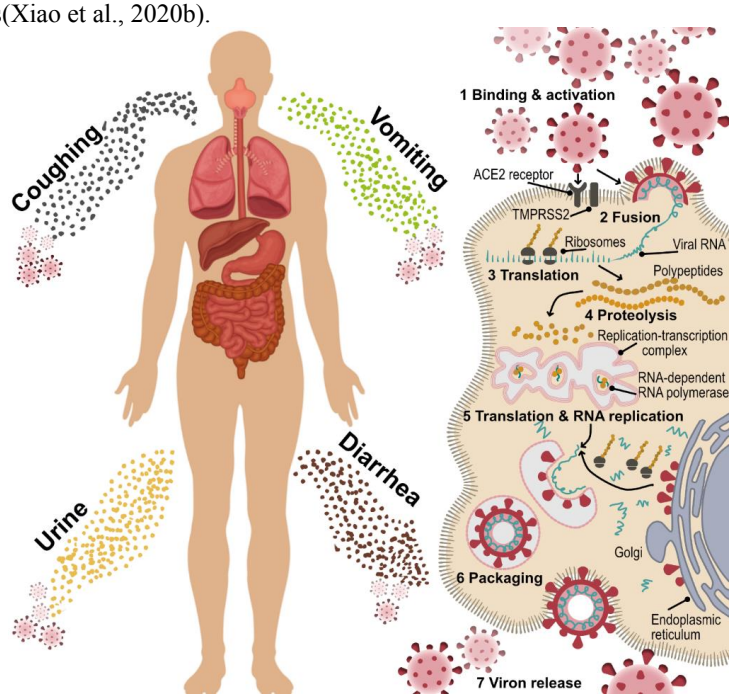

Fig. 4: Main routes by which SARS-CoV-2 leaves the body (left), and a summary of the mechanism of viral replication (right).

Once in the GI tract, the spike (S) protein, which is abundant in the viral lipid membrane, induces binding of the virus to the ACE-2 receptor on the host cell surface, the main point of cell entry(Tian et al., 2020). The S glycoprotein has 2 key functional domains, S1 and S2. S1 contains the receptor-binding domain, which directly binds to the peptidase domain of ACE-2, whereas S2 is responsible for binding to the cell membrane(Mönkemüller et al., 2020). These 2 domains need to become physically separated to induce cell binding (i.e. activated). This process is initially mediated by the host cell protein convertase, furin, which acts on the S1/S2 site to break open the S protein structure to allow simultaneous binding to the ACE-2 receptor (via S1) and cell membrane (via S2)(Bestle et al., 2020). This activation process is further facilitated by the host's type II transmembrane serine protease (TMPRSS2) which acts on the S2' domain to release the fusion peptide. Fusion and subsequent entry of the viral genetic material into the host cell then occurs (Fig. 4)(Hoffmann et al., 2020; Mönkemüller et al., 2020). Estimates suggest that this process takes from 10-15 min to complete(Ng et al., 2003). In addition to TMPRSS2, another mucosa-specific serine protease, TMPRSS4, also appears to enhance fusogenic activity and viral entry into the host cell(Zang et al., 2020). Once inside the cell, the uncoated viral RNA with 5′ cap structure and 3′ poly (A) tail, acts like mRNA, facilitating rapid translation of the replicase polyproteins(Pal et al., 2020). Once complete, viral replication proceeds, followed by RNA

packing and envelope packaging as described in detail elsewhere(Y. R. Guo et al., 2020)(Boopathi et al., 2020). The replicated virions are then released from the cell via exocytosis (i.e. continual budding rather than cell bursting) back into the GI tract to infect other cells(da Costa et al., 2020). This eclipse period (i.e. time taken from adsorption into the cell to the subsequent release of infectious progeny) is estimated to be 7-8 h(Harcourt et al., 2020; Schneider et al., 2012). Although not known for SARS-CoV-2, based on other viruses, each cell may be produce up to 10^2-10^3 virions(Hirano et al., 1976). Given the number of epithelial cells with ACE-2 receptors in the GI tract, even a mild infection may therefore lead to a rapid multiplication of SARS-CoV-2, with the potential to produce a high abundance of viral RNA in fecal matter. Once released, however, the survival of these virions may be extremely low. For example, it has been shown that vesicular stomatitis virus chimeras expressing SARS-CoV-2 spike protein are rapidly inactivated by human colonic fluids with viral titers decreasing 100-fold in 1 h(Zang et al., 2020), however experiments using wild type SARS-CoV-2 are required to validate this finding. That said, this may help to explain why the capacity to recover infectious virus from stool specimens of COVID-19 patients is highly variable. It is also possible that transit time through the GI tract (i.e. greater in diarrhea cases(Roy et al., 1991)) and pre-existing GI conditions (e.g. Crohn's disease, ulcerative colitis)(An et al., 2020) may influence viral recovery in feces. This potentially poor survival contrasts with other human enteric viruses that primarily spread via the fecal-oral route (e.g. norovirus, rotavirus) and which are capable of withstanding the harsh environment in the GI tract, including the low pH of gastric fluids, bile and digestive enzymes in the small intestine and exposure to multiple bacterial by-products(Zang et al., 2020)(Tung-Thompson et al., 2014) (Table 1).

Table 1. Comparison of the properties of SARS-CoV-2 with Norovirus, a virus with known fecal-oral transmission.

	SARS-CoV-2	**Norovirus**
Family	Coronaviridae	Caliciviridae
Type	+ssRNA	+ssRNA
Shape	Spherical	Icosahedral
Genome size (kbp)	29.9	7.5
Size (nm)	50-200	23-40
Coating	Enveloped	Non-enveloped
Human infections per year	>7 million (Nov. 2019-Jun. 2020)	685 million
Primary symptoms	Respiratory problems, fever, GI pain	Diarrhea, GI pain, vomiting
Prevalence of diarrhea (% of total cases)	11	88
Incubation period	5-7 d	1-3 d
Symptom duration	7-14 d	2-5 d
Death rate (% of total infections)[a]	1.40	0.003
Shedding rate in feces (gc/ml)	10^2-10^7	10^8-10^{10}
Shedding duration after symptoms have subsided (d)	14-28	14
Infectious dose (PFU)[b]	Unknown (estimate 10^2-10^3)	10^1-10^2
Vaccine available	No	No
Cases directly linked to fecal-oral transmission	None	Frequent
Links to consuming contaminated water	None	Infrequent
Links to consuming contaminated food	None	Frequent
Individuals most at risk of complications	Elderly	Elderly
Environmental durability	Low	High
Sensitivity to low pH	High	Low
Sensitivity to alcohol	High	Low
Sensitivity to chlorine	High	Medium-high

[a]Deaths after accounting for both confirmed cases and estimates of asymptomatic carriage.

[b]Infection mediated via the gastrointestinal tract. Only an estimate is available for SARS-CoV-2.

[c]Values from the main text and from published values(Li et al., 2021; Robilotti et al., 2015)(Hall et al., 2013)(Pfeiffer, 2010)(Kampf et al., 2020)(Siddharta et al., 2017).

In mild COVID-19 infections, no significant damage to the mucous epithelium of esophagus, stomach, duodenum and rectum cells has been reported(Xiao et al., 2020b). However, it is also clear that severe infection can result in prolonged diarrhea and inflammation of the GI tract in a significant proportion of clinical cases (Fig. 1). Although tissue and organ damage may be precipitated by the body's immune response to SARS-CoV-2 infection (leading to the 'cytokine storm', viral sepsis and organ failure)(di Mauro Gabriella et al., 2020; H. Li et al., 2020), it might also be caused by direct viral attack of absorptive enterocytes which can induce diarrhea by destroying the cells resulting in malabsorption, unbalanced intestinal secretion and activated enteric nervous system(Tian et al., 2020; H. Zhang et al., 2020). This is similar to that seen in porcine epidemic diarrhea (corona)virus (PEDV) infections where widespread histopathological damage to enterocytes occurs(Jung et al., 2014).

The role of the gut microbiome in the potential colonization of the GI tract remains unknown. Evidence from the upper respiratory tract, however, suggests that some commensal bacteria in the mucosal biofilm (e.g. *Proteobacteria*) express proteins which can bind to the viral S-protein. This may prevent viral interactions with cell surface ACE-2 receptors and which may help prevent severe infections from developing (i.e. bacterial decoys)(Honarmand Ebrahimi, 2020). Whether this occurs in the GI tract remains unknown, however, it should be noted that the overabundance of Proteobacteria in the GI tract is normally associated with dysbiosis(Shin et al., 2015). It does suggest that further investigations of the gut microbiome are needed to establish its role is viral infection and the development of symptoms. Ultimately, this may also lead to the development of therapies to reduce the severity of COVID-19(Kalantar-Zadeh et al., 2020).

5. Levels of SARS-CoV-2 in urine and feces

A range of PCR-based technologies (e.g. RT-qPCR, digital PCR) are available to quantify the amount of SARS-CoV-2 RNA present in tissue, fluid and stool samples with very high sensitivity (≤10 gc/sample). These assays typically target genes encoding the S, E and N structural proteins, the RdRp gene which encodes the RNA-dependent RNA polymerase or the replicase protein ORF1ab gene(van Kasteren et al., 2020). These quantitative assays, however, also have limitations that must be considered. For example, differences in sensitivity can occur depending on the PCR primer and probe sets used(Jung et al., 2020)(Pillonel et al., 2020). Poor sensitivity and PCR inhibitors in fecal material (e.g. bile salts, lipids) may also lead to underestimation of viral abundance, or the reporting of false negatives(Schrader et al., 2012). Loss of viral signal during sample pre-treatment (e.g. heat inactivation) may also occur(Y. Pan et al., 2020a)c. Further, the lack of extraction controls (i.e. surrogate CoVs to look at viral recovery from the sample) may lead to underestimates of viral abundance. The lack of standards has meant that only semi-quantitative results (i.e. Ct values) have been reported in most early studies, especially those examining the temporal dynamics of viral shedding. Lastly, these Ct values vary between platform, gene target assay, and template concentrations used, which causes issues of comparability between studies(Seong et al., 2016). It is also important to state that quantification of viral RNA by RT-qPCR or digital PCR does not necessarily equate to infectious viral particles(Atkinson and Petersen, 2020), as it is likely that a large proportion of viral particles are damaged during passage through the GI tract and are thus non-infectious(Pfeiffer, 2010)(Zhou et al., 2017)(Zang et al., 2020). Despite these limitations, there is strong evidence to suggest that

feces contain high viral RNA loads. For example, one study has shown that levels of SARS-CoV-2 RNA in stools can range from 5.5×10^2 to 1.2×10^5 copies/ml(Y. Pan et al., 2020b), while another has reported levels of 6×10^5 to 7×10^6 gc/ml in three patients(Zang et al., 2020) and two studies reporting fecal shedding of up maximum of 1.0×10^7 gc/ml(Han et al., 2020)(Wölfel et al., 2020). This wide variation in fecal viral RNA load (10^2-10^7 gc/ml) reflects differences in the severity of disease between patients and also the temporal dynamics of the disease(To et al., 2020b). It should be noted, however, that the abundance of SARS-CoV-2 RNA in feces are much lower than for other non-enveloped enteric viruses, such as norovirus (ssRNA virus; 10^8 to 10^{10}/g)(Lai et al., 2013; Lee et al., 2007), rotavirus (dsRNA virus; up to 10^9/g)(Bennett et al., 2019) and adenovirus (dsDNA virus; 10^6 to 10^{11}/g)(Srinivasan et al., 2015).

In comparison with feces, at the peak of infection, levels of SARS-CoV-2 in saliva have been shown to typically range from 10^3 to 10^8 gc/ml with averages of 3.3×10^6 gc/ml(To et al., 2020a), 5.7×10^5 gc/ml(To et al., 2020b), 8.4×10^6 gc/ml(Yoon et al., 2020) and 5.0×10^5 gc/ml(Han et al., 2020). Analysis of nasopharyngeal fluid has reported values ranging from 6.4×10^2 gc/ml to 1.3×10^{11} gc/ml (median of 8.0×10^4 in throat samples and 7.5×10^5 in sputum samples)(Han et al., 2020; Y. Pan et al., 2020b)(Yoon et al., 2020), while others have reported viral loads ranging from 10^6 to 10^8 gc/ml in pharyngeal mucosa and endotracheal aspirate(To et al., 2020b)(Fitzek et al., 2020). This implies that swallowing of sputum, saliva and nasopharyngeal fluids may contribute to the fecal SARS-CoV-2 RNA signal in some individuals. However, the fact that SARS-CoV-2 RNA cannot be found in feces from all infections (i.e. nasopharyngeal positive, fecal negative) suggests that its contribution might be small.

There are few reports of SARS-CoV-2 RNA in urine as this is not a common manifestation of COVID-19, even in severe infections(Lo et al., 2020)(D. Wang et al., 2020)(Wölfel et al., 2020); however, one study has reported levels of 3.2×10^2 gc/ml(Liang Peng et al., 2020) and in another a very short-lived peak of 6.1×10^5 gc/ml(Yoon et al., 2020). It should be noted that most of the reports of viral loads are for hospitalized patients with mild to severe COVID-19 symptoms and that this may not accurately reflect viral abundance in asymptomatic, pre-symptomatic or very mild cases where levels in feces are likely to be much lower. It is also expected that renal infections will not occur in these mild or asymptomatic cases, suggesting that urine is not a vehicle for disease transmission outside of clinical settings, or at all.

The between-person variability in viral load, even within severe cases, appears to be very large(To et al., 2020b). This likely reflects the wide variation in symptoms experienced by individuals and organs targeted by the virus (Fig. 1). Overall, evidence suggests that high levels of SARS-CoV-2 RNA in feces is consistent with a GI tract infection in some individuals. However, the possibility that GI tract symptoms in COVID-19 cases are caused by other organisms cannot be discounted. For example, antibiotics are often prescribed during treatment of severely ill patients, creating a niche for opportunistic GI bacterial pathogens, and has been directly linked to the incidence of diarrhea in some COVID-19 studies(Lin et al., 2020). An analysis of nasopharyngeal swabs also showed that 20% of the individuals (n = 116), who tested positive for SARS-CoV-2 also tested positive for other respiratory pathogens(Kim et al., 2020). The most common co-infections being rhinovirus/enterovirus (6.9%), respiratory syncytial virus (5.2%), and non-SARS-CoV-2 coronaviridae (4.3%). A similar study reported co-infection of the respiratory tract by SARS-CoV-2 and influenza A and B(Q. Ding et al., 2020). Similar work is therefore required to determine the level of co-infections in the GI tract, especially as this might impact on the severity of infection by SARS-CoV-2. The quantities of SARS-CoV-2 RNA in feces are also within the range reported for other respiratory viruses such as avian influenza H1N1 which has been detected in respiratory, stool, and urine samples at levels of 2.7×10^9, 7.2×10^6, and 7.24×10^4 copies/ml, respectively(To et al., 2010), and in the case of MERS-CoV where levels in urine ranged from 10^2-10^3 gc/ml, feces from 10^3-10^4 gc/ml and those in the respiratory tract from 10^6-10^7 gc/ml(Corman et al., 2015; Drosten et al., 2013) (Fig. 3). In contrast, the levels of SARS-CoV-

1 in feces, however, has been reported to be much higher than for SARS-CoV-2, ranging from 10^3 -10^9 gc/ml(Cheng et al., 2004; Hung et al., 2009). This latter result suggests that conclusions on fecal-oral transmission risk from SARS-CoV-1 should be extrapolated to SARS-CoV-2 with extreme caution.

6. Is SARS-CoV-2 in stool and urine infectious?

Of critical concern in evaluating the risk of a fecal/urine-oral or fecal/urine-ocular transmission pathway for SARS-CoV-2 is the degree of infectivity of fecal- and urine-derived virus particles. These studies require tissue culture with human (or other) cell lines where addition of SARS-CoV-2 leads to an increase in viral titer from 10^2 particles/ml in the culture medium to 10^6 particles/ml within 12 h(Matsuyama et al., 2020; Ogando et al., 2020). One of the first infectivity studies was undertaken from stool samples taken from a laboratory-confirmed COVID-19 severe pneumonia case, 15 days after the onset of symptoms. After viral isolation, VERO cell cultures were inoculated and virus multiplication was subsequently detected, suggesting that feces have the potential to transmit the disease(Y. Zhang et al., 2020). In a subsequent, more comprehensive study of COVID-19 cases, it was found that of the 153 stool specimens analyzed, 29% tested positive for SARS-CoV-2, from which infectious virus was recovered from 2 samples(W. Wang et al., 2020). Similar studies have also confirmed the recovery of infectious virus from stools in both VERO cells and human organoids(Xiao et al., 2020a; Zhou et al., 2020).

Other comprehensive studies have suggested that no infectious viral particles can be recovered from feces at the peak of infection, despite infectious virus being recovered from respiratory specimens(Wölfel et al., 2020). The recent isolation of infectious virus from urine raises the possibility for urine-based transmission(J. Sun et al., 2020), although given the low prevalence of this phenomenon, its significance outside of clinical settings is probably extremely low. Although these studies confirm that feces and urine may contain infectious viral particles, they also have various drawbacks. Firstly, it is evident that while viral recovery is possible from some samples, interestingly it is not from others, despite all the feces testing RT-qPCR or digital PCR positive for SARS-CoV-2 RNA. Similar observations have also been made for nasopharyngeal swabs from patients with lower viral load, suggesting viral nucleic acids might be detected for longer periods than the live virus in different sample types(NCIC-AMS, 2020). In addition, studies have only focused on feces with high viral loads (based on Ct values) and these may not be reflective of pre- or asymptomatic cases. The levels of SARS-CoV-2 RNA in the samples used in these infectivity assays are also not reported, preventing realistic quantitative risk assessments to be made for fecal/urine-oral transmission (and to account for the levels added in the source material itself). The lack of inclusion of positive controls is also problematic where no infectious virus is recovered from any samples; i.e. problems with local culturing protocols cannot be eliminated(Xin Wei Wang et al., 2005). Further, in plaque-based assays, co-contaminating (non-CoV) viruses may also lead to false-positive results, although metagenomics could be used to identify this. In such cases, it is essential that a quantitative increase in SARS-CoV-2 beyond the inoculum dose is confirmed by qPCR. It would also be advantageous to undertake dose response curves (i.e. serial dilution of fecal extracts) to allow determination of comparative levels of infectivity between samples with known viral titres(Matsuyama et al., 2020). Further, the virus is known to propagate poorly in some cell lines currently being used to assay the infectivity of SARS-CoV-2(Harcourt et al., 2020; Matsuyama et al., 2020; Ogando et al., 2020). Therefore, it is unclear whether negative infectivity results indicate a lack of infectious particles or just a poor choice of screening assay. Based on this we conclude that further work is needed to better evaluate the temporal dynamics of viral shedding and its infectious nature in feces and urine.

For disease transmission in the community it is important to know whether feces and urine contain infectious virus in the pre- and post-symptomatic phase. This is particularly pertinent given that clinical cases may still be shedding the virus after the relieving of symptoms and their

discharge back into the community. However, current evidence suggests that the infectious viral count will decline rapidly within a week of symptoms starting. Drawing on evidence from nasopharyngeal samples, which has shown a close correlation between viral abundance and infectivity, it is likely that viral shedding in feces in the post-symptomatic phase poses a much lower transmission risk(La Scola et al., 2020; Wölfel et al., 2020). In addition, even if infectious virus is detected in cell culture, it doesn't necessarily imply that it will cause infection in the upper respiratory tract of humans at the same dose, as physicochemical barriers (e.g. mucus, low pH) can further limit virus infectivity(Nis, 2020).

Overall, we conclude that while virus particles contained in respiratory droplets are known to be highly infectious, evidence suggests that feces and urine probably contain low levels to no infectious particles. In comparison to respiratory particles, they are also less likely to be spread during daily life, being confined largely to toilet and other enclosed environments. This may subsequently lead to contamination of hands, surfaces, food and water; however, in most cases the levels of contamination are likely to be low where good hygiene is practiced. Despite this, the possibility of infection by contamination of the oral cavity, respiratory mucosa and eyes cannot be entirely discounted. This risk of infection spread is most likely associated with those experiencing co-infections or frequent watery diarrhea(Peiris et al., 2003; Tsang et al., 2003). As shedding rates appear to be correlated with symptom severity and the peak of the infection cycle, this risk would be greatest firstly in intensive care units (i.e. nosocomial spread), followed by care facilities (e.g. elderly care homes) where residents with diarrhea need secondary assistance, and heavily used and poorly maintained public toilets. The potential for the virus to spread from domestic toilets is likely to be very low as these have restricted use, probably reflect persons with mild infections and those with the capacity to practice good personal hygiene unassisted. Subsequently, in developing regions, where access to safe and hygienic sanitation is limited, the risks associated with fecal transmission routes may be higher.

The survival of SARS-CoV-2 in feces after release from the body is poorly understood. However, this information is important to evaluate the potential for environmental transmission. The fecal-oral route has also been implicated in disease transmission during sexual contact, however, this risk is believed to be very low in comparison to disease transmission via respiratory droplets and the oral-oral route(F. Pan et al., 2020)(Cui et al., 2020)(D. Li et al., 2020). From the available evidence on SARS-CoV-1 it has been shown that the virus can survive for 3 h to 5 d depending on the watery nature of the diarrhea (positively related to water content), but numbers fall exponentially with time and survival rate is less than in nasopharyngeal or tracheal aspirate(Chan et al., 2004; Lai et al., 2005). More work is needed to understand the factors that influence the survival of fecal-derived SARS-CoV-2 on different matrices after release (e.g. bed sheets, towels, clothes, toilets).

7. Persistence of SARS-CoV-2 in sanitation facilities

One of the most likely points of disease transmission from feces and urine is via shared toilets (e.g. hospitals, workplaces). Based on the use of surrogate viruses and 10^6 viral particles per fecal event, work has shown that is unlikely that SARS-CoV-2 would reach high levels on contact surfaces via the aerosol route after flushing (e.g. $<10^3$ particles on either the seat, handle, floor, walls)(Sassi et al., 2018). In contrast, repeated use by people infected by SARS-CoV-2 might lead to a progressive accumulation of virus to higher levels, assuming infrequent cleaning. This is supported by studies in a dedicated SARS-CoV-2 outbreak center in Singapore where SARS-COV-2 RNA was recovered from the toilet bowl, sink and door handle(Ong et al., 2020). Another study also found elevated levels of the virus in a patient-dedicated mobile toilet in China(Y. Y. Liu et al., 2020), while others have detected contamination of toilet seats, exhaust grilles and taps in a COVID-19 dedicated hospital(Z. Ding et al., 2020)(Chia et al., 2020) and in households(Döhla

et al., 2020). The source of contamination could have been from urine and feces in the toilet, particularly in facilities used by patients with diarrhea(Chia et al., 2020). It is also likely that contamination on touch surfaces and walls was caused via respiratory droplets during coughing, or from transfer to surfaces from hands contaminated with nasopharyngeal fluids. Although each episode of diarrhea or vomit may spread low levels of virus, patients with GI symptoms often have several/frequent episodes of these symptoms, potentially increasing the virus load on those surfaces.

Vomiting also has the potential to spread the virus more widely than either defecation or urination events (i.e. vomiting onto floors, toilets and sinks) due to the greater potential for droplet formation and aerosolization(Kirby et al., 2016; Makison Booth and Frost, 2019). For example projectile vomit can contaminate an area of up to 8 m^2 (Makison Booth, 2014). Unfortunately, the levels of infectious SARS-CoV-2 in vomit remain unknown, but are likely to be low based on the low pH of vomit (mean pH of 3.8, range 2.5-5.0) and studies in other CoVs(Kirby et al., 2016)(Willumsen et al., 2004)(Cowen and Hitchner, 1975; Panon et al., 1988). Vomit is also likely to contain SARS-CoV-2 from nasopharyngeal fluids as well as from the GI tract. The potential for vomit-, fecal- and urine-derived SARS-CoV-2 to remain infectious on sanitation surfaces for long periods of time remains unclear and is probably highly dependent on the receiving surface (toilet bowl, walls, floor etc). Studies on other matrices, however, have shown that viable SARS-CoV-2 might persist for at least 3 h in aerosols after their formation, and for up to 2 or 4 days on plastic and stainless steel surfaces(van Doremalen et al., 2020)(Chin et al., 2020). In conclusion, there is evidence to suggest that viral contamination of toilet environments may occur, although levels of contamination are expected to be very low in most settings based on infectious viral loads in feces and urine. Although we cannot discount the potential for faecal-mucosal transmission when individuals touch their mouth, nose or eyes with contaminated hands, this would be largely preventable through handwashing and regular disinfection of sanitation facilities.

The discussion above mainly relates to countries with good levels of domestic sanitation; however, over 2.5 billion people worldwide lack access to improved water and sanitation (e.g. urban slums, rural locations, refugee camps)(Sommer et al., 2015). In these settings, infection control may be more challenging due to the lack of handwashing facilities and cultural issues (e.g. gender violence)(Poole et al., 2020; Sommer et al., 2015). Additionally, existing toilet and sanitation facilities tend to be less private, which leads to greater personal congregation near central facilities. Similar is true for community potable water sources, which often are only in a handful of locations, such as community water taps, for whole neighborhoods. To date, very little is known about the persistence and infectivity of SARS-CoV-2 in these contexts and further work is clearly needed in this area.

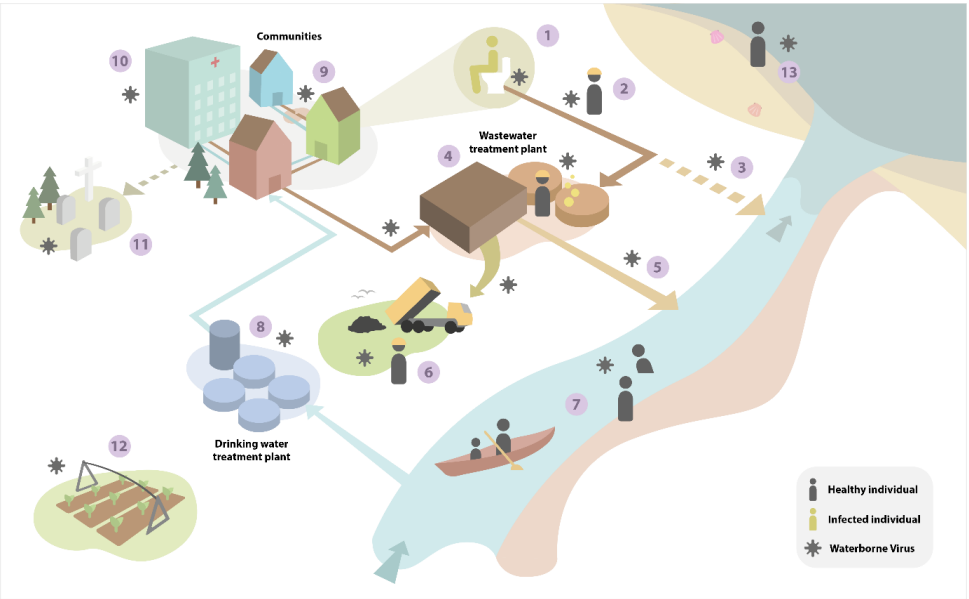

Fig. 5: Summary of the main infection pathways by which SARS-CoV-2 can theoretically contaminate the environment and cause secondary infections.
The numbers denote the major pathogen transport routes and exposure points: (1) contamination of toilets by infected individuals, aerosolization of feces/urine, faulty plumbing in buildings (2) pathogen transfer in the sewer network and potential exposure to sanitation workers in the sewer network, (3) discharge of untreated contaminated wastewater to rivers (sewer overflows), (4) release in bioaerosols from wastewater treatment plants and exposure of workers to potentially contaminated wastewater, (5) release of treated wastewater to rivers, (6) disposal of wastewater-derived biosolids to land, (7) transport in freshwater and exposure of individuals during recreational activities, (8) abstraction of river water for human consumption, (9) breaks in sewage pipes leading to groundwater contamination (10) hospital/medical centre release of wastewater, (11) contamination of groundwater from burial of infected bodies, (12) irrigation of crops with potentially contaminated water abstracted from rivers, (13) contamination of marine waters, dispersal in the coastal zone and potential contamination of fish/shellfish and people engaging in recreational activities.

8. Amount and persistence of SARS-CoV-2 in the sewer network
Once feces and urine enter the sewer network there are several points at which human exposure may occur (Fig. 5). However, significant dilution will occur in the drainage network due to inflow of water from other domestic and industrial sources. For example, at the peak of a severe infection, based on our analysis, an adult may be expected to lose ca. 1.0 l of fluid in diarrhea (during 3-6 events) and 0.8 l in urine per day(Aranda-Michel and Giannella, 1999)(L. Pan et al., 2020). Assuming a SARS-CoV-2 load of 8×10^6 gc/ml in feces and 3.2×10^2 gc/ml in urine and a flushing volume of 6.8 l per defecation/urination event (6 per d), this equates to a viral concentration in water leaving the toilet of 1.9×10^8 gc/l. In a single occupancy household setting, and assuming a total water use of 135 l/person/d, this will be further diluted, giving a maximum final effluent concentration of 5.9×10^7 gc/l and total viral excretion load of 8.0×10^9 gc/person/d. It is important to note that these calculations are based on genome copy numbers, which are significantly higher than infectious virus particle numbers, due to the production of defective viral genomes during

RNA virus replication(Vignuzzi and López, 2019). Studies of wastewater have yet to recover infectious virus, despite its genetic material being readily detected by PCR(Döhla et al., 2020).

The human minimal infectious dose of SARS-CoV-2 is not currently known. Estimates for SARS-CoV-1 range from 16 to 280 plaque forming units (PFU)(Watanabe et al., 2010). Unfortunately, the relationship between genome copies and PFU is also unknown for SARS-CoV-2, however, it is interesting to note that viable SARS-CoV-2 could not be isolated from clinical respiratory tract samples containing fewer than 10^6 gc/ml(Wölfel et al., 2020). For influenza virus, the ratio between TCID50 (TCID50 = PFU/0.7) and particle count is 1:100 to 1:1000(Yezli and Otter, 2011), whilst work with clinical influenza samples has demonstrated a 100-10,000 fold difference between TCID50 and genome copy number(Van Wesenbeeck et al., 2015). On this basis, it is likely that the human minimal infectious dose of aerosolized SARS-CoV-2 is in the order of 10^3-10^4 gc. The route of infection is also critical when considering the infectious dose. In influenza, the infectious dose of aerosolized virus appears to be several orders of magnitude lower than for virus that is deposited in droplets on the upper respiratory tract(Yezli and Otter, 2011). The infectious dose of SARS-CoV-2 if transmitted via the feco-oral route is therefore likely significantly higher than 10^3-10^4 gc. On this basis, exposure to raw sewerage from an infected household, elderly care home, or medical center could theoretically pose a small infection risk to sanitation workers, assuming the virus is still infectious. Parallels from SARS-CoV-1 investigations can also be drawn here. In the classic Amoy Gardens case study, raw sewage from one household entered vertically connected neighboring households, resulting in a localized infection hotspot(McKinney et al., 2006; Yu et al., 2014)(Stein, 2011). It should be noted, however, that this sanitation network was poorly maintained and would not represent those in most municipal buildings and should not be used to infer the risk of fecal-oral transmission of SARS-CoV-2. Furthermore, transmission in the Amoy Gardens case study was believed to be via the aerosolization and inhalation of infectious fecal matter, rather than via the feco-oral route.

Beyond the immediate point of entry into the sewer system point, the wastewater will be further diluted in the drainage network by the addition of sewerage from non-infected households. At the peak of infection in the UK in April 2020, it was estimated that 0.25% of the population was infected(Office for National Statistics, 2020). This would equate to an average community sewerage load of 1.75×10^5 gc/l reaching a centralized wastewater treatment plant. This is consistent with typical concentrations being reported in wastewater in many regions of the world ranging from 10^2 to 10^6 gc/l(F. Wu et al., 2020)(Ahmed et al., 2020; S Wurtzer et al., 2020; Sebastien Wurtzer et al., 2020)(Randazzo et al., 2020). At present, there are many uncertainties in the survival of SARS-CoV-2 during its passage through the sewer pipe network. CoVs are not thought to survive well in aqueous environments, especially in comparison with other viruses which can persist for months (e.g. poliovirus, norovirus)(Seitz et al., 2011). This is supported by studies in which SARS-CoV-2 RNA can be readily detected by qPCR in wastewater leaving hospitals, but which has yet to be found to contain infectious virus(Xin Wei Wang et al., 2005)(D. Zhang et al., 2020)(J. Wang et al., 2020). In fact a recent study suggests that levels of infectious virus were not significant in wastewater and receiving rivers, indicating the effectiveness of wastewater treatment, combined with the natural loss of viral integrity(Rimoldi et al., 2020). Additionally, viral particles are likely to become bound to biofilms in the pipes, degraded by other microorganisms and inactivated by xenobiotics (e.g. surfactants, disinfectants), all of which will lead to a progressive loss of qPCR RNA signal and degrade infectious virus (if any is present at all)(Cheng et al., 2004; Wigginton et al., 2015). However, when SARS-CoV-1 was inoculated into sewage at high titers (10^5-10^6 gc/l) it was found to still contain infectious material after 14 d at 4°C and 2 days at 20°C(X. W. Wang et al., 2005). These conflicting laboratory and field-based studies may reflect the different nature of the starting inoculum and failure of the lab conditions to reflect those in the field. This, however, may suggest that, if any live virus is present in the wastewater, some could survive during passage through the sewage network, based on typical

transit times from households to the wastewater treatment plant (1 to 24 h). But, current evidence suggests that the levels of SARS-CoV-2 are greatly lowered during wastewater treatment, suggesting that the virus is either degraded or becomes associated with the solids fraction during floculation(J. Wang et al., 2020). This is consistent with studies showing a 2 to 3 $\log_{10}$ removal efficiency in viral RNA abundance when comparing viral levels in influent and effluent(S Wurtzer et al., 2020) and the accumulation of SARS-CoV-2 in the sludge fraction(Peccia et al., 2020)(Alpaslan Kocamemi et al., 2020). If the sludge (biosolids) fraction is treated (e.g. pasteurized, heat-dried, alkali-lime treated), as per the legislative requirement in many countries, this should pose no further risk to human health. One potential area where a heightened risk of exposure may occur is during the release of bioaerosols from wastewater aeration ranks. However, based on current estimates of the infectious dose of SARS-CoV-2, the likelihood that this poses a risk to workers or local residents is extremely low based on the amount of sewage that would need to be inhaled by this route to cause infection. In addition, there is no evidence to suggest that wastewater plant operatives are at any greater risk to SARS-CoV-2 exposure via this route than that of the general population, particularly when standard issue personal protective equipment is worn(WHO, 2020).

9. Amount and persistence of SARS-CoV-2 in the wider environment

Given the reduced evidence on infectious virus in sewers at present and the possible degradation & treatment processes explained above, detection in the wider environment most likely reflects viral RNA, not infectious virus. Based on the available evidence and our own measurements, the quantity of SARS-CoV-2 RNA in the effluent from wastewater treatment plants at the peak of a community infection (< 0.5% of the total population) is unlikely to exceed 10^4 gc/l(S Wurtzer et al., 2020). Assuming that levels of viral infection decline in the community due to the implementation of successful control measures (e.g. lock-down and social distancing) then levels in wastewater are expected to fall below $<10^2$ gc/l. Based on the large dilutions of treated wastewater after discharge into adjacent freshwaters (ca. 5-100 fold dilution under low river flow conditions when the risk is greatest) or the coastal zone (ca. 10^5 fold dilution), it is highly likely that SARS-CoV-2 will pose very little threat to human health (e.g. during watersports, bathing, angling, consumption of shellfish etc)(Keller et al., 2014). This is supported by measurements of typical levels of water ingestion during recreational activities of 3-30 ml/person in rivers and lakes(Dorevitch et al., 2011), 34 ml/person during surfing(Stone et al., 2008), and 10-50 ml/person during swimming and bathing(Dufour et al., 2017)(Schets et al., 2011). Assuming a worst case human feco-oral infectious dose of 10^3 gc/person, this would necessitate that levels of infectious SARS-CoV-2 greater than 3.3×10^4 gc/l would be needed to cause concern. It should also be noted that while the eyes are often in contact with water during recreational activities, this route of SARS-CoV-2 entry into the body is thought to be minimal, particularly in comparison to ingestion of water and oral/nasopharanyx mucosal exposure(C. Bin Sun et al., 2020)(Deng et al., 2020).

In comparison to wastewater entering waterbodies, a greater source of potential risk to infection could be the presence of an infected individual within the water itself. It is likely that during swimming, a person may release ca. 30-60 ml of saliva into the water(Bretz and Carrilho, 2013). Given the highest recorded levels of virus in saliva (10^8 gc/ml), a swimming volume of 375,000 l ($25 \times 10 \times 1.5$ m), then the levels of SARS-CoV-2 in the water would be 1.2×10^4 gc/l. Assuming the inadvertent ingestion of 20 ml/person, this would result in a SARS-CoV-2 exposure dose of 2.4×10^2 gc/person. This risk would be most relevant in non-chlorinated waters as standard disinfection procedures (e.g. chlorination and UV treatment in swimming pools) should rapidly reduce levels of infectious virus in the water(WHO, 2020). It should be noted that natural UV irradiation is also likely to eliminate the virus in the water, however, the effect of this on SARS-CoV-2 in aqueous media remains unknown(Lytle and Sagripanti, 2005). Work on aerosolized SARS-CoV-2, however, that it will inactivated relatively quickly (within hours) by solar UV

irradiation(Sagripanti and Lytle, 2020). Further work is required to model the dispersal of SARS-CoV-2 in a range of aqueous environments (e.g. lidos, swimming pools, rivers, estuaries, coastal waters). Fundamental to this is a better knowledge of the persistence and infectivity of SARS-CoV-2 in these environments, the potential for zoonotic infection (secondary hosts for SARS-CoV-2), and establishing the infectious dose of the virus. Using these data, and currently known information on SARS-CoV-2, quantitative microbial risk assessments could be undertaken to inform on human health risks in different environmental exposure scenarios based on dose-response models (Beaudequin et al., 2015).

Unlike other viruses (e.g. norovirus), there is no evidence to suggest that SARS-CoV-2 can accumulate in marine and freshwater organisms destined for human consumption (e.g. fish, oysters, mussels). The low likelihood of SARS-CoV-2 accumulation in fish is supported by the low levels of ACE-2 receptors in these organisms(Damas et al., 2020). In the case of shellfish, it is known that norovirus readily accumulates in shellfish as it binds to a human-like intestinal type A histo-blood group antigen in the shellfish tissue(Tian et al., 2007). Evidence also suggest that oysters possess an ACE-2-like receptor (CgACE) suggesting that bioaccumulation may be possible, however, whether SARS-CoV-2 can bind to CgACE, and whether the receptor is present in sufficient amounts to induce bioaccumulation remains unknown(Riviere et al., 2011).

10. Conclusions and implications for public health

Our critical analysis of the available evidence and potential transmission routes suggests that the possibility of fecal/urine-oral/ocular transmission of SARS-CoV-2 is extremely low to negligible except where direct person-to-person contact occurs. This is consistent with the many millions of documented cases of COVID-19 documented worldwide, and the fact that none of these have implicated feces or fecal contaminated material as part of the infection pathway. Feces have been implicated in contamination of the healthcare environment/surfaces, however, the role of those in infection remains unclear. It should be noted that our conclusions are based on western-style sanitation networks and wastewater treatment. The risks may be higher in less economically developed countries and areas with poor sanitation; however, there is insufficient evidence to enable this to be critically evaluated. This is clearly an area that warrants further research. Assuming levels of SARS-CoV-2 remain relatively low in the population (<1%), our analysis also suggests that the risk of contracting COVID-19 from water supplies, wastewater, food, bathing/recreational waters, and the coastal zone remains extremely low. This is particularly the case if personal hygiene measures are maintained (e.g. handwashing) and communal sanitary facilities are regularly cleaned and disinfected(Lotfinejad et al., 2020)(Brauer et al., 2020). Following a precautionary principle, we would also recommend that households with an on-going infection, and particularly those exhibiting diarrhea, add sodium-hypochlorite or similar disinfectant prior to flushing to reduce further downstream risk of infection.

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Author contributions

DLJ conceived the project and led the writing. All other authors contributed to drafts of the article.

Declaration of interests

All authors declare no competing interests.

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