## MASTERS BY RESEARCH

# Use of passive samplers for the capture of SARS-CoV-2 and other viruses from wastewater

Lambert-Slosarska, Katy

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A thesis submitted to fulfill the requirements of a Master of Science by Research (MScRes)

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# Abstract:

Wastewater based epidemiology (WBE) is an area of science that has gained a lot of traction in recent years and been at the forefront of monitoring the pandemic. This work focuses specifically on the Severe Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic and how we can used more unexplored methods in WBE, to detect and quantify viral loads, specifically passive sampling. This thesis is comprised of a literature review, giving an overview into SARS-CoV-2, the current applications of wastewater-based epidemiology and the knowledge gaps within this field. Passive sampling is the use of different materials which adsorb viruses and water for their concentration and subsequent elution. There are two data chapters both focusing on the use of passive sampling for the detection of SARS-CoV-2. The first chapter compared two extraction methods (manual and automated systems), three different sampling materials (cotton-based swab, ion exchange paper and tampon) and seven different concentration processes (direct extraction, squeeze, phosphate buffered solution (PBS) elution into polyethylene glycol (PEG), PBS elution into ammonium sulfate, beef extract elution into PEG, beef extract into ammonium sulfate, no-elution into PEG). It also explored the relationship between passive sampling and grab sampling. A manual extraction showed higher RNA recoveries and fewer results below the detection limit. We found that cotton tampons recovered the highest amounts of RNA in comparison to an electronegative membrane and cotton swabs. The highest viral load and human faecal indicator bacteriophage (crAssphage) were recovered with the no-elution PEG precipitation method. We found that passive sampling has a lower variability over time compared to wastewater grab samples. These results demonstrated that passive sampling is a low-cost, effective alternative to traditional liquid-based WBE to monitor low level viral loads in

wastewater. The second data chapter investigated the use of passive sampling further for the detection of SARS-CoV-2 and other enteric viruses from wastewater (Influenza A and B (Flu-A & B), SARS-CoV-2, human adenovirus (AdV), norovirus GII (NoVGII), measles virus (MeV), pepper mild mottle virus (PMMoV), the faecal marker crAssphage and the process control virus *Pseudomonas* virus (phi6). This chapter compared the four highest viral recovery methods from the previous chapter; we found that the preferred viral recovery method from the tampon passive samplers was the no-elution/PEG precipitation method. We also compared three different tampon types and found that there were no significant differences between type and viral recovery. Furthermore, we demonstrated that non-enveloped viruses had higher percent recoveries from the passive samplers than enveloped viruses. This was the first study of its kind to assess passive sampler and viral recovery methods amongst a plethora of viruses commonly found in wastewater or used as a viral surrogate in wastewater studies. Overall, this thesis evidenced that tampons are the most suitable passive sampler material for the recovery of SARS-CoV-2, with different methods being preferential when detecting different viruses. Yet for all viral types, PEG based methods consistently recover the highest viral quantities. There are options to extend passive sampling further for the quantification of different pathogens such as bacteria, as well as options to extend these methods to other waterway systems.

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# 1. LITERATURE REVIEW

## 1.1 WHAT IS A VIRUS?

Viruses are biological entities that infect cells and replicate themselves, phages are viruses which infect bacteria (Breitbart and Rohwer, 2005). Viruses can exist outside of cells where RNA or DNA genomes are surrounded by a protein casing known as a capsid; these free particles are known as virions (Breitbart and Rohwer, 2005). There are an estimated $10^{31}$ to $10^{32}$ virus particles in the biosphere, which exceeds hosts by at least one order of magnitude (Bamford et al., 2005; Bergh et al., 1989). This means that almost all organisms are infected by viruses consistently, so much so that it is hypothesised that this could be one of the greatest selection pressures on cellular organisms, thus driving evolution (Bamford et al., 2005).

## 1.2 COVID-19 PANDEMIC

Coronaviruses are a group of highly diverse, positive-sense, single-stranded, enveloped RNA viruses, that infect mammals, including humans, and avian organisms (V'kovski et al., 2021). These viruses mainly result in respiratory and enteric infections (Corman et al., 2018). The most recent outbreak of coronavirus in humans was first detected in Wuhan, China in December 2019 (Wang et al., 2020), now known as the Severe Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), and which spread rapidly worldwide. This zoonotic disease seemed to originate from the natural host in bats and is the third documented case of animal coronavirus infecting humans

within the past two decades resulting in a major epidemic (Guo et al., 2020; “The species Severe acute respiratory syndrome-related coronavirus,” 2020).

Structurally, SARS-CoV-2 has four main proteins, 16 non-structural proteins (nsp) and eight accessory proteins (Jiang et al., 2020). The four main proteins, shared amongst the coronavirus family, are the spike glycoprotein (S), small envelope glycoprotein (E), membrane glycoprotein (M) and the nucleocapsid protein (N) (Astuti and Ysrafil, 2020; Forchette et al., 2021; Kaur et al., 2021). The S glycoprotein is a transmembrane protein, that forms homotrimers which protrude out of the viral surface. Each protein is comprised of two subunits (S1 and S2). There are three of these units in every S protein forming a trimer, and a single transmembrane (TM) anchor (Wrapp et al., 2020; Yao et al., 2020). The main entry receptor of the virus is the angiotensin converting enzyme 2 (ACE2) on host cells, where SARS-CoV-2 binds via its S proteins (Walls et al., 2020). ACE2 are located in the respiratory tract cells, and highly expressed in the lower tract. The S1 subunit holds the receptor-binding domains (RBD) and is responsible for the binding to ACE2, while S2 promotes membrane fusion (Lan et al., 2020).

The N protein binds to the nucleic acid material of the virus, forming a long, flexible, helical nucleocapsid (Chang et al., 2006; Troyano-Hernáez et al., 2021). This multifunctional protein is required for the optimisation of SARS-CoV-2 replication, enhancing the efficiency of viral transcription and assembly, where it interacts with the M protein (Arndt et al., 2010; Neuman et al., 2011). The N protein is also responsible for triggering the host cells response to a viral infection and plays an important role in viral pathogenesis. This protein assists the activation of Cyclooxygnase-2 (COX-2) which leads to lung inflammation (Yan et al., 2006). It also plays a role in the restriction of bodily immune responses via the inhibition of type I Interferon (Lu et al.,

2011). The M protein has three transmembrane domains and is the most abundant structural protein, playing a huge role in the virions assembly and encasing the virus (Arndt et al., 2010; Satarker and Nampoothiri, 2020).

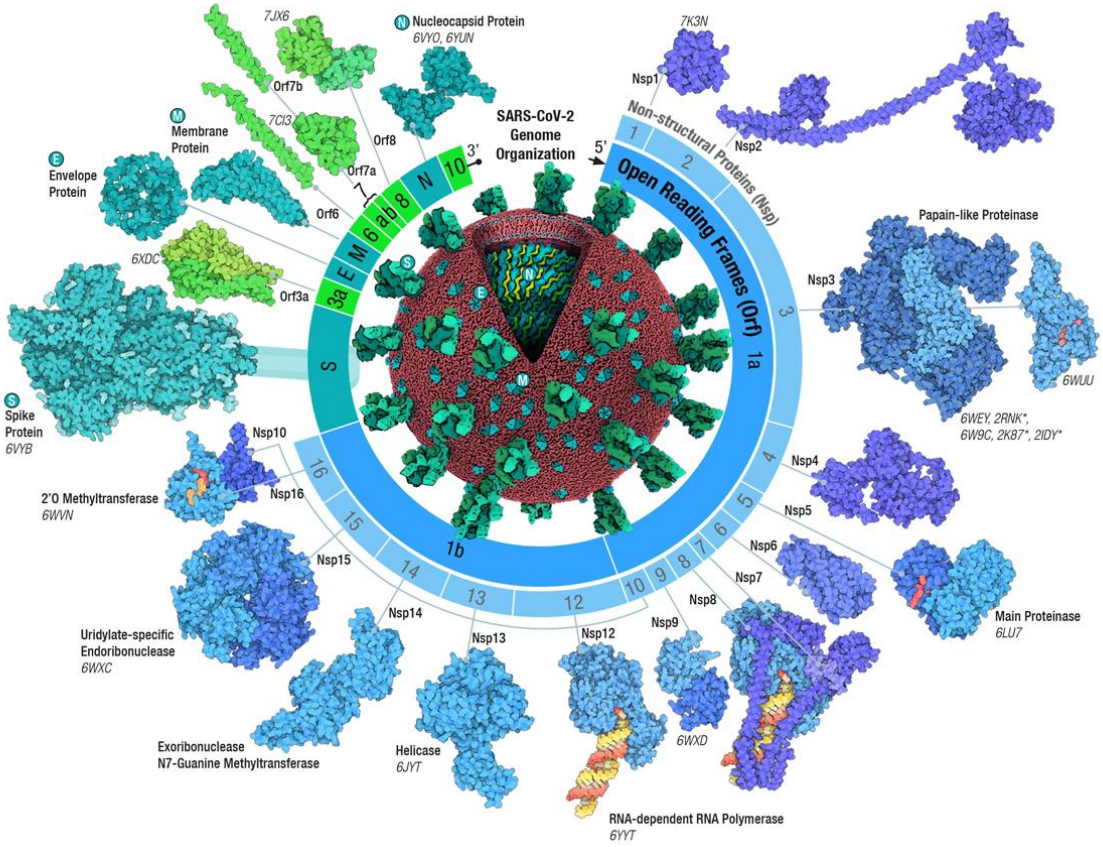


**Figure 1: SARS-CoV-2 genome and proteome, including virion structural proteins (teal), Nsps derived from polyproteins, pp1a, and pp1ab (shades of blue), open frame reading proteins (green). Taken from PDB-101, adapted from Lubin et al. (2022)**.

The Coronavirus Disease 19 (COVID-19) pandemic was declared by the World Health Organisation (WHO) in March 2020. Since the first detection of the virus it has subsequently spread to over 210 countries and territories worldwide (Ali and Alharbi, 2020; Li et al., 2020). Transmission of the virus is thought to occur before or near to symptom onset, this is due to the

short serial interval (3 – 5 days) compared to the incubation time of ~ 5 days (Cheng et al., 2020; Park et al., 2020; Quesada et al., 2021; Shah et al., 2022). This creates a high virulence of human-to-human infection rates, as the virus spreads faster than people present symptoms. COVID-19 is a wide-ranging infection on a clinical spectrum from asymptomatic infection to severe flu-like symptoms. Typically, most patients suffer from fever, dry cough and shortness of breath, with some severely symptomatic patients requiring hospitalisation that can ultimately result in death (Ali and Alharbi, 2020; Zhou et al., 2020). WHO reports (https:// covid19.who.int/) that as of the 23rd of August 2022 there have been 593,269,262 confirmed cases and 6,446,547deaths across the world.

There are certain risk factors associated with the severity and death rate of COVID-19 patients; including older aged patients (≥65) and those with at least one of several underlying health conditions, including respiratory and cardiovascular disease and cancer (CDC COVID-19 Response Team et al., 2020; Jordan et al., 2020; Mahase, 2020a). COVID-19 patients can suffer with 'Long-COVID', a term used to describe the long-term effects of the disease, lasting for more than 12 weeks post-infection (Mahase, 2020b; Sykes et al., 2021). The symptoms of which are wide-ranging causing many different ailments such as cardiopulmonary, glucometabolic and neuropsychiatric complications (Dasgupta et al., 2020; Sykes et al., 2021).

Since the emergence of SARS-CoV-2 in December 2020 there have been multiple different mutations within the genome creating different variants of the virus. These have caused controversy surrounding vaccine efficacy and effectivity (Forchette et al., 2021). Variants have been shown to produce different symptoms from the wild type. They have also been shown to

have effects on the virulence of the virus due to the way the mutations affect the overall structure of the virus. For instance, the Delta variant has 23 mutations, 12 of which are in the Spike protein, resulting in the S protein having a different shape (Shiehzadegan et al., 2021). This conformational change means that the immune system cannot identify the virus as easily, meaning that less antibodies can bind to the virions. This makes new variants more infectious and the most transmissible, hence why in the UK new variants such as Omicron BE and BF lineages are now responsible for the majority for the COVID-19 cases being reported.

In November 2021 a new variant of concern has emerged, Omicron. This variant has multiple descendant lineages all of which are classed as variant of concern lineages under monitoring (VOC-LUM) (Table 1). These are classified as variants that according to phylogenetic analysis belong to a VOC, show transmission advantages and have additional amino acids responsible for the changes in epidemiology in comparison to other circulating variants. Since Omicron was first detected in November 2021 there have been six VOC-LUMs classified by WHO. In October 2021, Delta comprised almost 90% of viral sequences on GISAID (https://gisaid.org/), however, by February 2022 Omicron was the dominantly sequenced variant representing over 98% of infections ("Tracking SARS-CoV-2 variants," 2022). The evolution of the SARS-CoV-2 and thus, changes to the virus' shape, properties, and infectious levels, will continue to change throughout time. This makes the SARS-CoV-2 an ever-changing virus resulting in differences with human infection that will continue to need monitoring for a long time. This has created the need for the world to continue to monitor not only the virus itself but also the infected individuals in order to understand the evolution of the virus and the design of vaccines.

**Table 1: List of all current and previous SARS-COV-2 (June 2022) Variants of Concern/Interest and those that have been previously according to WHO.**

| WHO name | Strain name | Country of first detection | First detection time | Variant of Concern or Variant of Interest and dates |
|---|---|---|---|---|
| Alpha | B.1.1.7 | UK | Sep-2020 | VOC |
| Beta | B.1.351 | South Africa | May-2020 | VOC |
| Gamma | P.1 | Brazil | Nov-2020 | VOC |
| Delta | B.617.2 | India | Oct-2020 | VOC |
| Kappa | B.617.1 | | | VOC |
| Lambda | C.37 | Peru | Dec-2020 | VOI |
| Mu | B.1.621 | Columbia | Jan-2021 | VOI |
| Kappa | B.1.617.1 | India | Oct-2020 | VUM |
| Iota | B.1.526 | USA | Nov-2020 | VUM |
| Eta | B.1.525 | Multiple countries | Dec-2020 | VUM |
| Epsilon | B.1.227/B.1.429 | USA | Mar-2020 | VUM |

*VUM only the variants under monitoring are listed that have previously been those of interest or concern.

## 1.3 MONITORING METHODS OF THE COVID-19 PANDEMIC

Monitoring the COVID-19 pandemic has been an interdisciplinary effort, relying heavily on clinical testing in healthcare facilities as well as home testing. Mass monitoring of the pandemic has also depended on the efforts of wastewater-based epidemiology (WBE). Diagnostic testing revolves around four main techniques: 1. RT-PCR (reverse transcriptase polymer chain reaction), 2. LAMP (loop-mediated isothermal amplification), 3. Lateral flow testing, and 4. ELISA enzyme-linked immunosorbent assay (Green et al., 2020).

The standard method used throughout the pandemic has been RT-(q)PCR. This method has been used for decades to detect genetic information and has proved sensitive, reliable and relatively quick to perform. However, it does require complex equipment and highly trained operators, which can delay efficiency and results as well as making it unfeasible for mass adoption in low middle income countries (Green et al., 2020; Thompson and Lei, 2020). PCR testing is the standard not only in clinical testing but also in WBE and is also the only detection method used to quantify virus levels within this study. LAMP assays are similar to conventional PCR testing, however, the process occurs at one temperature and is therefore quicker and easier to use (Thompson and Lei, 2020). The negatives of this technique is that, due to the novelty, it has not been as well validated as PCR, with some research suggesting that its diagnostic accuracy for the detection of SARS-CoV-2 is still unproven (Inaba et al., 2021).

Lateral flow tests are also referred to as antibody tests, due to their detection of antibodies in blood. Lateral flow tests are different to lateral flow devices that test for viral antigens within saliva, blood or mucus. This technology is more novel and mainly works on

patients with high viral loads, this is the least accurate testing type (Peto et al., 2021). However, it is the cheapest method and has been used for at home testing throughout the pandemic.

## 1.4 WASTEWATER-BASED EPIDEMIOLOGY (WBE)

### 1.4.1 Overview

Wastewater-based epidemiology (WBE) is relatively new field compared to wastewater analysis with new advancements being continuously made, especially during recent years (Choi et al., 2018). Wastewater provides a complex matrix of different biological and chemical markers relating to human activity (Choi et al., 2018; Warwick et al., 2013). WBE can be defined as the normalisation of analyte influent concentrations per capita, using the daily flow and catchment population of specified wastewater treatment plants (WwTP), providing population-scale data on human activity within these catchment areas (Choi et al., 2018). The relative concentrations of these 'waste' markers can be quantified to inform on the activities and health of the inhabitants of the given WwTP catchment. WBE provides a cost-effective, accessible, non-invasive alternative to clinical community-level assessments for mass monitoring via the qualifying and quantifying of various 'waste' markers (Choi et al., 2018; Shah et al., 2022). These markers can be chemical; illicit and licit drugs, pharmaceuticals, chemical exposure or biological; diet markers, pathogens, and overall human health (Bowes and Halden, 2019; Escolà Casas et al., 2021; Feng et al., 2018; Gracia-Lor et al., 2018; Zhang et al., 2019).

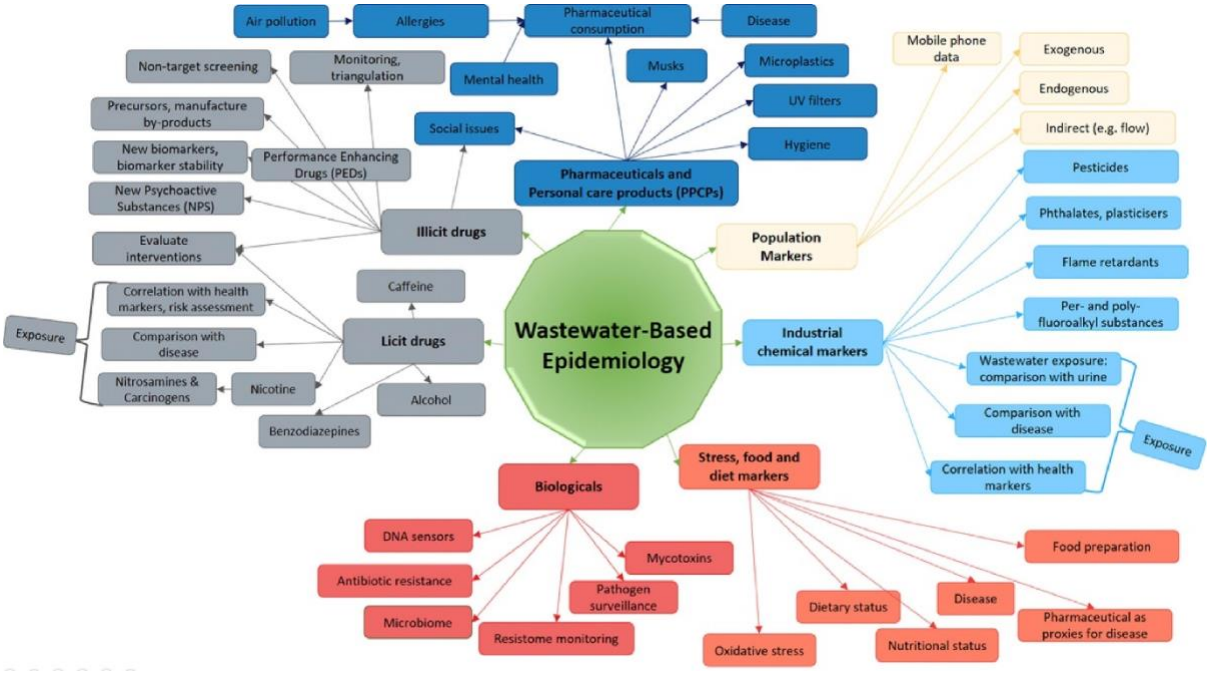


**Figure 2: Mind map of the current and future uses of wastewater-based epidemiology. Each colour represents a different area of use. Taken from Choi et al. (2018).**

Wastewater surveillance is made up of the WBE approach combined with current clinical testing to fully quantify any findings. Wastewater surveillance has previously been used to provide an early warning sign for viruses such as Hepatitis A and Norovirus outbreaks in Sweden in 2013 (Hellmér et al., 2014), while in Israel, 30-40% of the population was monitored by WBE detecting the early stages of an outbreak of paralytic polio, which in response the country implemented a mass vaccination program (Anis et al., 2013; Manor et al., 2014; Schmidt, 2020). The technique used in Israel was paramount in terms of eradicating paralytic polio infections; this worked so well due to three main factors, all which SARS-CoV-2 have in common, making it perfect for wastewater surveillance: (i) both viruses are excreted in large amounts of faecal matter, (ii) some people are asymptomatic resulting in undiagnosed populations, (iii) it allows for early detection and surveillance in wastewater creating a faster actionable dataset (Schmidt,

2020). WBE can provide a good overview of outbreaks occurring in any given catchment by testing a single sample. This contrasts to clinical surveillance which requires large numbers of samples entailing more time and costs incurred for a similar monitoring outcome.

### 1.4.2 SARS-CoV-2 wastewater-based monitoring

The first publication confirming positive detection of SARS-CoV-2 in untreated wastewater was in Australia published August 2020. Since then, many governments and companies around the world have employed WBE as a tool for monitoring COVID-19 (Ahmed et al., 2020a; Hillary et al., 2021; Medema et al., 2020; Shah et al., 2022). The comparatively new way of using WBE to provide an early warning system to monitor SARS-CoV-2 has left the field with a lack of clarity on the best sampling and processing methods for viral detection, with the least amount of work being done on passive samplers (Zhou et al., 2021). The process of WBE consists of five main steps consisting of (i) sampling, (ii) sample transport/storage, (iii) concentration of sample, (iv) extraction, and (v) detection. There are multiple methods of detection, including reverse transcriptase quantitative and digital droplet polymer-chain reaction (RT-qPCR, dd PCR), nested PCR, LAMP and CRISPR assays; all these techniques are high in sensitivity and specificity (Alygizakis et al., 2021; Eftekhari et al., 2021; Jalandra et al., 2020).

The sampling of wastewater used for viral detection will be explained in detail later. Sample storage is best at temperatures of 0-4°C with processing taking place within a few days of collection. Longer-term sample storage is best at much low temperatures (≤-80°C), even though this is still being debated within the literature (Markt et al., 2021; Simpson et al., 2021). Sample concentration can be done using four broad method types, based on a variety of studies

conducted using various water types and viral quantification approaches: (i) two-phase separation/precipitation, (ii) particle exclusion, (iii) viruses adsorption elution (VIRADEL), and (iv) ultrafiltration (Lu et al., 2020). The two methods that are recommended by the World Health Organisation (WHO/V-B03.3) are the two-phase (PEG) and VIRADEL methods (Lu et al., 2020). However, the two-phase method is the most commonly and widely accepted method, with solutions such as PEG (polyethylene glycol) being the WHO standard method for environmental viral surveillance used in previous disease outbreaks (WHO, 2003). For this method, salt and metallic oxides can also be used. Particle exclusion uses soluble filtration via membranes to concentrate down viral matter. VIRADEL also works via filtration namely using cotton gauze pads, electronegative membranes and electropositive membrane filtration (Haramoto et al., 2004; Hata et al., 2015; Hsu et al., 2007; Liu et al., 1971). This method works well for high volumes of water. Yet the presence of organic matter leads to a reduction in the efficiency of viral concentrations. Ultrafiltration does not require the water samples to be preconditioned before extraction, unlike the PEG and electropositive membrane filtration methods, and allows for a varying range of water qualities to be used (Hill et al., 2007). Simply put, this method works by concentrating pathogens via size exclusion (Liu et al., 2012).

Wastewater samples positive for SARS-CoV-2 have been detected as early as two months prior to the first clinical case being identified (Hata and Honda, 2020). Viral shedding in faeces occurs around five days before symptom onset and has been reported to last up to 17.2 days (Cevik et al., 2021; Jones et al., 2020; Shah et al., 2022). Reports showed high levels of persistent faecal shedding of viral RNA in up to 89% of asymptomatic, presymptomatic and symptomatic people infected with SARS-CoV-2; with stool densities ranging 0.8 – 7.5 $\log_{10}$ gene copies (gc) per

gram of faeces. The level of viral shedding is seemingly independent of the infection severity (Bivins et al., 2020; Cheung et al., 2020; Gerrity et al., 2021; Shah et al., 2022; Song et al., 2021; Zheng et al., 2020) which allows most infected people to be monitored via WBE methods whether they are symptomatic or not.

### 1.4.3 Grab sampling

Grab sampling consists of taking a single point sample of wastewater at a specific time, usually untreated influent water. Grab sampling has been applied to many different studies due to the relatively low costs, ease of deployment and convenience of collection (Ahmed et al., 2020b; Liu et al., 2020a). This simple method is somewhat limited because samples are only representative of a single time point. The viral load of each sample will also be highly variable depending on the time of day, associated human actives, meaning that peak levels of shedding could be missed, while rainwater and other factors can also affect viral loads. Overall, grab sampling leads to a high level of uncertainty in RNA load as sample are rarely truly representative of the whole area. Consequently, other sampling techniques have been used to provide a more robust analysis of the levels of viral RNA in wastewater from a given population.

### 1.4.4 Composite sampling

Composite sampling is considered a more representative method, because it consists of the collection of numerous samples over a certain time period to form a homogenous sample representative of different time points (Johannessen et al., 2012). This means that due to the high number of smaller samples taken over a prolonged period the composite is unlikely to miss peaks and troughs in viral loads due to varying human activities within a wastewater shed. Both

grab and 24 h composite sampling are the two methods that are the most commonly used for WBE (Wilson et al., 2022). An automated sampling device like an autosampler can be used where you can set time points and sample volumes to fit your exact needs and to capture the highest viral load consistently of a catchment area, or just apply consistent timings so that data can be comparable for the same time over the study period.

Composite sampling either requires someone to stand and take multiple samples over the period or use a costly automated sampling device. Autosamplers can cost between £2,000-£6,000 each (Bivins et al., 2022) and can be costly to install and maintain. They also present their own set of challenges including the need for a large area and power supply. As such, these devices are not ideal for near-source monitoring in most cases (Liu et al., 2022). Overall, because of the high costs and maintenance, autosamplers may not be suitable for smaller catchment areas, low resource settings or near-source monitoring, meaning that cheaper alternatives are required. Finer spatial scales such as the analysis of specified buildings and residential areas may not hold enough space or have sewer networks suitable for composite sampling, requiring alternative methods of sampling that encompasses both the merits of the ease and low cost of grab sampling with the reliability and robust data that comes from composite sampling.

### 1.4.5 Passive sampling

Passive sampling is a technique that has been used worldwide for decades in environmental monitoring/surveillance and the investigation of pathogen outbreaks. The technique was originally adapted from Moore Swab sampling, where a cotton swab/gauze is tied by rope and suspended in flowing water to capture pathogens (Moore et al., 1952). The premise of this

technique is based on the different charges of the materials and through hydrostatic bonds and different ion charges different materials will attract different pathogens (Castaño et al., 2020). The Moore Swab sampling was first used to detect *Salmonella Paratyphi B* from effluent wastewater sewage in the South of England, to find the source of sporadic outbreaks of paratyphoid fever (Sikorski and Levine, 2020). This type of sampling has been revived in recent years for the detection of *Salmonella* bacteria (Sikorski and Levine, 2020).

This technique works by continuously filtering waster through the materials membrane to 'trap' microorganisms, this can be physically, or through chemical interactions between pathogens and the material itself. The number of pathogens in wastewater and environmental waters can be very dilute, so the sampler effectively concentrates the virus increasing the sensitivity for detection (Vincent-Hubert et al., 2017, 2021). These microorganisms or viruses are then isolated and quantified, or their presence determined by various laboratory methods (e.g. culturing), depending on the pathogen of interest, the passive sampler used and the availability of cost/equipment/resources. Since the first use of Moore Swab sampler, there has been little innovations within the method. The notable two are the use of various materials with contrasting physical and chemical properties that will adhere to different pathogens, creating a wide range of potential applications for passive sampling. The other innovation has been the use of housings to hold the passive sampler materials facilitating easy deployment and which minimizes the risk of 'ragging' (Rusina et al., 2007; Schang et al., 2021). Ragging is defined as the accumulation of solids (>5 mm) within wastewater for instance toilet roll or food scraps (Schang et al., 2021). Hayes et al. (2021) developed a COVID-19 Sewer Cage (COSCa) for the housing of passive

samplers within pipelines which are designed to allow water to flow through them but withstand high temperatures and can be 3D printed to allow for public access of use.

Passive sampling is also ideal for areas of low income and resources as passive samplers are usually use readily available materials, making it a cost-effective alternative to traditional grab and composite sampling methods (Amereh et al., 2021). Previous studies have also reported that Moore-type sampling is more sensitive to changes in viral loads especially in low prevalence areas, when the virus is at an early stage of infection within a certain defined community (i.e. hospital, residential area, stadium etc) (Liu et al., 2020b). Studies have also shown that passive sampling has a higher rate of detecting low level viral loads, another reason why it is a superior method for near-source sampling. Passive sampling can also be deployed anywhere in comparison to composite sampling which has to either have someone on site for the entire sampling period or have a large heavy piece of equipment to capture the samples. This provides all sorts of requirements from the influent pipe not being too far away to the logistics of different manhole covers being accessible, meaning that passive sampling, if done correctly, could be more advantageous for near-source monitoring.

One study suggests that passive sampling is more in line with composite sampling compared to grab samples in terms of the viral quantification levels from manhole covers assessing small communities, meaning that this method of sampling could provide a cheaper more accessible type of COVID-19 WBE monitoring (Rafiee et al., 2021). Lui et al. (2022) also compared grab samples to passives from a university hospital and determined that passive sampling was more sensitive for the detection of SARS-CoV-2.

Studies have previously investigated passive sampling for the detection of SARS-CoV-2. These have included the analysis of much of the overall process, including the deployment of the samplers, various materials used as well as concentration methods. A variety of materials have been reviewed including various cotton materials (gauze, buds, Q-tips and cheesecloth), cellulose sponges, electronegative membrane, ion exchange paper, glass wool and tampons (Bivins et al., 2021; Corchis-Scott et al., 2021; Hayes et al., 2021; Schang et al., 2021; Habtewold et al., 2022). Due to the different methods of collection and viral concentration and extraction, direct comparison between the suitability of materials is difficult. This provides a large gap in knowledge surrounding different materials suitability for the process and which methods of processing are optimal for different materials.

## 1.5 Aims and Objectives

This study aims to help close the knowledge gap surrounding passive sampling and its suitability for the detection of SARS-CoV-2 and other viruses from wastewater. In order to achieve this, the present study aimed to:

1. Find the best method for passive sampling to monitor and detect SARS-CoV-2 from wastewater, by:
    a. Comparing manual versus automatic extractions of passive samples
    b. Comparing multiple materials for the use of passive sampling
    c. Comparing multiple methods that concentrate and extract RNA from the optimum material for passive sampling

2. Optimise the use of passive sampling by comparing different methods of concentration and extraction of viral matter for multiple viruses.
    a. To achieve this, the study will compare different passive sampling materials to find the optimum material for viral recovery. In addition, it will compare different viral concentration and RNA extraction methods for the detection of multiple viruses in the context of WBE.
    b. We will also look at some of the characteristics of these viruses and see how they affect their recovery from passive samplers.

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# Chapter 2: Passive sampling a method for detecting SARS-CoV-2: A comparison of materials and processing methods

**(IN PREP)**

**Authorship declaration statement:**

KLS, CP, JK, KF and DLJ conceived the project. KLS and JK undertook the experimental work. KLS led the writing of the manuscript, with contributions from CP. All other authors contributed to the final draft of the manuscript.

# 2. PASSIVE SAMPLING A METHOD FOR DETECTING SARS-COV-2: A COMPARISON OF MATERIALS AND PROCESSING METHODS

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## 2.1 ABSTRACT

Wastewater-based epidemiology (WBE) has been at the forefront of many national monitoring programmes evaluating the spread of COVID-19 within urban communities. This has led to the rapid development of many WBE methodologies for recovering, quantifying and sequencing SARS-CoV-2 RNA within wastewater. One such low-cost approach has been the adoption of passive sampling, particularly for near-source surveillance. However little research has been conducted comparing different passive sampler materials and methods for the recovery of SARS-CoV-2 RNA from the sample after deployment. This paper aims to address this knowledge gap by comparing three different sampling materials, two extraction methods and seven different concentration processes. We found that cotton tampons recovered the highest amounts of RNA in comparison to an electronegative membrane and cotton swabs. A no-elution PEG precipitation method recovered the highest viral load and human faecal indicator bacteriophage (crAssphage), with a manual extraction showing higher RNA recoveries and fewer negative results. Our results demonstrate that passive sampling is a low-cost, effective alternative to traditional liquid-based

WBE to monitor low level viral loads in wastewater. This study is the first of its kind to compare both the methodology of viral recoveries and materials used within passive sampling at a wastewater treatment site.

***Keywords**:* Passive sampling; Sewage surveillance; COVID-19 pandemic; Public health monitoring; Viral capture

## 2.2 INTRODUCTION

Since the COVID-19 pandemic was declared in March 2020, researchers have been developing new methods for treating and tracking the causative agent, SARS-CoV-2. SARS-CoV-2 is an enveloped, positive sense, single-stranded RNA virus, that infects humans through their respiratory and gastrointestinal tract (Qian et al., 2021), resulting in viral shedding in saliva and excreta; mainly faecal matter (Wang et al., 2020). Shedding through faecal matter has been shown to occur in ca. 50% of cases with shedding often persisting for longer than in nasopharyngeal fluids. Those infected with SARS-CoV-2 typically shed between 2 to 8 $\log_{10}$ genome copies (gc) per gram in both their saliva and stool which can occur before symptom onset (Cheung et al., 2020; Zheng et al., 2020). Symptomatic individuals are often confirmed as positive cases by measuring respiratory shed with lateral flow or reverse transcription polymerase chain reaction (RT-PCR) testing, but up to 50% of positive cases are asymptomatic, and are thus less likely to seek out testing ("Coronavirus (COVID-19) Infection Survey, characteristics of people testing positive for COVID-19, UK - Office for National Statistics). Viral shedding in stool occurs in both symptomatic and asymptomatic cases, so can be measured in wastewater to monitor the epidemiology of SARS-CoV-2 at a community level (Wade et al., 2022). Wastewater-based

epidemiology (WBE) can, thus, be used to assess asymptomatic cases that go undetected by mass clinical testing, especially as the rate of viral shedding in faeces appears to be independent of infection severity (Gerrity et al., 2021; Zheng et al., 2020). WBE therefore represents a cost-effective, unbiased, and non-invasive complementary approach to clinical testing for monitoring community-level viral infections over prolonged periods of time (Choi et al., 2018).

Sampling for WBE is ideally carried out using an autosampler that recovers a 'composite' wastewater sample taken incrementally over the course of 24 hours. However, the use of autosamplers can be costly as well as requiring specialist expertise, a power supply and continual maintenance. In addition, they are often not suitable for confined spaces or for use where sewer manholes are located (e.g. roads), are prone to vandalism and theft in public places and may not be legally compliant in some situations. Due to these issues, 'grab' or 'spot' sampling is frequently carried out, where a sample is collected at one instant in time, which can result in a high degree of intrinsic variability depending on the relationship between sampling and facility use in the community (Wade et al., 2022). Passive sampling is a promising alternative which is currently being explored for SARS-CoV-2 and human pathogen detection (Li et al., 2022). Stemming from Moore Swab sampling (Moore et al., 1952), passive sampling is an environmental surveillance tool where a material, usually gauze or cotton, is suspended into flowing water to gradually accumulate virus over time, making it effective for low viral load detection (Blanco et al., 2019; Vincent-Hubert et al., 2021). This method has been used for decades for the detection of enteric viruses in public and environmental health studies (Barrett et al., 1980; Sbodio et al., 2013; Sikorski and Levine, 2020). The ease of use in confined areas and low cost make passive sampling a great alternative to autosamplers, especially in low economic resource areas (Liu et al., 2020).

Due to their low cost and ease of use, passive samplers also provide an opportunity to measure smaller catchment areas or near-source environments (e.g. prisons, residential blocks), which may prove useful if the prevalence of COVID-19 subsides and the monitoring of local areas and individual organisations and critical infrastructure (e.g. military installations) becomes more important (Hayes et al., 2021).

Passive sampler materials contain surfaces which can electrostatically bind SARS-CoV-2, as well as possessing properties that facilitate physical entrapment of wastewater solids to which viruses may be attached. The overall binding ability and net charge of the virion are largely dependent on the spike proteins size, abundance, and composition (Adamczyk et al., 2021). In a wastewater environment with pH range of 7.0-8.0, SARS-CoV-2 is suggested to have a net electro-negatively charged envelope and spike proteins (Scheller et al., 2020). Free RNA released from virions are also present in wastewater are also expected to be highly negatively charged (Draper, 2004). Thus, materials used for passive sampling should preferentially be electro-positively charged. This, alongside different hydrophobic and van der Waals interactions are how viruses bind to different passive samplers. This phenomenon is well documented within the sampling of airborne viruses and their adhesion to passive samplers (Robotto et al., 2021; Verreault et al., 2008). The principles of these adhesions are still very much relevant despite the change in media, therefore the virus type as well as the physio-chemical properties of the sampling material will affect the recovery of different viruses (Rzeżutka and Carducci, 2013).

Despite the promising application of passive samplers, research into viral processing methods has mainly been focused on the optimisation of techniques applied to grab and composite samples (Ahmed et al., 2020; Kumblathan et al., 2021). Little attention has been paid

to passive sampling for wastewater virology, with it only gaining attention during the COVID-19 pandemic (Schang et al., 2021). Passive sample materials tested across multiple studies include cotton materials (e.g. gauze, buds and cheesecloth), cellulose sponges, electronegative membranes, ion exchange paper, glass wool and tampons. These have used a range of laboratory processing methods to recover viruses from their surface, including direct extraction, no-elution methods, elution buffer (lysis buffer, phosphate buffered saline, PBS) processing and the ultrafiltration of sorbates (Bivins et al., 2021; Corchis-Scott et al., 2021; Habtewold et al., 2022; Hayes et al., 2021; Schang et al., 2021). However, most studies only apply individual recovery approaches when comparing the effectiveness of passive sampler materials for viral capture (Bivins et al., 2021; Corchis-Scott et al., 2021; Habtewold et al., 2022; Hayes et al., 2021; Lambertini et al., 2008; Schang et al., 2021). An exception to this is a recent study by Kevill et al. ( 2022a) which tested a range of passive sampler materials, processing methods and viral targets under laboratory conditions. Studies have also focused on minimizing issues that can arise when using passive sampling approaches, such as ragging. Ragging is the accumulation of debris on the sampler itself which can lead to sewer blocking and inefficient viral capture due to the covering of viral capture surfaces.

This study, therefore, aims to close the research gap by deploying three contrasting passive sampler materials (Cotton swabs, SG81 Whatman ion exchange paper and Tampax super compact) at a centralised wastewater treatment works (WwTW) at a major urban centre (Chester) and investigating appropriate methods for laboratory processing, including sample concentration and extraction of nucleic acids from passive samplers, as well as identifying the best material for SARS-CoV-2 recovery. We also measured the levels of crAssphage (a human

DNA virus) used as a human faecal marker which is often used to normalise for wastewater dilution (Langeveld et al., 2021). We compared seven concentration methods: squeeze in PEG (glycol-8000) precipitation, PBS elution into PEG precipitation, PBS elution into ammonium sulfate precipitation, beef extract/glycine elution into PEG precipitation, beef extract/glycine elution into ammonium sulfate precipitation, no-elution into PEG precipitation, and direct extraction. Alongside two extraction methods, one being an automatic extraction using a Kingfisher Flex system and the other conducted manually, with the best material being further specified. The overall aim of this study was to determine the most compatible material and method for monitoring SARS-CoV-2 in wastewater using passive sampling as a method of detection.

## 2.3 Materials and Methods

### 2.3.1 Sampling sites and passive sampler materials.

Four separate experiments were conducted over the course of July2021 to October 2021. The samples for the nucleic acid extraction were collected from a principal wastewater treatment plant in Chester (northwest of England) in July 2021 and the samplers were deployed for varying amounts of time (0.5, 1, 3,6 and 24 h), with two materials used (Tampax Compak regular tampons and cotton swabs).

The experiment identifying the optimal passive sampler material was conducted at a principal wastewater treatment plant in the northwest of England (Chester) in August 2021. Three different materials were used: Tampax Compak super tampons, cotton swab and paper (SG81 ion exchange Whatman paper), these were used based on their previous use for

viral recovery in different aqueous environments (Barrett et al., 1980; Bivins et al., 2021; Zou et al., 2017). These materials have been previously tested for their basic physical and chemical characteristics (Jones et al., 2022). These passive samplers were deployed in the crude influent stream behind the primary screen grate for 24 h.

For the assessment of grab sampling compared to a passive sampling approach tampons were used based on the outcome of the passive sampler material comparison experiment. Grab samples taken hourly for 24 h, three passive samplers were deployed for 24 h over four six days in August 2021. Each sampler was placed behind the primary screen gate and tied using fishing wire.

For the comparison of the concentration of passive sampler materials and nucleic extraction only tampons were used, as these were identified as having the highest viral recovery. Three tampon types (Asda regular, Tampax Compak super and Tampax Pearl super) were used to assess the impact of tampon type and best method for the SARS-CoV-2 and crAssphage recovery. This experiment was conducted at a large urban wastewater treatment plant in Bangor (North Wales). Each passive sampler type was placed in a separate rectangle of polypropylene mesh and deployed into the influent stream of wastewater for 1 h. Each sample was run in triplicate.

For all experiments the passive samples were transferred into a plastic Ziplock® bag whilst the liquid samples were stored in 500 ml Nalgene polypropylene copolymer (PPCO) centrifuge bottles. All passive samplers and liquid samples were stored and transported on ice at 4°C to the laboratory for processing. Upon arrival samples were all stored at 4°C until sample processing on the same day or the day after.

### 2.3.2 Initial testing to identify the optimal passive sampler material and extraction method for viral recovery

For the two initial experiments, a direct extraction method was performed. Samples of each passive sampler (1 $cm^2$) were placed in 15 mL centrifuge tubes containing 2 mL of NucliSens lysis buffer (BioMerieux, Marcy-l'Étoile, France; Cat No. 280134 or 200292). The tubes were then vortexed and prepared for manual nucleic acid extraction as detailed in Farkas et al. (2021). The concentrated solution was extracted both manually and automatically, using the NucliSens MiniMag Nucleic Acid Purification system (BioMerieux SA, Marcy-l'Etoile, France) (manual) and a Kingfisher 96 Flex system (Thermo Scientific, Waltham, MA, USA) (Automatic), both as detailed in Farkas et al. (2021) and Kevill et al. (2022b). A magnetic bead extraction was used due to the ease and reproducibility of the results as well as the ability of this method to be used for high-throughput samples in comparison to other methods available such as spin columns. The NucliSense magnetic extraction (BioMerieux) procedure developed by CEFAS is an ISO accredited method for shellfish virus recovery and have a long history of use for recovery of viruses from environmental samples (Le Guyader et al., 2009; Lees et al., 2012). The BioMerieux bead were also one of the kits recommended by the Centre for Disease Control and Prevention for sample preparation for COVID-19 RT-PCR testing (Ravi et al., 2020). The manual system is performed in 1.5 mL Eppendorf microcentrifuge tubes, with a magnetic rack used (BioMerieux SA, Marcy-l'Etoile, France), manual aspiration used to remove buffers and elute extracted sample. The Kingfisher 96 Flex System is an automated system that moves the magnetic beads (BioMerieux SA, Marcy-l'Etoile, France) via magnets between the various extraction buffers, the same Nuclisense kit was used for both manual and automated extractions. A 0.1 mL extraction volume is eluted from the automated system compared to a 1 mL one from the manual method.

### 2.3.3 Assessment of grab sampling compared to a passive sampling approach

To compare passive and grab sampling approaches, grab samples were collected at hourly intervals from the influent wastewater stream alongside the passive samples deployed for 24 h, over six days. Grab samples were concentrated by a PEG-based precipitation method as described in Farkas et al., (2021) and extracted using an automated Kingfisher 96 Flex system (Thermo Scientific, Waltham, MA, USA) system prior to quantification with qPCR (Kevill et al., 2022b). The grab samples were taken to allow for a comparison of baseline SARS-CoV-2 in the wastewater and enabled the assessment of SARS-CoV-2 accumulation on the tampon passive sample materials over time, tampons were used as the comparison due to the results found in section 2.7.2.

### 2.3.4 Passive Sample concentration and nucleic acid extraction

After tampons were identified as the passive sampler material that recovered the highest viral loads, three separate tampon types (Asda regular, Tampax Compak super and Tampax Pearl super) were used to assess the impact of tampon type and best method for the SARS-CoV-2 and crAssphage recovery.

Viruses were concentrated from passive samplers using one of seven methods. These methods were the squeeze method, Phosphate Buffered Saline (PBS) elution into polyethylene glycol and NaCl (PEG8000; Sigma-Aldrich, St. Louis, MO, USA) precipitation, PBS elution into ammonium sulfate- precipitation, beef extract/glycine elution into PEG precipitation, beef extract/glycine elution into ammonium sulfate precipitation, no elution into PEG precipitation, direct extraction of 1 $cm^2$. Each of the seven methods were run using all three

different tampons, all run in triplicate. A negative processing control of deionised water was also included for each method.

#### 2.3.4.1 Squeeze method

During the squeeze method each tampon was squeezed to remove all the absorbed wastewater into a sterile tube. A 500 µL aliquot was taken for extraction and added to 2 mL of lysis buffer prior to a manual nucleic acid extraction.

#### 2.3.4.2 Direct extraction method

The direct extraction method is the same as detailed in Section 2.3.2.

#### 2.3.4.3 Phosphate buffered saline (PBS) elution into Polyethylene glycol (PEG) method

Passive samplers were saturated with 20 mL of PBS and hand massaged for 2 min in individual Ziploc® plastic bags (Bivins et al., 2021). The eluent and wastewater were then squeezed out of the sampler material and into a sterile tube and the pH of the supernatant was adjusted to 7.0 - 7.5, and then mixed with PEG to form a 10 % (w/vol) PEG8000 and 2 % (w/vol) NaCl solution, samples were inverted to mix. Samples were then left to incubate at 4 °C overnight. The samples were then centrifuged at ×10,000 g for 30 min, the supernatant decanted off and the viral pellets resuspended in 900 µL of lysis buffer, and manually extracted.

#### 2.3.4.4 PBS elution into ammonium sulfate method

The passive samplers were saturated with 20 mL of PBS and hand massaged for 2 min in individual Ziploc® bags (Bivins et al., 2021). The eluent and wastewater were then squeezed out of the sampler material and into a sterile tube. c.a. 30 mL of sample and eluent added to 14 g of ammonium sulfate and dissolved. This was incubated at 4 °C for 1 h. The samples were then

centrifuged at ×10,000 g for 30 min, the supernatant removed and the viral pellets resuspended in 900 µL of lysis buffer, and manually extracted.

#### 2.3.4.5 Beef extract/Glycine elution into PEG method

Passive samplers were saturated, and hand massaged for 2 min in individual Ziploc® bags; with 20 mL of a beef extract/glycine solution with an adjusted pH of 9.5 (Lab Lemco beef extract (Oxoid, USA) and 2 M glycine to reach the final concentration of 3% and 0.5 M, respectively). The eluent and wastewater were then squeezed out of the sampler material into a sterile tube and the pH of the supernatant adjusted to 7.0 - 7.5, mixed with PEG to form a 10% PEG 8000 and 2 % NaCl solution, and the samples inverted to mix. Samples were then left to incubate at 4 °C overnight. The samples were then centrifuged at ×10,000 g for 30 min, the supernatant removed and the viral pellets resuspended in 900 µL of lysis buffer, and manually extracted.

#### 2.3.4.6 Beef extract/Glycine elution into ammonium sulfate method

Passive samplers were saturated, and hand massaged for 2 min in individual Ziploc® bags; with 20 mL of a beef extract/glycine solution with an adjusted pH of 9.5 (Lab Lemco beef extract (Oxoid, USA) and 2 M glycine to reach the final concentration of 3% and 0.5 M, respectively). The eluent and wastewater were then squeezed out of the sampler material into a sterile tube. ca. 30 mL of sample and eluent was then added to 14 g of ammonium sulfate (to create a final molarity of 0.0035 M) and dissolved, incubated at 4°C for 1 h, centrifuged (×10,000 g, 30 min) and the supernatant discarded. The viral pellets were then resuspended in 900 µL of lysis buffer, and manually extracted.

#### 2.3.4.7 No-elution into PEG

Each passive sample was placed into a Ziploc® bag and the wastewater squeezed out of each sample into ~20 mL of PEG (adjusted for varying sample amounts to form a 10 % (w/vol) PEG 8000 and 2 % (w/vol) NaCl solution), samples were then inverted to mix and left to incubate at 4 °C overnight. The samples were then centrifuged (×10,000 g, 30 min), the supernatant discarded and the viral pellets resuspended in 900 µL of lysis buffer, and manually extracted.

A manual extraction method was used for all concentration methods as described in Section 2.3.2. Virus concentration methods, nucleic acid extractions, and q(RT-)PCR assay preparation were all carried out in separate laboratories conducted in class II microbiological safety cabinets to minimize contamination risks.

## 2.4 Q(RT-)PCR AND QPCR ANALYSIS

The q(RT)-PCR assays for SARS-CoV-2 N1 detection were carried out using an RNA Ultrasense One-step q(RT)-PCR system (Life Technologies, Carlsbad, CA, USA, Cat. No. 11732927), in a Quant Studio Flex 6 (Applied Biosystems Inc., Waltham, MA, USA). Samples for all experiments were run on a MicroAmp Endura optical 384 well clear plate (Applied Biosystems Inc., Cat. No. 4483273), with a 20 µl reaction volume. The mastermix comprised RNA UltraSense™ 5× Reaction Mix with ROX, 10 pmol forward, 20 pmol reverse primers and 5 pmol probe, RNA UltraSense™ Enzyme Mix, 16 nmol $MgSO_4$, 1 µg bovine serum albumin (BSA), molecular grade water and 2 µl of extracted wastewater RNA/standard/control (Table S1). The primers and probes used are presented in the supplementary material. The settings for the q(RT)-PCR were: reverse transcription hold step at 55 °C (60 min), reverse transcriptase inactivation at 95 °C (5 min),

followed by 45 amplification cycles of 95 °C (15 s), 60 °C (1 min), 65 °C (1 min) at increments of 1.6 °C/s.

Detection of crAssphage with qPCR was performed using QuantiFast probe PCR with ROX mix (Qiagen, Hilden, Germany. Cat No. 204354). Samples were also run on with a 20 µl reaction volume, on a 384 well clear plate, using Quant Studio Flex 6. The mastermix contained QuantiFast no Rox, QuantiFast with Rox with 10 pmol of forward, 10 pmol reverse primers and 5 pmol probe, 16 nmol $MgSO_4$, 1 µg bovine serum albumin (BSA), molecular grade water and 4 µL of extracted wastewater RNA/standard/control. Settings for the qPCR were 98 °C (5 min), followed by 40 cycles at 95 °C (15 s) and 60 °C (1 min) with increments of 1.6 °C/s.

PCR no-template controls (molecular grade water) were used to determine the absence of contamination during the process. Samples, standards and controls were all run in duplicate. The mean values for each extraction were used for further analysis. Only samples that yielded a Ct mean <40 were included in the quantitative analysis. Samples were assessed against an ssRNA N1 (Kevill et al., 2022b) or plasmid DNA CrAssphage (as described in Farkas et al., 2019) standard curve serial dilution series of the target gene ranging from $10^5$-$10^0$ gc/µL. The N1 standard slope ranged from -3.01 to -3.31, the $R^2$ range was 0.990 to 0.998 and the efficiency (%) ranged from 100.4 to 114.8. The crAssphage target slope was -3.512, the $R^2$ was 0.998 and the efficiency (%) was 92.6. The N1 and crAssphage limit of detection (LOD) were determined previously by Farkas et al. (2021) and Farkas et al. (2019), respectively. 10 replicate dilutions of N1 and crAssphage low concentrated spiked wastewater samples were used. The LOD is defined as the minimum concentration where all replicates are positive, 1.7 gc/µL for the N1 gene and 2 gc/µL for the

crAssphage marker. Quantities can be detected below this level but are susceptible to false negatives.

## 2.5 DATA ANALYSIS AND VISUALISATION

QuantStudio™ Real-Time PCR Software, version 1.3 (Applied Biosystems, USA) was used to analyse the data. Manual adjustments to the baseline (cycle threshold; Ct) were made, when the standards needed alterations. The viral concentrations were expressed as genome copies (gc)/µL wastewater per qPCR reaction; these were then scaled for each experiment. For the extraction and three material comparison gc/$cm^2$ was used for viral load comparisons, while for the method and tampon type comparison this was scaled up to be representative of each tampon's recovery (gc/tampon). This was done so that all the results for the different experiments were comparable. Direct extractions were scaled up to be representative of each tampon. This was done by measuring the volume of each of the three specified tampons and scaling the 1 $cm^3$ sections to each tampon volume (Supplementary Data).

## 2.6 STATISTICAL ANALYSIS

Statistical analyses and data visualisation was performed in RStudio v1.4.1717 (R Core Team, 2020; Wickham, 2016), using the packages "dplyr" for data manipulation, "ggplot2", "ggbeeswarm", "patchwork" for data visualisation. Data was tested for normality using Sharipo-Wilk test, and Levene test for homogeneity of variance. The experimental datasets were not-normally distributed; thus, non-parametric tests were performed.

The comparison for manual vs automatic extractions determined and undetermined were done using a Pearson's Chi-squared test with a simulated p-value, based on 2000 replicates. The

median gene recovery comparisons were computed with a Wilcoxon rank sum test with continuity correction.

Once manual extraction was identified as the preferred RNA recovery extraction method, results from automated extractions were removed from the dataset and only manually extract samples were analysed from here onwards. To statistically compare the viral recoveries from each sampler material a Kruskal-Wallis rank sum test was used, a Pairwise Wilcoxon rank sum test with continuity correction was then used to determine which material was significantly different to the others, a Holm-Bonferroni adjustment method was used. A Pearson's Chi-squared test with simulated p-value (based on 2000 replicates), was used to establish differences between the determined and undetermined samples according to each material.

Comparison of viral recovery between the seven methods were carried out using a Kruskal-Wallis rank sum test, followed by pairwise Wilcoxon rank sum tests to compare each method against another for further investigation.

## 2.7 RESULTS

### 2.7.1 Viral RNA Extraction comparison of manual vs. automatic

After collection, all three types of passive samples were extracted both manually and using an automated a Kingfisher 96 Flex system (summary results in Table S1). Manual extractions had significantly fewer undetermined results when detecting SARS-CoV-2 N1 gene (Pearson's Chi-squared test with simulated p-value (based on 2000 replicates): X-squared = 9.52, p-value < 0.01; Figure 1b). Median N1 gene recoveries were significantly higher for the manual extraction (Wilcoxon rank sum test with continuity correction: W = 298, p-value < 0.05; Figure 1a).

Furthermore, median crAssphage gene recoveries were significantly greater using a manual extraction (Wilcoxon rank sum test with continuity correction: $W = 139$, p-value $< 0.001$; Figure 1c); however, both methods positively identified crAssphage in all samples (Figure 1d). These results indicate manual extractions are likely to increase gene recovery and reduce the number of undetermined results when viral load is low.

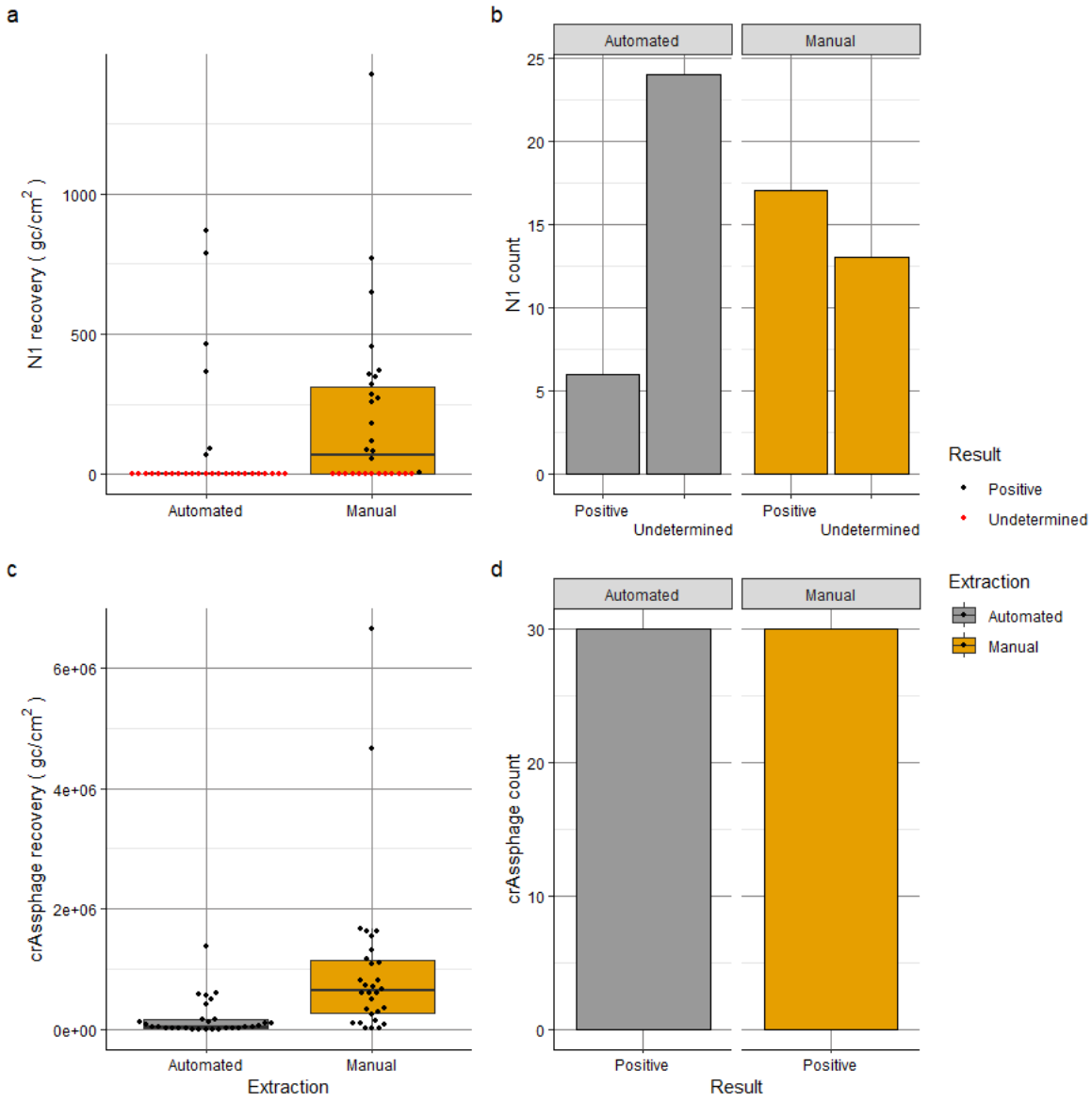


**Figure 1: Comparison of gene recovery and undetermined results between manual and automated extractions. (a) SARS-CoV-2 N1 gene recovery (gc/cm$^2$) between extraction methods. (b) SARS-CoV-2 N1 positive and undetermined samples between extraction methods. (c) crAssphage gene**

**recovery (gc/cm$^2$) between extraction methods. (d) crAssphage positive and undetermined samples between extraction methods.**

### 2.7.2 Effect of passive sample material on the comparison for viral recovery

After identifying manual extraction as the preferred RNA recovery method, results from automated extractions were removed from the dataset and only manually extract samples were analysed from here onwards. Passive samples were constructed with three materials (Artibetter medical grade non-woven gauze cotton swab (Barrett et al., 1980), SG81 ion exchange paper, and tampax compak super) before identical assessment (summary results in Table S2). Median SARS-CoV-2 N1 gene recoveries differed between materials used to construct the passive samplers (Kruskal-Wallis rank sum test: Kruskal-Wallis chi-squared = 10.19, df = 2, p-value < 0.01; Figure 2a), and pairwise comparisons found tampax to have significantly greater recovery compared to both cotton (Artibetter cotton swab) and paper (SG81 ion exchange paper) (Pairwise Wilcoxon rank sum test with continuity correction: Tampax ~ Cotton swab: p-value < 0.05; Tampax ~ Paper: p-value < 0.05 (Holm-Bonferroni (H-B) adjustment method)). The frequency of negatives could not be assumed different for any materials (Pearson's Chi-squared test with simulated p-value (based on 2000 replicates): X-squared = 5.75, p-value > 0.05; Figure 2b). Similarly, median crAssphage gene recoveries did vary between the three materials (Kruskal-Wallis rank sum test: Kruskal-Wallis chi-squared = 8.02, df = 2, p-value < 0.05; Figure 2c), pairwise comparisons found only tampax and paper to be significantly different (Pairwise Wilcoxon rank

sum test with continuity correction: Tampax ~ Paper: p-value < 0.05 (H-B adjustment method)).

All materials positively identified crAssphage in all but one cotton swab sample (Figure 2d).

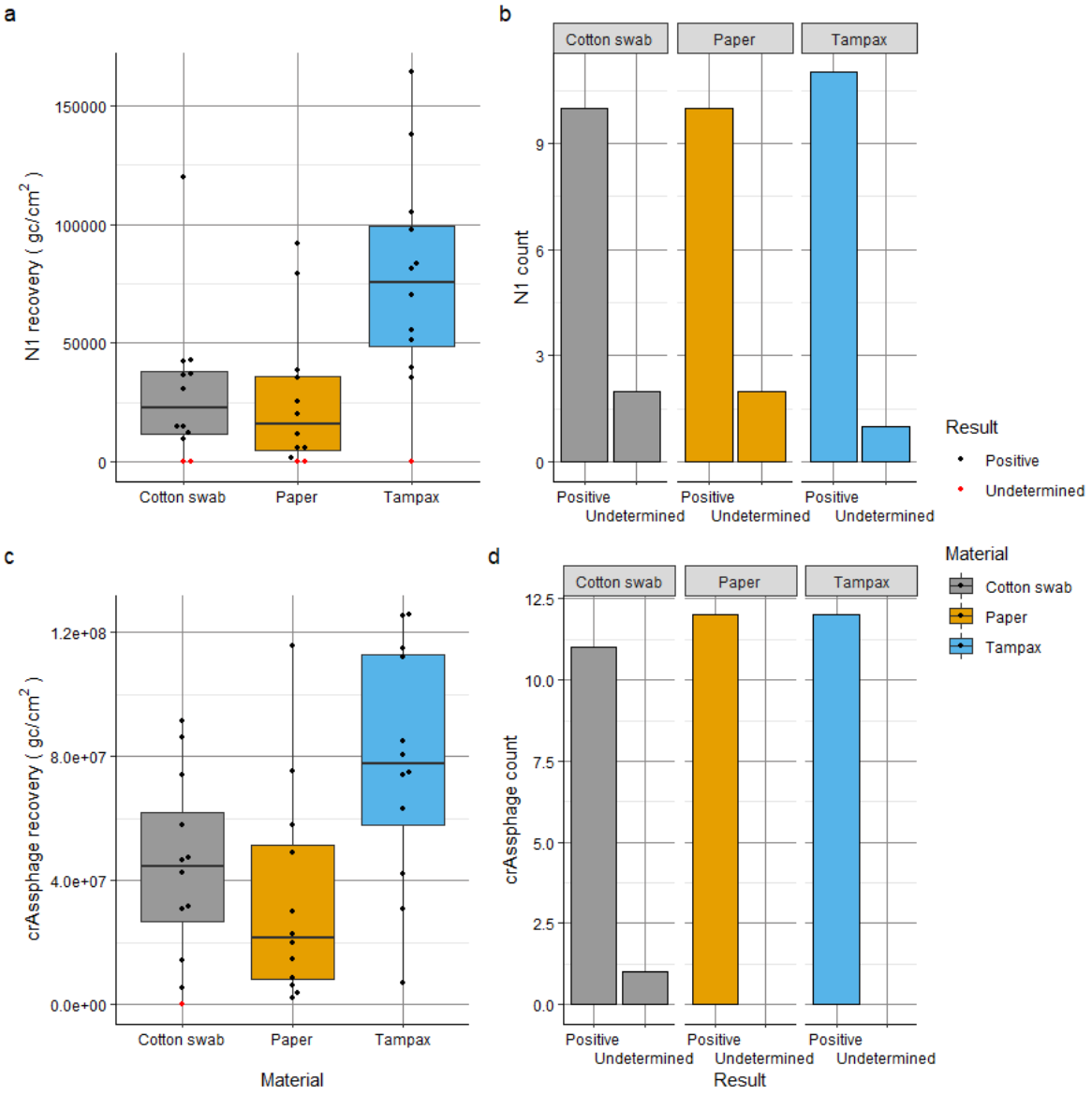

**Figure 2: Comparison of gene recovery and undetermined results between passive sampler material. (a) SARS-CoV-2 N1 gene recovery between sample material. (b) SARS-CoV-2 N1 positive and undetermined samples between sampler material. (c) crAssphage gene recovery between sampler material. (d) crAssphage positive and undetermined samples between sampler material**

### 2.7.3 Comparison of crAssphage and N1 gc recovered from grab samples and passive samplers

Grab samples, with a concentration step, recovered more genome copies per reaction compared to passive samples (Figure 3). Viral copies of the grab samples were in the range of 179 – 73,773 and 1.96 – 330 gc/L for crAssphage and SARS-CoV-2 N1, respectively. This can be compared to the passive samples which ranged from 203 - 12,556 and 0.15 – 16.39 gc/L for crAssphage and SARS-CoV-2 N1, respectively.

Furthermore, the number of crAssphage and SARS-CoV-2 N1 positives varied between sampling type, as grab samples has 82 % and 93% positives for crAssphage and SARS-CoV-2 N1, respectively, compared to passive samples which were 98% and 86% positive. This indicated that that passive samplers were more reliable at detecting crAssphage, however they captured a much lower variability. Overall passive samples had lower variability over time compared to grab samples (Figure 3).

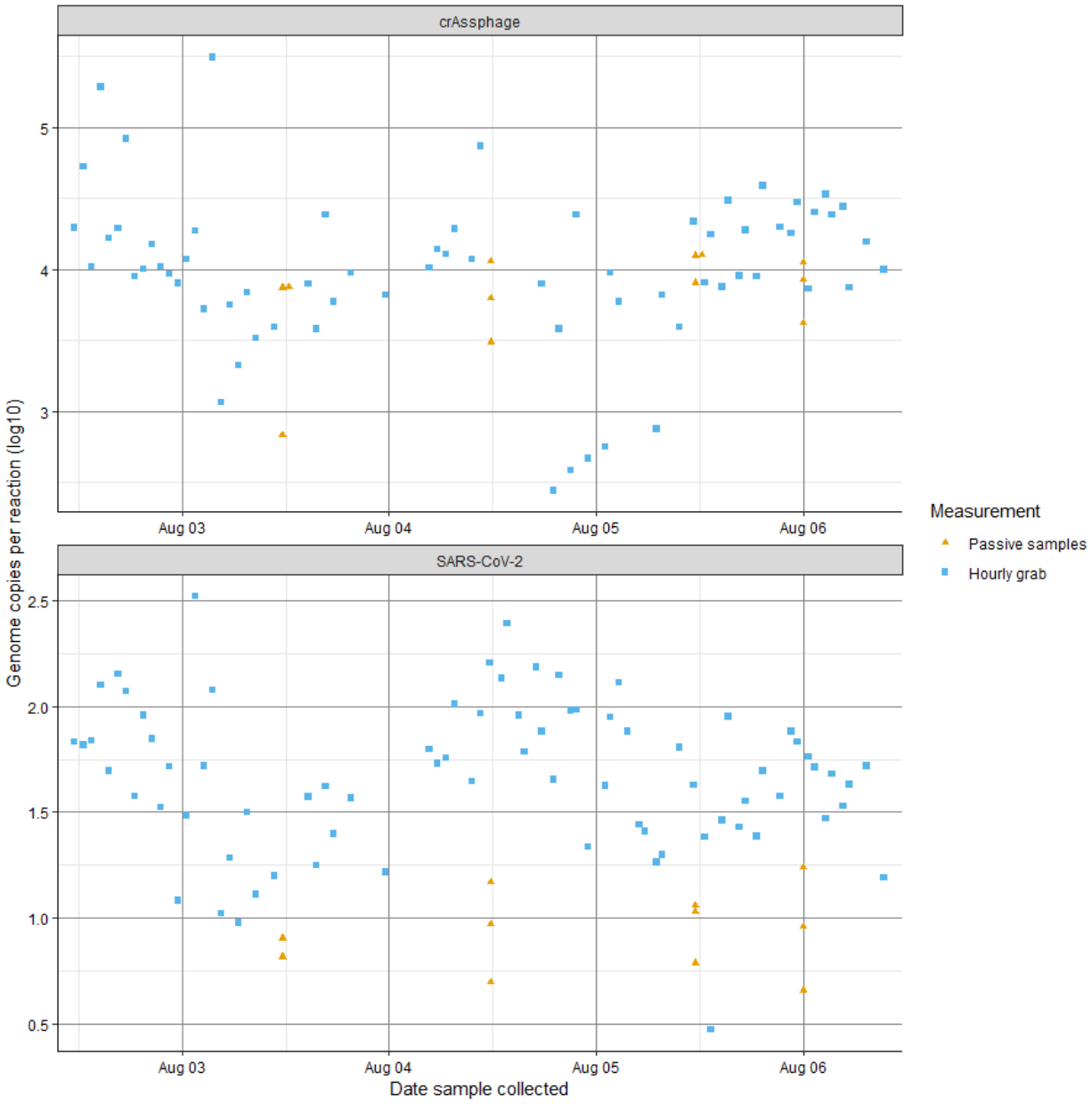


**Figure 3: Comparison between autosampler and passive sample results. Autosampler results are plotted as hourly grab samples. Passive samples are plotted as the final composite retrieved after 24 hours exposure. Undetermined results are plotted as transparent points with zero gene copies per reaction.**

### 2.7.4 Comparison of viral recovery concentration methods for passive samples

There were differences found between the seven concentration methods and their viral median recoveries. For this dataset, all tampon types were analysed under the 'tampon' banner head. The SARS-CoV-2 N1 gene recovery differed the most between methods used (Kruskal-Wallis rank sum test: Kruskal-Wallis chi-squared = 64.139, df = 6, p-value < 0.001; Figure 4), Wilcoxon pairwise comparisons found that over half of the methods were significantly different (Figure 4). No-elution in PEG had the highest SARS-CoV-2 N1 recovery mean (1483 gc/tampon), the second highest mean recovery was PBS elution in PEG (1224 gc/tampon). These two methods are significantly ($p < 0.001$, Figure 4) different from each other, even though the ranges overlap. Overall, the no-elution with PEG precipitation method recovered the most SARS-CoV-2 RNA.

Similarly, crAssphage median recoveries differed between method used (Kruskal-Wallis rank sum test: Kruskal-Wallis chi-squared = 31.946, df = 6, p-value < 0.001; Figure 4). A Wilcoxon pairwise comparison found that over half of the methods were significantly different (Figure 4). As for SARS-CoV-2, no elution with PEG precipitation proved the best method for crAssphage recovery (mean, 1580616 gc/tampon), while the Direct extraction method had the next highest mean recovery (mean, 1267332 gc/tampon).

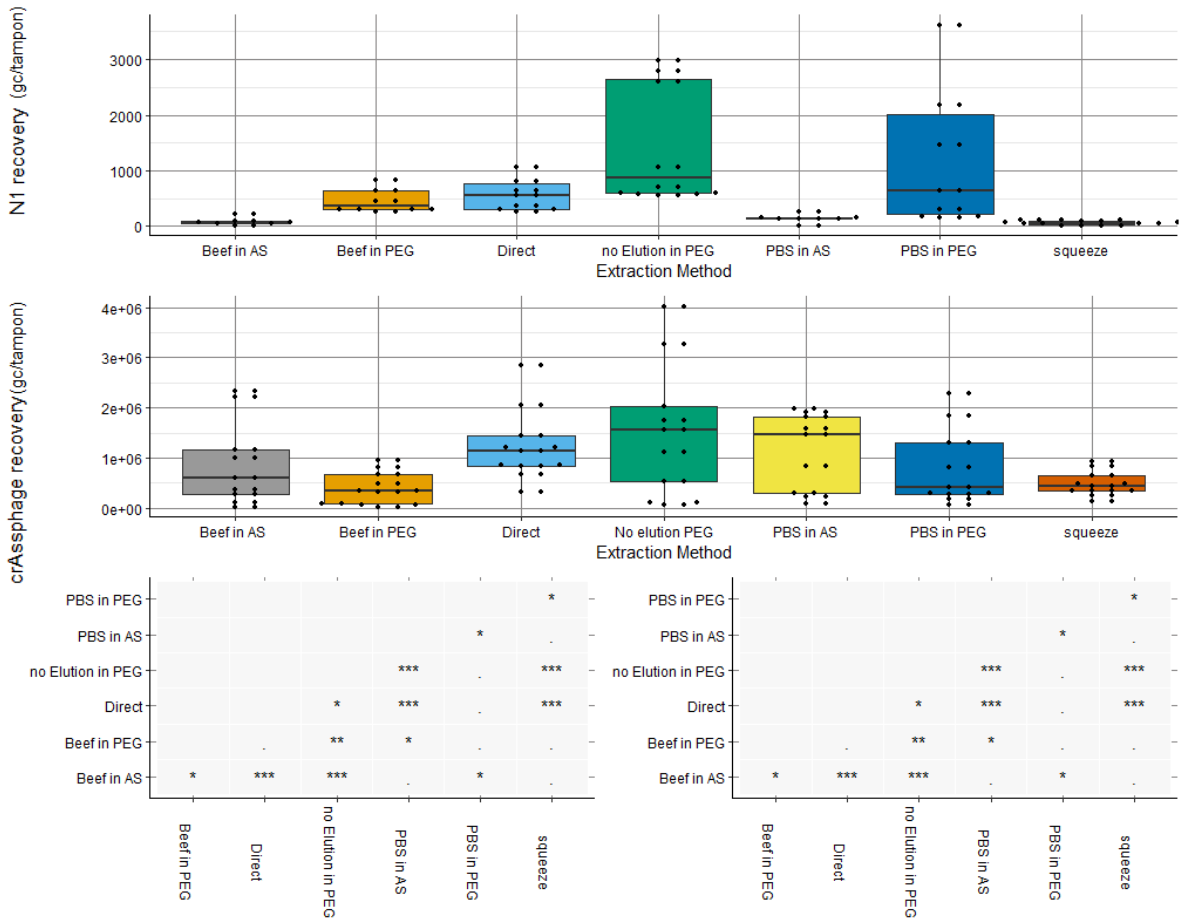


**Figure 4: Comparison of gene recoveries per tampon between processing methods, carried out on passive samplers suspended in an influent wastewater stream for 1 h. Panel (a) compares concentration methods for the SARS-CoV-2 N1 gene, panel (b) compares concentration methods for the bacteriophage crAssphage, while panel (c) and (d) show p-values (*p*-value: < 0.001 [***]; < 0.01 [**]; < 0.05 [*]; > 0.05 [.]) of Wilcoxon pairwise comparisons of the processing methods for SARS-CoV-2 N1 and crAssphage, respectively.**

### 2.7.5 Comparison of viral recovery from different tampon types

SARS-CoV-2 N1 gene median recoveries were not significantly different between tampon types (Kruskal-Wallis rank sum test: Kruskal-Wallis chi-squared = 7.22, df = 2, p-value > 0.05; Figure 5). Pairwise Wilcoxon test showed that no tampon type was significantly different from another (Figure 5). CrAssphage median recoveries differed between tampon types (Kruskal-Wallis rank sum test: Kruskal-Wallis chi-squared = 11.72, df = 2, p-value < 0.005; Figure 5). A pairwise Wilcoxon test found that Asda tampons are significantly ($p < 0.05$) different to Tampax's Pearl and Compact (Figure 5). These results show that Asda tampons have a lower crAssphage gene recovery compared to the branded tampons.

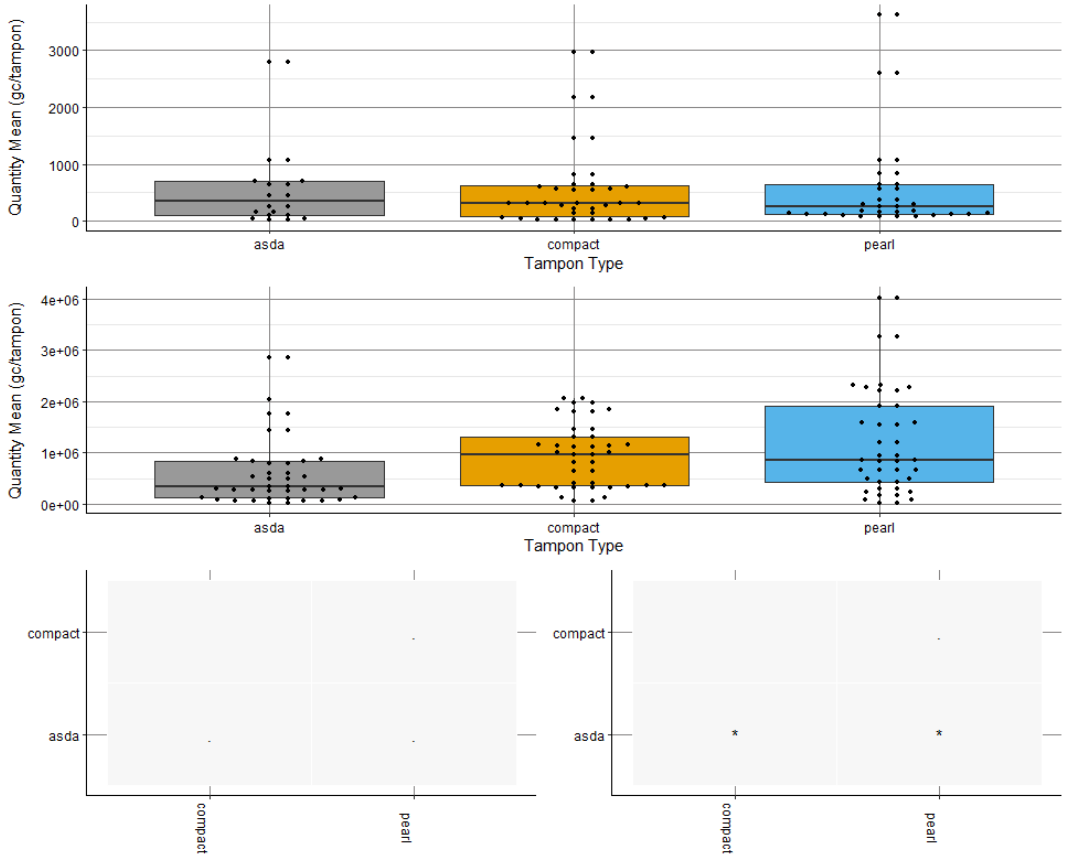

**Figure 5: Comparison of gene recoveries per tampon between different tampons, carried out on samplers suspended in an influent tank for one hour. Panel 'a' compares concentration methods for the SARS-CoV-2 N1 gene, panel 'b' compares concentration methods for the bacteriophage crAssphage, while panel 'c' and 'd' show p-values (*p*-value: < 0.001 [***]; < 0.01 [**]; < 0.05 [*]; > 0.05 [.]) of Wilcoxon pairwise comparisons of the processing methods for SARS-CoV-2 N1 and crAssphage, respectively.**

## 2.8 DISCUSSION

### 2.8.1 SARS-CoV-2 detection in wastewater

Even with the advancements and implementation of passive sampling in WBE for SARS-CoV-2 since the start of the COVID-19 pandemic, there is still a lot of uncertainty surrounding the optimal methodology, including sampler type, concentration methods and quantification (Bivins et al., 2021; Corchis-Scott et al., 2021; Habtewold et al., 2022; Hayes et al., 2021; Schang et al., 2021). Previously, a few studies have examined sections of the whole process, but none fully investigated the whole process, or multiple sections. This study investigated the effectiveness of passive sampling for SARS-CoV-2 detection, in which we compared different sampler materials (n=3), extraction (n=2) and concentration (n=7) methods for passive sampling to optimise the detection of SARS-CoV-2 in wastewater. Previous studies have commented on the processing methods for SARS-CoV-2 recovery being imperative to the amount of viral recovery (Vincent-Hubert et al., 2021). This novel study is the first of its kind to compare passive sampler material, sample processing method for viral recovery and the nucleic acid extraction method with an aim

to improve methodology with the aim of developing a standardised protocol for the effective use of passive sampling for monitoring of SARS-CoV-2 within a WBE context.

### 2.8.2 Manual extractions compared to automatic extractions

Our first experiment evidenced that in the case of passive sampling, manual extractions yielded a higher gene recovery in comparison to the automated system adopted in many high throughout laboratories. These extractions recovered significantly more gene copies for both SARS-CoV-2 N1 and crAssphage, indicating that this extraction method would increase gene recovery overall for passive sampling. These results signify that manual extractions reduce the number of undetermined results when the viral load is low. Both manual and automatic extractions use the same reagents and volumes. One reason for the higher extraction yield could be the larger volume of concentrated sample that is extracted within the manual extraction (1.5 ml) meaning that the inhibitors are more diluted through the process as well as there being a higher volume for virus to be concentrated from. Another reason could be that manual extractions allow for more precise washing reagent removal. This is because the magnets are on the side of the Eppendorf microcentrifuge tubes, instead of the base like with automatic extractions, meaning that when the beads are magnetized they move laterally creating a space in the base of the tube. This equates to a higher removal of washing reagents in the washing step as you can visibly see the liquid being removed. The volume of reagents being removed and will be slightly different for each sample, which can be detected manually where as with an automated system the same volume is removed each time. This is especially relevant when passive samples release fibres which attach to the beads in the extraction process meaning these

can be removed during extraction whereas the automatic system cannot catch these minor changes in processing.

These results coupled with passive samplers' accumulation of virus over time concentrating low viral loads, means that passive sampling is an optimum method for use of viral monitoring in areas with low viral prevalence, such as a specified building or community (Bivins et al., 2021, Schang et al., 2021). Examples of these include prisons, healthcare facilities such as hospitals or high-density housing areas, i.e., a housing estate. This is because unlike liquid samples, the passive sampler materials will adsorb as much virus as able until the viral saturation point is reached, meaning that a higher level of virus could be eluted compared to the liquid samples. Going forward, there could be options to extend passive sampling to detect outbreaks or areas where case rates begin to increase from low case rates or vice versa, it can also be applied to other human pathogens (Kevill et al., 2022a). Other human enteric pathogens that could be detected are sexually transmitted diseases such as hepatitis C, or other respiratory viruses (i.e. influenza). Passive sampling as a method could also be optimized for the detection and quantification of infection causing bacteria like the original use of the method (Moore et al., 1952).

### 2.8.1 Comparison of grab samples and passive samples viral recovery

Although multiple proof of concept studies have been conducted, they failed to compare passive sampling with liquid sampling as a whole. We compared grab sampling as a method against passive sampling. When compared passive samples with grab samples there was a greater variability in-between the grab samples compared to the passive samplers. This variability shows that passive samplers can be used in environments where the viral load is lower. As grab

samples are 'point in time' samples, variability in viral presence and absence as well as viral load is one limitation of their use and using a passive sampler suspended for 1 hour may provide more opportunity to detect pathogens of human health concerns in wastewater. Passive sampling may avoid undetermined results still seen in concentrated grab samples, even though grab recover higher gc/reaction, the passive samplers have lower variability in this study. This indicates an improved sensitivity when detecting SARS-CoV-2 as previously supported by other work (Liu *et al.* 2022).

### 2.8.2 Identification of the optimum passive sampler material

Overall, our study showed that cotton-based tampons are the best material to use for passive sampling in wastewater. Tampons yielded a higher viral recovery of SARS-CoV-2 when compared to ion exchange paper and cotton swabs. Our results indicate that Tampax will likely have improved recoveries over cotton swabs and ion exchange paper when viral loads are low. Previous work has commented on viral adsorption onto materials being driven by electrostatic, hydrophobic and van der Waals interactions primarily (Castaño et al., 2020). The contribution of these forces are tempered by the surrounding environment namely pH (Castaño et al., 2020, Vega et al., 2008), as this work was all conducted within the same influent WWT system it is assumed that pH and other environmental factors are relatively stable, thus viral interactions are largely reliant on the materials. All three materials (Cotton swabs, SG81 ion exchange paper and Tampax super compak) have electronegative charges. However, between tampons and cotton the main compositional difference is the rayon content in tampons, rayon is a semi-synthetic cellulosic fibre. It has been assumed that at wastewater pH levels (7-8), SARS-CoV-2 and its free

RNA will mainly be negatively charged. This implies that electropositive membranes should be tested in the future.

### 2.8.3 Comparison of passive sampler concentration methods

The no elution in PEG concentration method had the highest gene recovery mean for both SARS-CoV-2 N1 and crAssphage genes, suggesting that this method is the most effective at SARS-CoV-2 recovery from the sample, with the normalization of the human population of each catchment area using crAssphage as an indicator (Figure 5). The PEG precipitation method works on the reduction of solubility of proteins in the solution, which precipitates out viral matter from the solution (Machado-Moreira et al., 2020); this implies that the release of the wastewater liquid from the tampons is the most important factor to the levels of virus recovered. This implies that the physical entrapment may be more important than the bonds between the virus and the materials by differing chemical interactions, this is a statement that has previously been hypothesized previously (Brown et al., 2021)

Comparisons between our data and those already published are difficult due to the vast differences in methods (both processing and conditions in which the studies took place) for viral recovery (Corchis-Scott et al., 2021; Habtewold et al., 2022; Hayes et al., 2021; Liu et al., 2022; Schang et al., 2021). Previous studies have eluted virus from cotton gauze based passive samplers (Habtewold et al., 2022; Hayes et al., 2021; Liu et al., 2022; Rafiee et al., 2021; Schang et al., 2021), whereas electronegative filters and certain membranes (nylon etc) have used more direct extraction methods for viral detection (Hayes et al., 2021; Li et al., 2022). Hayes et al. (2021), found that elution methods yielded higher concentrations of viral recovery compared to direct

methods on certain passive sampler materials (cheesecloth and electronegative filters). This conclusion contradicts our results for elution methods, bar the no elution in PEG method. However, Hayes et al. (2021) performed a laboratory-based study that did not stimulate field conditions fully, the lack of a high flowing stream of water would mean that the materials would be more likely to hold together. Our study also used different elution buffers creating the need for further work within the method development to further optimise the elution step from various materials. Because PBS was used on the basis that it has been shown to reduce PCR inhibition in comparison to other elution buffers with a phosphate base, which is a known issue within wastewater based qPCR (McMinn et al., 2012). Phosphate also desorbs negatively charged viruses and free RNA from the surfaces of materials meaning it is an optimum solution to use for passive sampling.

The main limitation with the direct extraction method is the fact that the data needs to be scaled up from 1 $cm^2$ to a whole tampon size. For this method, usually only the end of the tampon is cut, if this end is submerged in the influent stream for longer this would affect recovery rates. Therefore, more work needs to be done comparing direct extractions with other methods using the same amount of material per method.

Overall, the use of a PEG-based method creates a multi-use of both passive and liquid wastewater testing for large scale testing. Although the whole processing time is lengthy in comparison to other methods tested here the actual bench time is still relatively short in comparison to other methods. For instance, the Beef Extraction methods contain the costly time-consuming process to adjust the pH of samples multiple times, and the direct extraction

introduces a larger variability in the reliability of the data produce due to the scaling of results and the precision required to cut the sampler in the first place (Table 1).

**Table 1: Summary of the overall performance and ranking of the seven different passive sampler concentration methods.**

| Scoring criteria | Direct extraction | Squeeze | PBS in PEG | PBS in ammonium sulfate | Beef in PEG | Beef in ammonium sulfate | No elution in PEG |
|---|---|---|---|---|---|---|---|
| Viral capture/recovery | | | | | | | |
| Ease of use | | | | | | | |
| Processing time | | | | | | | |
| Cost | | | | | | | |
| Overall score | 17 | 16 | 16 | 15 | 11 | 8 | 17 |
| Ranking | =2 | =4 | =4 | 5 | 6 | 7 | =2 |

| Score | |
|---|---|
| 1 | Low |
| 2 | |
| 3 | Medium |
| 4 | |
| 5 | High |

### 2.8.4 Further comparisons between passive sampler materials

Furthermore, when specified brands of tampons were compared, we found that Tampax super compak and pearl tampons recover more viral RNA than ASDA own brand tampons tested, however, there was no statistical significance between them for SARS-CoV-2, however crAssphage recovery was significantly difference between ASDA and the leading brands. There is little evidence as to whether this is solely based on a larger size. More work is needed to investigate if this is because the materials have a difference in charge or because of differences in material composition. These results could vary greatly due to differing materials presenting

different levels of viral and material interactions (electrostatic, hydrophobic and van der Waals) (Castaño et al., 2020).

## 2.9 Limitations of Passive sampling within WBE

Near-source WBE monitoring of pathogens, such as passive sampling, has a range of useful applications, however, one potential limitation is the high incidence of ragging during near-source monitoring. However, as this work used short deployment times (1 h) and mesh cages to protect the samplers, reducing the propensity for ragging. More work is therefore needed looking at minimizing ragging issues over longer deployment times, especially at near source locations. High ragging rates reduce the interactions between wastewater viruses and the material of passive samplers (Schang et al., 2021).

Another limitation to passive sampling is the difficulties in quantifying the infection rates of SARS-CoV-2 in an area because the total volume of wastewater flowing through and past the sampler is unknown. Work by Corchis-Schott et al. (2021), calculated the SARS-CoV-2 concentration based on a ratio of target virus to the faecal indicator PMMoV, much like our data utilises crAssphage, with the flow rate, wet faecal mass and the clinical case data for the number of infected people contributing to that community (Corchis-Scott et al., 2021). However, the overarching purpose of wastewater is to complement clinical data, so the use of this statistic in the method defeats the purpose. This only further demonstrates the difficulties faced within determining the quantity of infected people within the given area. Furthermore, it has not been noted within these calculations that previous work has denoted infection severity to be unrelated to faecal shedding rates (Gerrity et al., 2021; Zheng et al., 2020).

## 2.10 Supplementary Material

**Table S1***: Primers and probes used for viral detection with qPCR within this study.*

| Target | Reference | Primers and probe name | Primer and probe sequence (5'-3') | Target sequence (5'-3') |
|---|---|---|---|---|
| **SARS-CoV-2** | (Centers for Disease Control and Prevention, 2020) | N1-Forward | GACCCCAAAATCAGCGAAAT | GTGAAATGGTCATGTGTGGCGGTTCACT ATATGTTAA ACCAGGTGGAACCTCATC AGGAGATGCCACAACTGCTTATGCTAATA GTGTTTTTAA CATTTG |
| | | N1-Reverse | TCTGGTTACTGCCAGTTGAATCTG | |
| | | N1-Probe | ACCCCGCATTACGTTTGGTGGACC | |
| **CrAssphage** | (Stachler et al., 2017) | CrAss-Forward | CAGAAGTACAAACTCCTAAAAAACGTAGAG | CAGAAGTACAAACTCCTAAAAAACGTAG AGGTAGAGGTATTAATAACGATTTACGT GATGTAACTCGTAAAAAGTTTGATGAAC GTACTGATTGTAATAAAGCTAATGGCTTG TTTATTGGTC |
| | | CrAss-Reverse | GATGACCAATAAACAAGCCATTAGC | |
| | | CrAss-Probe | AATAACGATTTACGTGATGTAAC | |

**Table S2**: *Tampon sizes used for scaling direct extractions*.

| Tampon | Length (cm) | Width (cm) | Water Holding Capacity (ml) |
|---|---|---|---|
| ASDA own brand Regular | 6 | 3.5 | 32.8 |
| Tampax Compact Super | 6.2 | 3.6 | 34.2 |
| Tampax Pearl Super | 5 | 6 | 33.1 |

**Table S3**: *Summary statistics for passive sample extraction method (Figure 2).*

| Target | Extraction | Mean (gc/l) | Median (gc/l) | positive | undetermined |
|---|---|---|---|---|---|
| SARS-CoV-2 N1 | Auto | 88.1 | 0.0 | 6 | 24 |
| SARS-CoV-2 N1 | Manual | 201.0 | 68.1 | 17 | 13 |
| crAssphage | Auto | 172642 | 42912 | 30 | 0 |
| crAssphage | Manual | 1007677 | 638202 | 30 | 0 |

***Table S4**: Summary statistics for passive sample material.*

| Target | Material | Mean (gc/l) | Median (gc/l) | positive | undetermined |
|---|---|---|---|---|---|
| SARS-CoV-2 N1 | Cotton swab | 197.2 | 97.0 | 18 | 9 |
| SARS-CoV-2 N1 | Paper | 262.8 | 159 | 10 | 2 |
| SARS-CoV-2 N1 | Tampax | 501 | 368 | 20 | 7 |
| crAssphage | Cotton swab | 601510 | 473741 | 26 | 1 |
| crAssphage | Ion exchange paper | 337814 | 214332 | 12 | 0 |
| crAssphage | Tampax | 1059697 | 741626 | 27 | 0 |

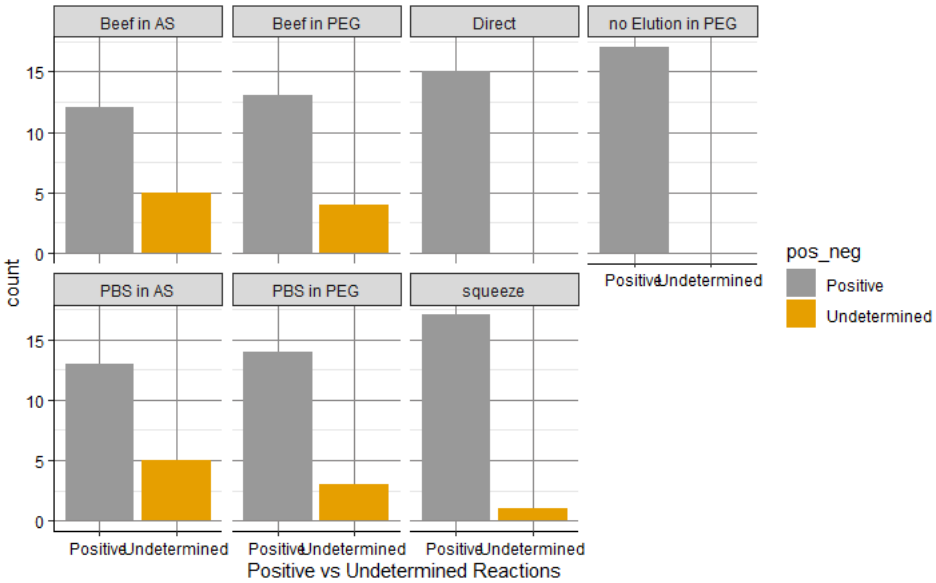

Figure S1: Comparison of number of tampons that recovered positive and undetermined results for each of the seven different concentration methods

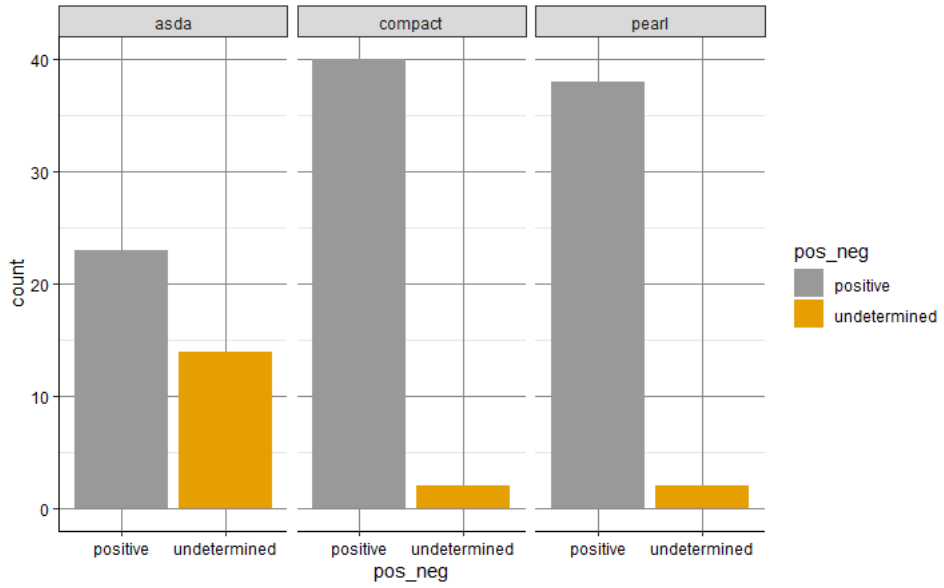


**Figure S2:** Comparison of number of tampons that recovered positive and undetermined results for each of the three different tampon types.

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## 2.13 Declaration of interests

All authors declare no competing interests.

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# Chapter 3: Assessment of two types of passive sample for the efficient recovery of SARS-CoV2 and other viruses from wastewater.

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# 3. ASSESSMENT OF TWO TYPES OF PASSIVE SAMPLER FOR THE EFFICIENT RECOVERY OF SARS-COV-2 AND OTHER VIRUSES FROM WASTEWATER

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**Keywords: COVID-19 surveillance, Sewage sampling**, **Viral capture method, Public health risk, Environmental monitoring**.

## 3.1 ABSTRACT

Wastewater-based epidemiology (WBE) has proven to be a useful surveillance tool during the ongoing SARS-CoV-2 pandemic and has driven research into evaluating the most reliable and cost-effective techniques for obtaining a representative sample of wastewater. When liquid samples cannot be taken efficiently, passive sampling approaches have been used, however, insufficient data exists on their usefulness for multi-virus capture and recovery. In this study, we compared the virus-binding capacity of two passive samplers (cotton-based tampons and ion exchange filter papers) in two different water types (deionised water and wastewater). Here we focused on the capture of wastewater-associated viruses including Influenza A and B (Flu-A & B),

SARS-CoV-2, human adenovirus (AdV), norovirus GII (NoVGII), measles virus (MeV), pepper mild mottle virus (PMMoV), the faecal marker crAssphage and the process control virus *Pseudomonas* virus phi6. After deployment, we evaluated four different methods to recover viruses from the passive samplers namely, (i) phosphate buffered saline (PBS) elution followed by polyethylene glycol (PEG) precipitation, (ii) beef extract (BE) elution followed by PEG precipitation, (iii) no-elution into PEG precipitation, and (iv) direct extraction. We found that the tampon-based passive samplers had higher viral recoveries in comparison to the filter paper. Overall, the preferred viral recovery method from the tampon passive samplers was the no-elution/PEG precipitation method. Furthermore, we evidenced that non-enveloped viruses had higher percent recoveries from the passive samplers than enveloped viruses. This is the first study of its kind to assess passive sampler and viral recovery methods amongst a plethora of viruses commonly found in wastewater or used as a viral surrogate in wastewater studies.

## 3.2 INTRODUCTION

The COVID-19 pandemic, declared in March 2020, is responsible for millions of deaths worldwide (World Health Organization, 2021) and has caused major disruption to world trade and social wellbeing (Wei et al., 2021). The causative agent of COVID-19 is SARS-CoV-2, an enveloped, spherical, positive sense, single-stranded RNA (+ssRNA) virus that causes a wide-ranging infection, on a clinical spectrum from asymptomatic infection to severe flu-like symptoms.SARs-CoV-2 infected symptomatic people suffer from fever, dry cough, and shortness of breath, with some severely symptomatic patients requiring hospitalization, sometimes resulting in death (Ali and Alharbi, 2020; Zhou et al., 2020).

Positive SARS-CoV-2 cases are most often confirmed in individuals via lateral flow testing or by reverse transcription quantitative polymerase chain reaction (RT-qPCR), conducted in a diagnostic laboratory. Testing is often conducted when the infected person becomes symptomatic, therefore asymptomatic cases may go undiagnosed (Kronbichler et al., 2020). For population-level disease surveillance, wastewater-based epidemiology (WBE) may be used. WBE enables the sampling of large areas (Agrawal et al., 2021; Karthikeyan et al., 2021; Kumar et al., 2021; Saguti et al., 2021) or can be used for small scale monitoring (Corchis-Scott et al., 2021). Either way, WBE, and is a non-invasive alternative to clinical disease monitoring (Wade et al., 2022). Detecting human pathogens through sewage is not an entirely new science and has been used for the past 75 years, mainly focusing on enteric bacteria and viruses transmitted via the faecal-oral route, for example typhoid, poliovirus, enterovirus, and adenovirus (Gell et al., 1945; Moore, 1951; Sinclair et al., 2008). SARS-CoV-2-infected individuals have been evidenced to shed virus in their faeces at $10^1$ to $10^8$ genome copies (gc) $g^{-1}$ (Cheung et al., 2020; Jeong et al., 2020;

Zheng et al., 2020), several days before symptoms commence (Zhu et al., 2021). The shedding of SARS-CoV-2 viral RNA through faeces is independent of infection severity, and all infected individuals, including asymptomatic and pre-symptomatic cases shed through their faeces (Gerrity et al., 2021; Zheng et al., 2020), whilst viral shedding in urine is rare (Huang et al., 2020; Lo et al., 2020). Therefore, WBE has now been successfully introduced to monitor SARS-CoV-2 on a spatial and temporal level in many communities across the globe (Agrawal et al., 2021; Ahmed et al., 2021; Gerrity et al., 2021; Gonzalez et al., 2020; Hillary et al., 2021; Kumar et al., 2020; La Rosa et al., 2020; Sherchan et al., 2020).The importance of WBE in viral monitoring has led to the development of different methods for sampling wastewater. Most of the research and surveillance has been conducted using grab or composite sewage samples (Shah et al., 2022), taken either manually or with an autosampler device. However, grab samples may not be representative due to diurnal variations in virus titres in wastewater, and the deployment of autosamplers is often not feasible for logistical or economic reasons. Therefore, alternative techniques for wastewater sampling are needed.

Passive sampling is a method stemming from (Moore, 1951) where an absorbent material is immersed in the wastewater stream to capture human pathogens (Schang et al., 2021)(Schang et al., 2021; Sikorski and Levine, 2020). However, their use is not limited to biological agents, as passive samplers are also used to monitor chemical agents and pollutants in the environment (Greenwood et al., 2007). Passive sampling works on the premise that the sampler material surface charge attracts and holds viruses over a prolonged period of time (Blanco et al., 2019). The use of passive samplers, such as gauze, cotton or paper, could reduce the cost of WBE, by reducing sampling machinery and requires no electrical power or running costs. Over the course

of the SARS-CoV-2 pandemic different passive samplers have been explored, such as cotton gauze (Habtewold et al., 2022; E. K. Hayes et al., 2021; Li et al., 2022b; Liu et al., 2022; Rafiee et al., 2021; Schang et al., 2021; Wang et al., 2022), cotton buds (Habtewold et al., 2022; Li et al., 2022b; Schang et al., 2021), cheese cloth (E. K. Hayes et al., 2021), electromagnetic membranes (Habtewold et al., 2022; E. K. Hayes et al., 2021; Emalie K. Hayes et al., 2021; Li et al., 2022a, 2022b; Schang et al., 2021), cellulose sponge (E. K. Hayes et al., 2021)and tampons (Bivins et al., 2022a; Corchis-Scott et al., 2021; Li et al., 2022b) and their effectiveness varies dependent upon a number of factors, such as surface volume of the sampler, length of exposure time and material composition, as reviewed by (Bivins et al., 2022a). As viral loads in wastewater can be low, the sampler should be designed to effectively concentrate the virus increasing detection sensitivity (Vincent-Hubert et al., 2021, 2017). This increased sensitivity may also allow early identification of low-level disease outbreaks, including SARS-CoV-2 as hypothetically discussed by (Jiang et al., 2022). Furthermore, passive sampling has shown to outperform grab samples, being more consistent with composite sampling in well designed studies (Bivins et al., 2022a), and thus more sensitive to changes in viral loads over time; however, this has only been evidenced for SARS-CoV-2 using Moore swab sampling (Rafiee et al., 2021). Recent studies have highlighted the use of *in-situ* passive samplers to detect enterovirus, human adenovirus (hAdV), and pepper mild mottle virus (PMMoV) and SARS-CoV-2 from wastewater (Li et al., 2022b). While these studies investigated different sampler types, with an aim to develop cheaper, less time consuming sampling methods (Li et al., 2022b). The effectiveness of viral recovery and samples processing methods from passive samplers for a range of viruses has not been explored. Therefore, assessing

the recovery of multiple viruses present in wastewater, using different methodologies and sampler type is needed to move WBE beyond SARS-CoV-2 monitoring.

## 3.3 METHODS

## 3.4 STUDY AIMS

This study aims to evaluate two passive sampler materials Tampax Super Compak Tampon (Procter & Gamble Inc., Cincinnati, OH) and Whatman SG81 Si-cellulose ion exchange paper (Global Life Sciences Solutions USA, Marlborough, MA), alongside four different viral concentration and extraction methods (phosphate buffered saline (PBS) elution into polyethylene glycol (PEG) precipitation, beef extract (BE) elution into PEG precipitation, no elution in PEG precipitation, and direct extraction), with two water types (wastewater and deionized water ($dH_2O$)), to identify the best material and method for viral recovery of SARS-CoV-2, Influenza A and B (Flu-A & B), measles (MeV), norovirus GII (NoVGII), adenovirus (AdV),. human faecal markers (crAssphage, pepper mild mosaic virus (PMMoV)), and one processing control virus (*Pseudomonas* virus phi6 (Phi6)). To the authors knowledge this is the first time that a comprehensive study, looking at multiple viral targets, samplers and recovery methods has been undertaken.

## 3.5 SAMPLE COLLECTION

On the 15$^{th}$ November 2021, 20 liters of untreated sewage influent (hereby referred to as wastewater) was collected at the central wastewater treatment plant (WwTP) at 09.00 h located in Bangor, North Wales, UK (53°12'34.04''N, 4°10'58.56''W). This sampling time was chosen to

reflect peak flow and aimed to capture the highest faecal load (Hillary et al., 2021). The WwTP serves a population of 40,000 people and is mainly composed of domestic wastewater with few industrial inputs. Samples of crude influent wastewater were taken from behind the primary screen (flow 285 l $s^{-1}$) in polypropylene bottles and immediately transported to the laboratory at 4°C for experimentation (within 5 km of the WwTP). At the time of collection, the pH of the wastewater was 7.28, the electrical conductivity was 605 µS $cm^{-1}$, the turbidity was 226 NTU and the ammonium and phosphate concentrations were 1.3 mg N $l^{-1}$ and 2.46 mg P $l^{-1}$, respectively.

## 3.6 VIRAL STOCKS AND SPIKING

For spiking, we used inactivated SARS-CoV-2 (kindly provided by Prof Andrew Weightman, Cardiff University), inactivated influenza A/California/07/2009 (H1N1), B/Lee/40 (kindly provided by Eleanor Gaunt, University of Edinburgh), norovirus GII in diluted and filtered faecal matter from a patient with confirmed norovirus infection (kindly provided by Dr Lydia Drumwright, University of Cambridge) and measles virus in the form of a vaccine (VWR International, USA). We also used the *Pseudomonas* spp. phi6 bacteriophage, which we cultured in-house as described in (Kevill et al., 2022).

We created four groups of samples: spiked wastewater, unspiked wastewater, spiked $dH_2O$, and unspiked $dH_2O$. To achieve this, we prepared 2 × 3000 mL $dH_2O$ and 2 × 3000 mL wastewater aliquots. Subsequently, one set of each water type was spiked with SARS-CoV-2, Flu-A, Flu-B, MeV, NoVGII and Phi6 to reach the final concentration of approx. $10^4$-$10^5$ genome copies (gc)/mL per virus. We used this specific concentration for spiking to enable the calculation of recoveries as low as 0.1% for the precise comparison of methods. Wastewater was spiked as the water

matrices is representative of a typical wastewater sample, and $dH_20$ was spiked to allow for inhibition to be compared between the two sample types. The unspiked wastewater samples were used to determine the baseline of viruses that occur in wastewater (SARS-CoV-2, NoVGII, MeV, Influenza A and B). The wastewater sample used for spiking was negative of MeV, Influenza A and B, yet positive for SARS-CoV-2 and NoVGII, albeit at levels much lower than spiking concentrations ($10^3$ gc / sampler). The unspiked $dH_2O$ were used as negative controls. Aliquots of 100 mL of wastewater and $dH_2O$ were placed in sterile polypropylene copolymer (PCCO) centrifuge jars for experimentation. Each aliquot was made in triplicate, in four groups for each of the four viral recovery methods and passive sampler type (Tampax Super Compak Tampon and SG81 ion exchange Whatman paper).

## 3.7 VIRAL RECOVERY METHODS

All samples were processed in a Containment level/Biosecurity level 2 (CL/BSC2) laboratory, in biological safety cabinets. For each of the methods and sample type (spiked or unspiked wastewater or $dH_2O$) a Tampax Super Compak Tampon or 3cm diameter circular, SG81 ion exchange Whatman paper (both now referred to as passive sampler) was placed into the corresponding 100 ml aliquot and left at room temperature (20°C) for one hour. The passive samplers were then recovered and transferred to plastic Ziploc® bags (SC Johnson & Son Inc., Racine, WI), and processed immediately as per the methods detailed below. This resulted in direct comparisons between spiked/unspiked sample and tampon/Whatman paper samplers.

Subsamples of 200 µl spiked and unspiked wastewater and $dH_2O$ were taken in triplicates at the start of the experiment. The nucleic acids were also extracted from these to allow baseline quantification of the viruses present.

### 3.7.1 Phosphate buffered saline (PBS) elution into polyethylene glycol (PEG) precipitation (PBS-PEG method)

Each of the passive samplers per sample type were saturated with 20 ml sterile PBS, pH 7.4. The PBS was then hand massaged into the passive sampler, the corner of the Ziploc® bag was cut and the liquid squeezed into a sterile 50 ml tube. The volume of each eluent was adjusted to 30 ml with PBS, this step ensured that the final concentration of PEG is consistent between samples. Samples were centrifuged at 3000 *g* at 4°C for 30 mins. The supernatant was then poured into a new sterile 50 mL centrifuge tube, without disturbing the pellet. A 10 ml aliquot of 40% PEG8000 with 8% NaCl (PEG-NaCl) solution was then added to each eluent to reach a final concentration of 10% PEG and 2% NaCl. The tubes were inverted several times to mix, followed by a 4°C incubation for 16 h. After incubation, the samples were precipitated at 10,000 *g* for 30 min at 4°C. The supernatant was then discarded without disturbing the pellet. The pellet was then resuspended in 800 µl of NucliSens lysis buffer (bioMérieux SA, Marcy l'Etoile, France). The viral nucleic acids were extracted using the NucliSens extraction reagents (bioMérieux SA), as described previously (Farkas et al., 2021). The final volume of the eluent was 0.1 ml.

### 3.7.2 Beef extract (BE) elution into PEG precipitation (BE-PEG method)

The BE-PEG method is identical to the PBS-PEG method described above except the 20 mL sterile PBS used to saturate the passive sampler has been replaced with 20 mL 3% beef extract

containing 0.5 M glycine (Lambertini et al., 2008). The BE solution was freshly prepared on the day of use and pH of adjusted to 7.0 using NaOH, prior to the saturation of the passive sampler. The nucleic acid extraction method is also the same as that described for the PBS-PEG method.

### 3.7.3 No elution in PEG precipitation (No elution-PEG method)

The no elution method follows the PBS-PEG method except that PBS was not added to the passive sampler. Instead, the passive sampler was massaged and the solution it contained directly squeezed into a sterile 50 ml tube. The rest of the protocol remains the same. The nucleic acid extraction method is also the same as that described for the PBS-PEG method.

### 3.7.4 Direct extraction method

The direct extraction method differed between tampon and Whatman paper sampler. A 1 $cm^2$ area was cut from the tampon while for the Whatman paper all of the 3 cm diameter circle was used. Subsequently, each material was placed into 50 ml sterile polyproylene tubes alongside 2 mL of NucliSens lysis buffer (bioMérieux SA). The samples were then vortexed for 10 secs and incubated at room temperature for 10 min. The tampon fragment or Whatman paper was then removed from the solution and viral nucleic acid were extracted as described previously (Farkas et al., 2021).

## 3.8 QUANTIFICATION OF VIRAL RNA/DNA

Due to the number of PCR targets, differing reaction chemistry and cycling conditions, details of each qPCR method and kit are displayed in Table 1 while the primers and probes used are presented in Table 2. All reactions were run on a QuantStudio Flex 6 (Applied Biosystems Inc.,

Waltham, USA), at a reaction volume of 20 µl. Samples were run in duplicate, against a ssRNA (SARS CoV-2 N1, Phi6, influenza A and B, and measles), ssDNA (PMMoV) or plasmid DNA (CrAssphage, Adenovirus and Norovirus) standard curve dilution series of the target sequence in the range of 1–$10^5$ copies $\mu l^{-1}$ per reaction. The standard curve concentration was determined by a qubit fluorometer (Invitrogen, Waltham, USA), prior to preparing the 10-fold standard dilution series for N1 and Phi6, as these RNA standards were made in-house as previously described (Kevill et al., 2022),The remaining targets were were purchased at $10^6$ copies /µl (Influenza A and B (Twist bioscience, San Francisco, CA, USA)) or $10^{12}$ copies / µl (Measles, PMMoV, CrAssphage, Adenovirus and Norovius (IDT, Iowa, USA)) from commercial companies. PCR no-template controls (molecular-grade water) determined the absence of contamination during the PCR set-up. For RNA targets (Table 1), the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems Inc., USA) was used with 4 × Reaction Mix with ROX, 10 pmol of the forward, 20 pmol of the reverse primers and 5 pmol probe, 16 nmol $MgSO_4$, 1 µg bovine serum albumin (BSA), molecular grade water and 4 µl sample/standard/control. For DNA targets (Table 1) the QuantiNova Probe qPCR and QuantiFast SYBR reactions are previously described (Farkas et al., 2019, 2018).

**Table 1**. Viral target, genome structure, kit and qPCR cycling conditions.

| Viral Target | Genome | Kit | Cycling conditions |
|---|---|---|---|
| **SARS-CoV-2** | RNA | TaqMan Fast Virus 1-Step Master Mix | 50°C 30 min, 95°C 20 sec, x 45 cycles of 95°C 0.03 sec, 60°C 3 min |
| **Phi6** | | | |
| **Influenza-A** | | | |
| **Influenza-B** | | | |
| **Norovirus GII** | | | |
| **Measles** | | | |
| **CrAssphage** | DNA | QuantiNova | 95°C 2 min, x 40 cycles of 95°C 15 sec, 60°C 1 min |
| **Adenovirus** | DNA | Quantifast SYBR | 95°C 5 min, x 40 cycles of 95°C 15 sec, 55°C 1 min, melt at 95°C 15 sec, 60°C 1 min, 95°C 15 sec |

**Table 2.** Primers and probes used for qPCR viral detection within this study.

| Target | Reference | Primers and probe name | Primer and probe sequence (5'-3') | Target sequence (5'-3') |
|---|---|---|---|---|
| **SARS-CoV-2** | (Centers for Disease Control and Prevention, 2020) | N1-F | GACCCCAAAATCAGCGAAAT | GTGAAATGGTCATGTGTGGCGGTTCACTATATGTTAA ACCAGGTGGAACCTCATC AGGAGATGCCACAACTGCTTATGCTAATAGTGTTTTTA A CATTTG |
| | | N1-R | TCTGGTTACTGCCAGTTGAATCTG | |
| | | N1-P | ACCCCGCATTACGTTTGGTGGACC | |
| **Norovirus GII** | (ISO, 2019) | NoVGII-F | ATGTTCAGRTGGATGAGRTTCTCW GA | ATGTTCAGATGGATGAGAT TCTCAGATCTGAGCACGTGGGAGGGCGATCGCAATCT GGCTCCCAGTTTTGTGAATGAAGATGGCGTCGA |
| | | NoVGII-R | TCGACGCCATCTTCATTCACA | |
| | | NoVGII-P | AGCACGTGGGAGGGCGATCG | |
| **Pepper mild mottle virus** | (Haramoto et al., 2013) | PMMoV-F | GAGTGGTTTGACCTTAACGTTTGA | GAGTGGTTTGACCTTAACGT TTGAGAGGCCTACCGAAGCAAATGTCGCACTTGCATT GCAACCGACAA |
| | | PMMoV-R | TTGTCGGTTGCAATGCAAGT | |
| | | PMMoV-P | CCTACCGAAGCAAATG | |
| **CrAssphage** | (Stachler et al., 2017) | CrAss-F | CAGAAGTACAAACTCCTAAAAAAC GTAGAG | CAGAAGTACAAACTCCTAAAAAACGTAGAGGTAGAGG TATTAATAACGATTTACGTGATGTAACTCGTAAAAAGT TTGATGAACGTACTGATTGTAATAAAGCTAATGGCTTG TTTATTGGTC |
| | | CrAss-R | GATGACCAATAAACAAGCCATTAG C | |
| | | CrAss-P | AATAACGATTTACGTGATGTAAC | |
| **Influenza A** | (Shu et al., 2021) | Influ-A-F | CAAGACCAATCYTGTCACCTCTGAC CAAGACCAATYCTGTCACCTYTGAC | AAAGACAAGACCAATCCTGTCACCTCTGACTAAGGGG ATTTTAGGATTTGTGTTCACGCTCACCGTGCCCAGTGA GCGAGGACTGCAGCGTAGACGCTTTGTCCAAAATGCC CTAAATGGG |
| | | Influ-A-R | GCATTYTGGACAAAVCGTCTACG GCATTTTGGATAAAGCGTCTACG | |
| | | Influ-A-P | TGCAGTCCTCGCTCACTGGGCACG | |
| **Influenza B** | (Shu et al., 2021) | Influ-B-F | TCCTCAAYTCACTCTTCGAGCG | GGATCCTCAACTCACTCTTCGAGCGTTTTGATGAAGGA CATTCAAAGCCAATTCGAGCAGCTGAAACTGCGGTGG GAGTCTTATCCCAATTTGGTCAAGAGCACCGATT |
| | | Influ-B-R | CGGTGCTCTTGACCAAATTGG | |
| | | Influ-B-P | CCAATTCGAGCAGCTGAAACTGCG GTG | |
| **Measles virus** | (Hummel et al., 2006) | MeV-F | TGGCATCTGAACTCGGTATCAC | TGGCATCTGAACTCGGTATCACTGCTGAGGATGCAAG GCTTGTTTCAGAGATTGCAATGCATACTACTGAGGACA |
| | | MeV-R | TGTCCTCAGTAGTATGCATTGCAA | |

| | | | | |
|---|---|---|---|---|
| | | MeV-P | CCGAGGATGCAAGGCTTGTTTCAGA | |
| **Phi6 phage** | (Gendron et al., 2010) | Phi6-F | TGGCGGCGGTCAAGAGC | TGGCGGCGGTCAAGAGCAACCCGGTCGTCGCAGGTCTGACACTCGCTCAGATCGGAAGCACCGGTTATGACGCCTATCAGCAGCTTCTGGAGAATCATCC |
| | | Phi6-R | GGATGATTCTCCAGAAGCTGCTG | |
| | | Phi6-P | CGGTCGTCGCAGGTCTGACACTCGC | |
| **Adenovirus** | (van Maarseveen et al., 2010) | AdV_R | CCGGCCGAGAAGGGTGTGCGCAGGTA | CATGACTTTTGAGGTGGATCCCATGGATGAGCCCACCCTGCTTTATCTTCTTTTCGAAGTCTTCGACGTGGTCAGAGTGCACCAGCCACACCGCGGCGTCATCGAGGCCGTCTACCTGCGCACACCGTTCTCGGCCGG |
| | | AdV_F | CATGACTTTTGAGGTGGATC | |

## 3.9 DATA ANALYSIS

qPCR data analysis and quality control were performed using the QuantStudio Real-time PCR software v1.7 (Applied Biosystems, Inc., USA). Viral concentrations were expressed as gc/µl nucleic acid extract and were converted to gc/sampler (wastewater or $dH_2O$) by multiplying gc/µl by final nucleic extract elution volume. The data for direct extraction method using tampon passive samplers were multiplied up to one whole tampon, as the PSB-PEG, BE-PEG, and no elution methods recover viruses from one whole tampon, this allowed for direct comparisons between methods. Triplicate 200 µl unconcentrated subsamples also underwent total nucleic acid extraction. These subsamples provided the baseline gene copies spiked per 100 ml of sample and enabled the calculation of viral recoveries. The gc/100 ml were calculated by multiplying gc/ul nucleic extract by volume of eluent (100) and then multiplying by the sample volume extracted (500). Viral recoveries were calculated by taking the average gc/sampler and then dividing by the average baseline viral concentrations. This data was then used for statistical analysis.

Statistical tests were carried out in R (R Core Team, 2020); the full script and data are provided in a dedicated repository (https://github.com/CameronPellett/spiked-passive-Bangor).

Passive sample material (tampon and Whatman paper), concentration and extraction method (BE-PEG, direct extraction, no elution PEG, and PBS-PEG), water type (wastewater WW, and deionised water DW), and virus envelope (enveloped, and non-enveloped) were selected as factors and co-variates of viral recovery. A sequential approach to analysis was adopted,

removing data after each test to ensure later results were representative of the expected environmental conditions and best sampling practices. A multiple linear model with interaction effects was considered but deemed unacceptable due to unrepresentative groups of data after initial tests. First, viral recovery was compared between water types to clarify the effect of inhibitors; water type comparisons were made with only spiked samples, due to a lack of naturally present virus in unspiked deionised water. After assessing water type, results for deionised water were removed from later assessment as they would not mimic expected environmental conditions in WBE. Second, the viral recovery using tampon and Whatman paper passive sample materials were compared. Following the material comparison, results using the material with significantly lower recovery were removed so that later comparison would not be skewed by a less effective material. Third, after selecting the best passive sampler material, viral recovery between laboratory processing methods were compared for samples suspended in wastewater. Then finally, the effect of a viral envelope was compared using results from the best passive sampler material suspended in wastewater with results from all processing methods. For statistical tests the recovery percentile was log transformed to meet assumptions of a Gaussian distribution (see supplementary materials Figure S1-S5 for quantile-quantile plots). Equality of variances were tested with F tests. Statistical comparisons of features with two levels and non-equal variance were made with Welch two sample t-tests. Comparisons with three or more levels and non-equal variance were made with a Welch ANOVA (one way comparison of means), followed by pairwise two sample t-tests without pooled standard deviations, adjusting p-values with the Holm-Bonferroni method. Paired tests were not selected due to missing data created by removal of undetermined results and sample removal during qPCR quality control.

## 3.10 RESULTS

### 3.10.1 Wastewater reduces recovery of spiked virus compared to deionised water

To clarify the effect of inhibitors, recovery of virus from passive samples suspended in wastewater and deionised water ($dH_2O$) were compared. Passive samples had a greater median recovery of spiked viruses when they were suspended in deionised water (1.26%; n = 121) compared to suspension in wastewater (0.26%; n = 136). The difference between water types was found to be significant when comparing log transformed recovery (Figure 1a; Welch Two Sample t-test (log y): t = 2.1, df = 198, *p*-value < 0.05); this trend was seen in comparisons between all individual viruses (Figure 1b). These results suggest chemicals or other materials in wastewater influence uptake of virus on the passive sampler or inhibit later processing and quantification of the viral nucleotides collected in the sample. After identifying the differences in recovery between water types, only data for wastewater suspended samples were taken forward for further analysis, as these better reflected real-world conditions. Details of sample type, sampler type and viral recovery are also individually presented in supplementary figure 1.

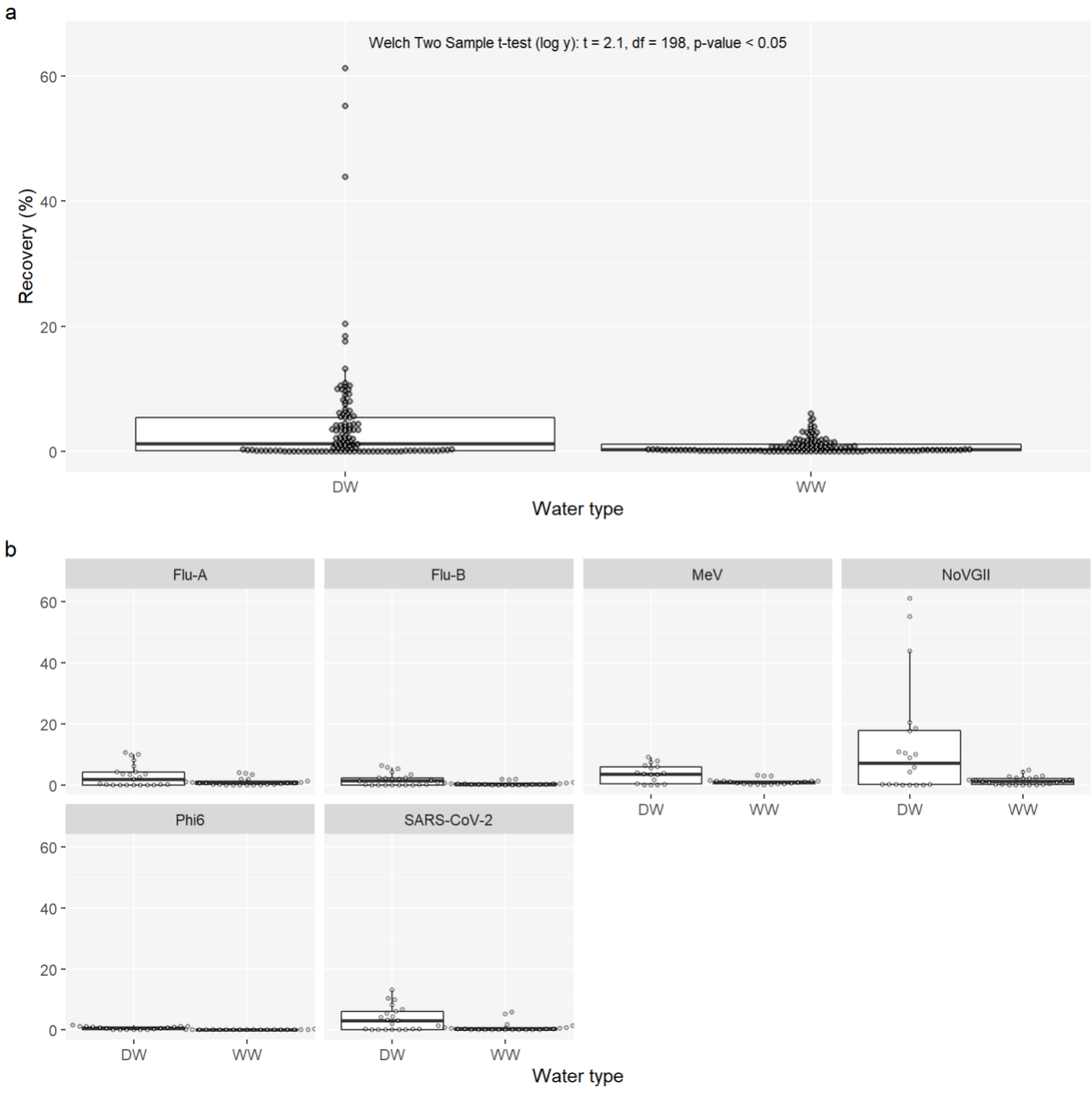
a
Welch Two Sample t-test (log y): t = 2.1, df = 198, p-value < 0.05
Recovery (%)
60
40
20
0
DW
WW
Water type
b
Flu-A
Flu-B
MeV
NoVGII
Phi6
SARS-CoV-2
60
40
20
0
DW
WW
Water type

Figure 1: Comparison of spiked virus recovery using passive samplers in wastewater (WW) and deionised water (DW). Panel 'a' combines data for all viruses, whilst panel 'b' separates recovery by each virus, such as influenza A and B (Flu-A and Flu-B), measles virus (MeV), SARS-CoV-2 (N1), norovirus GII (NoVGII) and bacteriophage phi6 (Phi6). A Welch two sample t-test was used to compare log transformed recoveries. Biological replicates were not averaged.

### 3.10.2 Tampon passive samplers have improved recovery over Whatman paper in wastewater samples

To identify the optimum material of passive samplers, tampon and Whatman paper samplers were compared. The median recovery of Tampon passive samplers (0.59%; n = 136) were greater than the recoveries observed for Whatman paper (0.16%; n = 140). This was found to be significant when comparing log transformed recovery (Figure 2a; Welch Two Sample t-test (log y): t = 11.6, df = 248, *p*-value < 0.001), the same trend was seen in all individual viruses (Figure 2b). These results indicate Whatman paper is not suitable as a wastewater passive sampler. Therefore, all further data analysis was performed on the tampon passive sampler data.

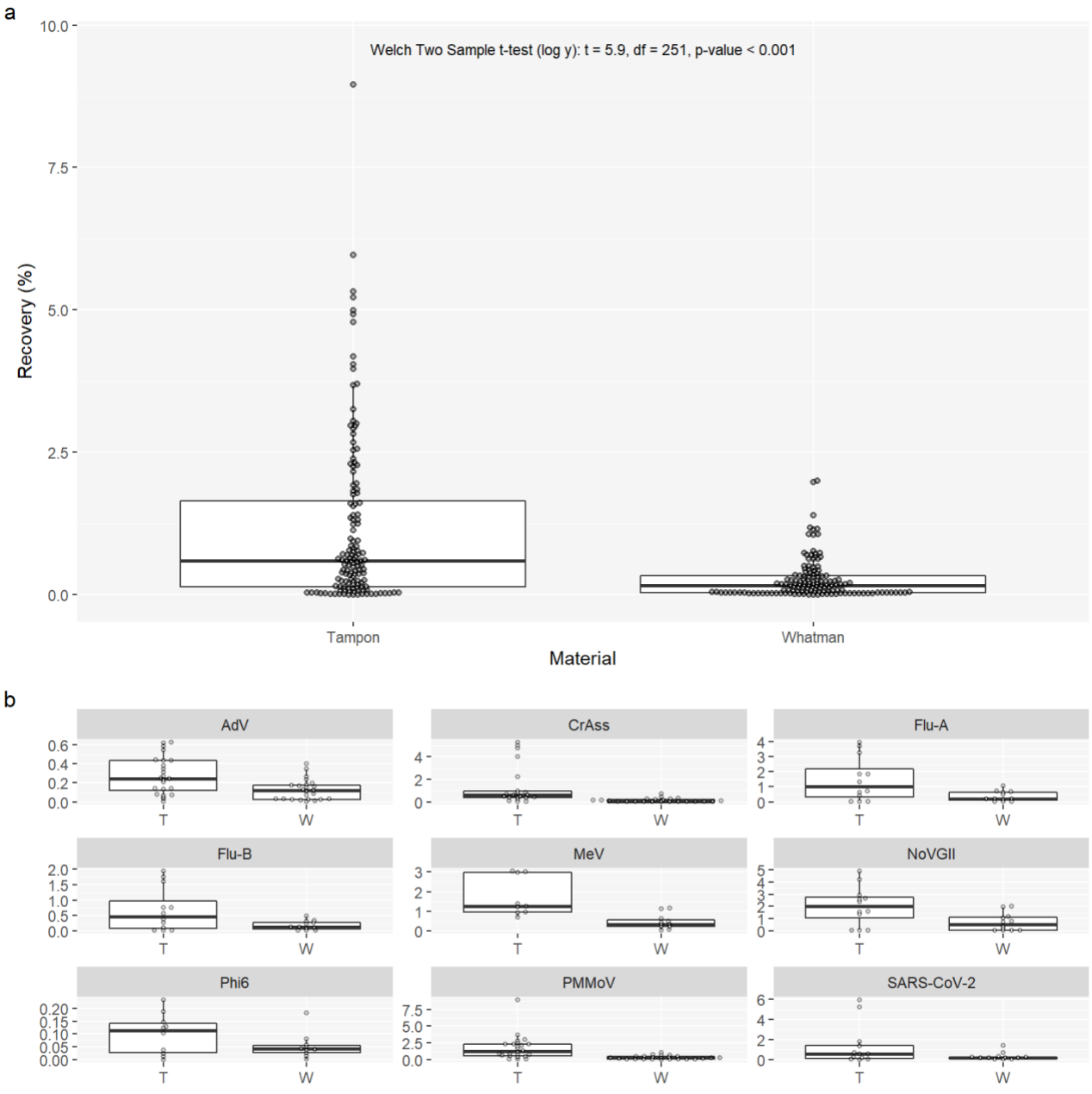
a
Welch Two Sample t-test (log y): t = 5.9, df = 251, p-value < 0.001
Recovery (%)
10.0
7.5
5.0
2.5
0.0
Tampon
Whatman
Material
b
AdV
CrAss
Flu-A
Flu-B
MeV
NoVGII
Phi6
PMMoV
SARS-CoV-2
T
W

Figure 2: Comparison of viral recovery of tampon (T) and Whatman (W) paper passive samplers suspended in wastewater. Panel 'a' combines data for all viruses, whilst panel 'b' separates recovery by each virus such as human adenovirus (AdV), crAssphage (CrAss), Flu-A and Flu-B, MeV, SARS-CoV-2, NoVGII, Phi6 and pepper mild mottle virus (PMMoV). A Welch two sample t-test was used to compare log transformed recoveries.

### 3.10.3 No elution PEG and direct extraction methods have improved viral recovery

To select the most efficient passive sample viral concentration and extraction method, four processing methods were compared. The no elution PEG concentration method had the highest median viral recovery (1.97%; n = 36), followed by direct extraction (0.81%; n = 36), BE-PEG (0.43%; n = 36), then PBS-PEG methods (0.03%; n = 28). Significant differences between the log transformed viral recovery of the methods were found (Figure 3a; Welch ANOVA (log y): $F = 28.1$, $df = 3$, $p$-value < 0.001), though pairwise comparisons found no significant difference between the no elution PEG and direct extraction methods (Figure 3c). This was likely due to some viruses (AdV and Phi6) having greater recovery with direct extraction compared to no elution PEG (Figure 3b). These results suggest the no elution PEG method is generally preferred for processing passive samples, but if AdV or Phi6 are the primary targets, the direct extraction method may be selected.

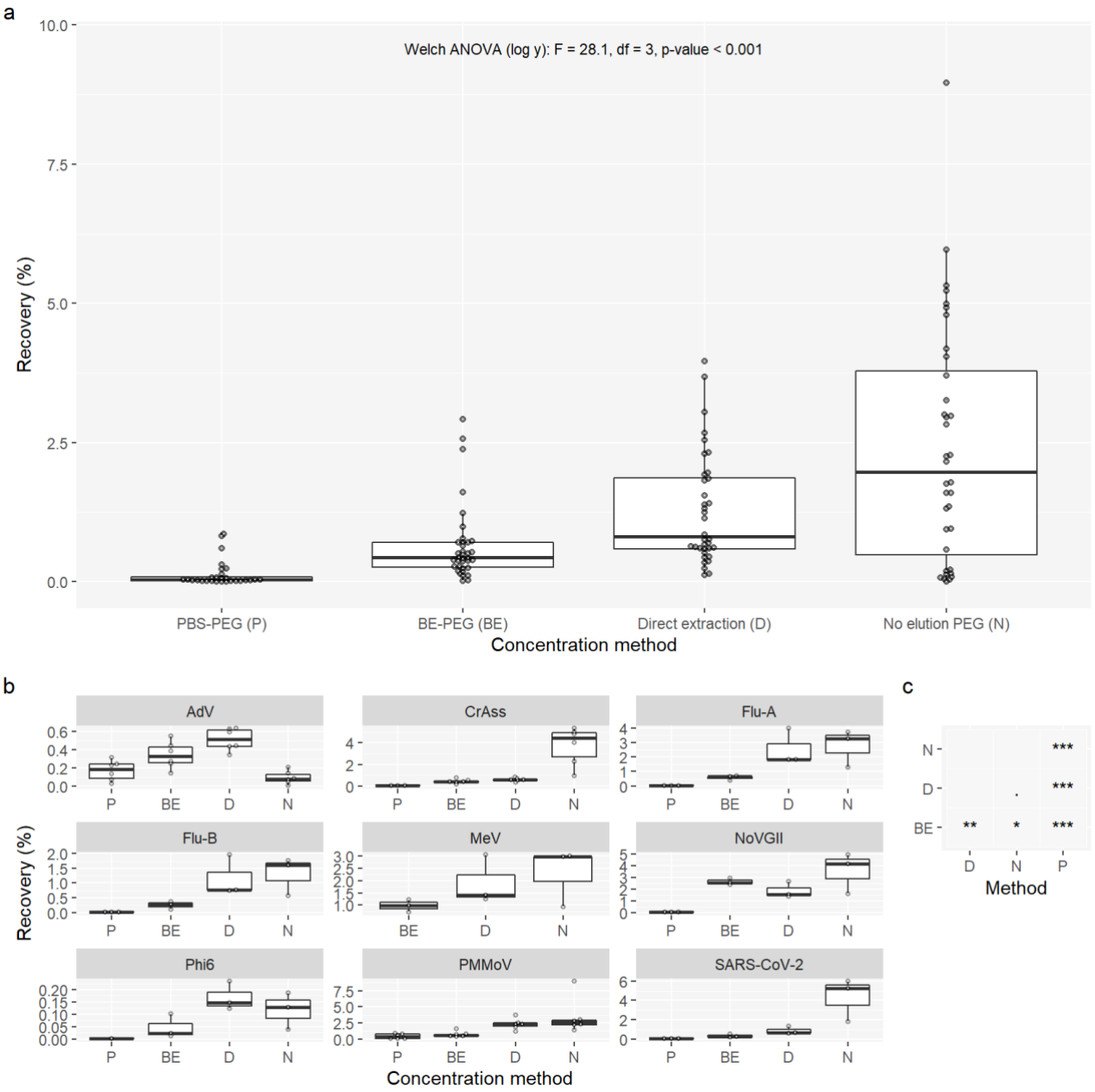


Figure 3: Comparison of viral recovery between processing methods carried out on tampon passive samples suspended in wastewater. Panel 'a' combine's data for all viruses, panel 'b' separates recovery by each virus, and panel 'c' shows p-values (*p*-value: < 0.001 [***]; < 0.01 [**]; < 0.05 [*]; > 0.05 [.]) of pairwise t-tests without pooled standard deviations adjusted with the Holm-Bonferroni method.

### 3.10.4 Enveloped viruses have reduced recovery using passive samplers

The viral envelope was identified as a potentially key virus characteristic that may impact viral recovery. Enveloped and non-enveloped viruses were, grouped and their recovery compared. Non-enveloped viruses had a greater median recovery (0.63%; n = 90), compared to enveloped viruses (0.32%; n = 46). The difference in mean log transformed recovery between enveloped and non-enveloped viruses was found to be significant (Welch Two Sample t-test (log y): t = 2.5, df = 71, *p*-value < 0.05; Figure 4a). These results indicate the viral envelope may be influencing the uptake of viral particles by the passive sampler.

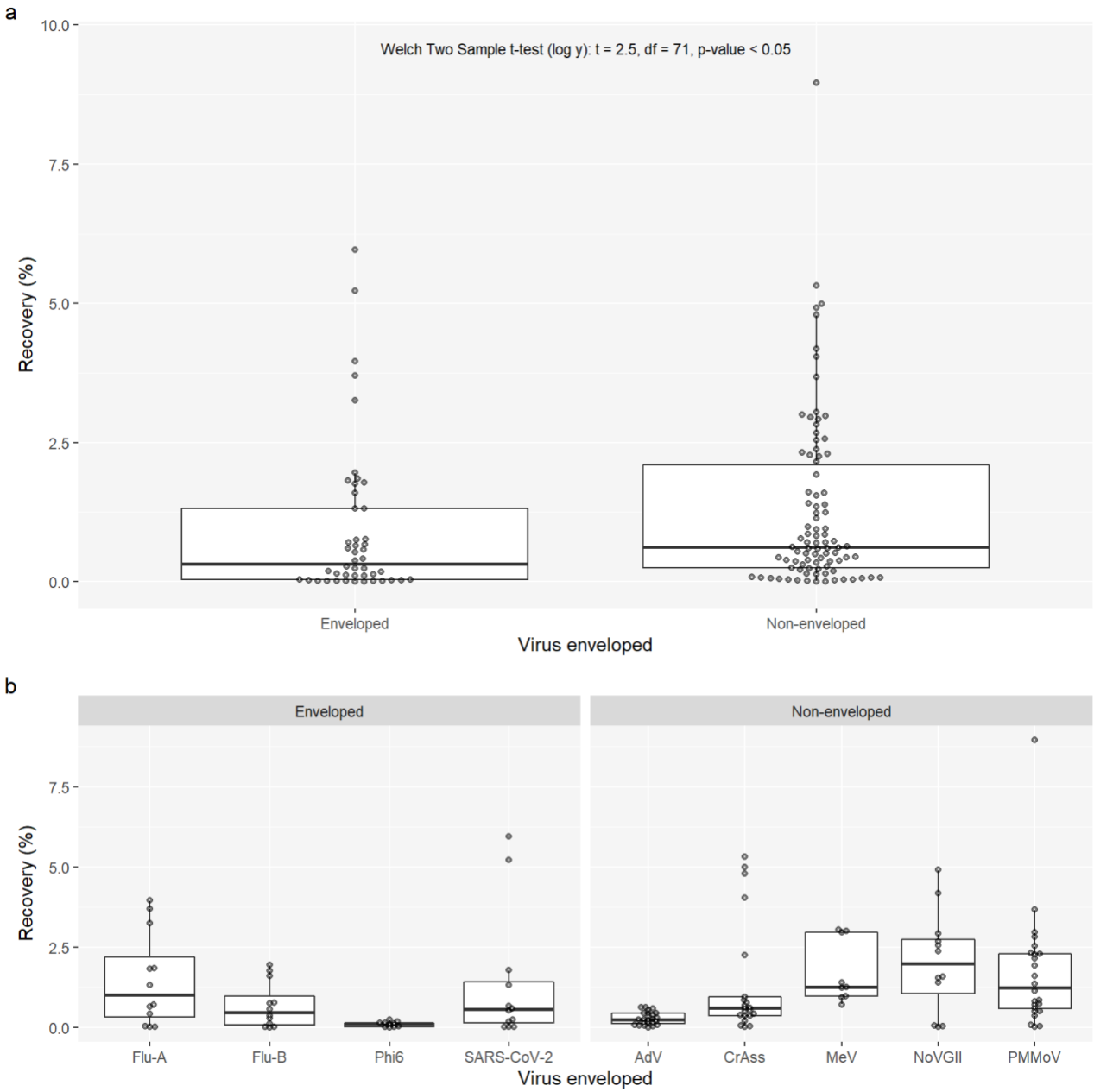


Figure 4: Comparison of enveloped and non-enveloped virus recovery. Panel ‘a’ combines data from all viruses, whilst panel ‘b’ separates viruses individually. A Welch two sample t-test was used to compare log transformed recoveries.

## 3.11 DISCUSSION

Cotton, tampon-based passive sampler devices have been used for wastewater viral infection surveillance in previous studies (Bivins et al., 2022b; Corchis-Scott et al., 2021; Li et al., 2022b) and have proven successful. A study of different passive sampler types show tampons are more effective than some traditional (Moore swab) and novel (cotton-based medical gauze swab) materials for the recovery of SARS-CoV-2 from wastewater *in-situ* (Lambert-Slosarska et al., 2022, paper in prep). This study compares the two best passive sampler methods; Tampon and Whatman filter Paper as per Lambert-Slosarska et al. (2022, paper in prep), for the recovery of eight viruses commonly detected in wastewater and one internal control. This study evidenced that viral recoveries from Whatman paper were poor compared to that of tampons. Both the tampons and Whatman paper were saturated in sample for one hour, showing that short sampling regimes allow viral absorption in tampon passive samplers, yet viruses were barely recovered using the Whatman paper. We recognise that one limitation of this study is that passive sampler materials were submerged in a constant concentration of viruses, in a laboratory setting, which does not reflect the field scenario for passive sampling. Therefore, we could not assess the recovery of low abundant viruses or the effect of flow rate upon the passive sampler material. In this study we saturated the passive sampler material for one hour, which was efficient for viral capture. One hour was selected as tampon deployment into the main wastewater stream in field experiments showed that tampons reached saturation within <3 hrs (unpublished data). Furthermore, short sampling regimes (<8 hrs) using tampons have been recommended, whilst other passive sampler types such as electronegative membranes may be better for longer continuous sampling (48 hrs) (Li et al., 2022b).

A range of viruses commonly detected in wastewater (Ahmed et al., 2020a; Eftim et al., 2017; Elmahdy et al., 2019, 2020; Heijnen and Medema, 2011; Hewitt et al., 2011; Kazama et al., 2016; Rosario et al., 2009; Tiwari et al., 2021), as well as viral surrogates (phi6) were selected to determine viral affinity for passive sampler type. Understanding viral affinity for passive sampler type is crucial for future experimental design in a world beyond SARS-CoV-2 surveillance where other viral targets begin to be monitored. The method used to recover/precipitate viruses from tampon passive samplers impacted percent recovery, and we found that the no-elution and direct extraction methods were best for viral recoveries from tampons. Viral recoveries were no greater than 10% when using tampons as passive samplers, while other studies evidence far higher viral recoveries of viruses precipitated directly from wastewater (Ahmed et al., 2020c; Brinkman et al., 2013; Farkas et al., 2018; Ikner et al., 2012) Farkas et al., 2022, paper in prep). Passive samplers allow for ease of sampling, are cheaper than using autosamplers, and in the case of the direct extraction method provide much faster sample to data turn around. Therefore, whilst recoveries are lower than some precipitation methods, passive samplers maybe preferred due to constraints such as short reporting times, lack of funds or expensive equipment. Furthermore, alternative passive samplers such as electromagnetic membranes have been shown to comparable to composite samplings (Habtewold et al., 2022; Schang et al., 2021) and may be a better alternative to tampon passive samplers if readily available.

The target virus may influence the choice of method used for viral recovery/precipitation from tampon passive samplers. The no elution PEG method is preferred, as viral recoveries were slightly higher (albeit non-significantly so) when compared to the direct extraction method. The

no elution PEG method had consistently higher viral recovery for all viruses except AdV. If AdV is the target virus, then the direct extraction method is preferred for viral recovery from tampon passive samplers. However, In addition to consideration of viral type and method for viral recovery from tampons, it is also worth considering the viral structure (enveloped or unenveloped), as unenveloped viruses had significantly higher mean recovery than enveloped ones. This may be explained by the fact that enveloped viruses are considered more fragile in the environment, as the phospholipid bilayer envelope and its associated proteins are more likely to be affected by changes in temperature, pH, and some disinfectants (Dvorak et al., 2005; Saadatpour and Mohammadipanah, 2020) than non-enveloped viruses (Firquet et al., 2015). Similarly, enveloped viruses are also likely to become inactivated in wastewater than non-enveloped viruses (Casanova et al., 2009; Gundy et al., 2009; Ye et al., 2016), this is particularly true for SARS-CoV-2 (Ahmed et al., 2020b; Rimoldi et al., 2020; Tran et al., 2021; Wang et al., 2005). For this reason, the use of inactivated, enveloped viruses for this study is appropriate; However, it is worth noting that enveloped viruses are diverse and little knowledge is available about their fate and persistence in wastewater environments. The alternative is that non-enveloped viruses have a higher affinity to tampon passive samples than enveloped viruses and hence cannot be eluted efficiently, however, this is yet to be evidenced.

The comparison between viral recoveries from $dH_2O$ and wastewater indicate that inhibitors are also present in samples recovered/precipitated from passive samplers, which ultimately affect qPCR and potentially other downstream applications. Inhibitors are naturally found in substances that make up the wastewater matrices such as bile salts in faeces (Lantz et al., 1997), complex polysaccharides found in faeces and plant material (Demeke and Adams,

1992; Monteiro et al., 1997), humic substances found in soils and plant materials (Tsai and Olson, 1992; Watson and Blackwell, 2000), and urea (Khan et al., 1991). Efforts can be made to reduce inhibitor levels, such as precipitating the solids from liquid via the initial centrifugation of the sample and including multiple wash steps during nucleic acid extraction (as per our protocols), however, inhibitor presence in wastewater samples are often unavoidable. Furthermore, the effect of surfactants originating from cleaning products upon passive samplers' ability to retain viral particles and nucleic acids is unknown and may be a potential route for viral loss/degradation; further research into this is suggested.

## 3.12 Conclusion and future research

Overall, tampons as passive samplers were more effective than Whatman Si-cellulose ion exchange filter papers for the recovery of viruses from wastewater. Our data suggest that viruses can be recovered from passive samplers by simply draining and concentrating the liquid from the samplers or by extracting viral nucleic acids directly from the passive sampler material. The deployment and process of passive samplers are simple, affordable and can be implemented at any WBE surveillance laboratory. Therefore, we recommend the use of tampons as samplers in areas where composite sampling is not feasible.

Viral structures had a significant effect on viral recoveries, and further work is needed to understand the mechanisms behind this. Future studies into the effect of temperature, pH, and disinfectants upon viral recovery from tampon passive samplers are also needed to fully understand the impact of wastewater matrices on viral recovery. In addition, the housing of the

passive samplers also requires further consideration as this may also influence the efficiency of viral capture.

## 3.13 Author contributions

Conceptualisation; KF, JLK. Laboratory work; KLS, NW, IRO, IP, NAS. Data analysis and curation; CP, KLS, JLK. First manuscript draft; JLK, KLS, CP. Final suggested edits; KF, DLJ. Funding acquisition; DLJ, KF.

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## 3.15 Conflicts of interest

The authors declare no conflict of interest.

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# 4. CONCLUSION AND FUTURE WORK

## 4.1 CONCLUSION

This work helped to advance the knowledge on passive sampling in relation to SARS-CoV-2, by testing multiple different concentration and extraction techniques as well as various materials to discover the pitfalls and merits of each method, so that their suitability for various programs can be assessed. This thesis was conducted during the SARS-CoV-2 pandemic, where the traditional method of monitoring viral infections within wastewater was from liquid sampling, namely grab and composite sampling. This is where either a single volume of liquid is collected at various time points and assessed individually (grab sampling) or where these volumes of liquid are collated over a certain time period (composite sampling). Although both methods are affective composite sampling was shown to offer a more complete picture of the viral levels over the whole time period rather than just a spot level insight like with grab sampling. However, these methods can not only be expensive but also require more infrastructure in place than passive sampling. This means that these methods are not available to those in low economic areas, as well as those that are prone to theft or in difficult to reach locations. This is where the main idea behind passive sampling as a solution to other more costly and resource intensive methods of viral detection starts.

At the time of writing little was known about the use and effectiveness of passive samplers for the detection and quantification of SARS-CoV-2. Previous papers had used tampons as a detection method however these methods were not yet compared to other passive sampling

materials that were available. Therefore, this thesis aimed to bridge these knowledge gaps within Chapter two.

This work found that passive sampling can be used for the detection and quantification of SARS-CoV-2 and other human viruses from influent wastewater. The data presented in the chapter two suggest that manual extractions yielded a higher viral recovery compared to an automated system. Furthermore, we found that tampons were the material that recovered the highest quantity of SARS-CoV-2 when compared to cotton swabs and an ion exchange paper material. The method that is the best at recovering SARS-CoV-2 from influent wastewater is no-elution in PEG. Passive samplers, specifically tampons, recover more virus at lower viral loads then traditional grab samples.

Following on from this work Chapter 3 was aimed at assessing the passive sampling material – tampons - in a wider context in terms of environmental human infectious viral detection. This was to see how effective passive sampling as a technique was for the detection of other human viruses from environmental samples. Chapter three investigated the use of passive sampling for the detection of SARS-CoV-2 and other viruses (Influenza A and B (Flu-A & B), SARS-CoV-2, human adenovirus (AdV), norovirus GII (NoV GII), measles virus (MeV), pepper mild mottle virus (PMMoV), the faecal marker crAssphage and the process control virus *Pseudomonas* virus phi6) from wastewater. All samples were extracted manually, and tampons were used as the sampler materials, as these were found to recover the highest viral load for SARS-CoV-2 detection as found in Chapter two. For this we tested the four viral recovery methods that yielded the highest viral recoveries from the second chapter. We found that the preferred

viral recovery method from the tampon passive samplers were the no-elution/PEG precipitation method. We also evidenced that non-enveloped viruses had higher percent recoveries from the passive samplers than enveloped viruses. Through this experiment we compared three different brands of tampons and found that there was no significant difference between viral recoveries and specified brands of tampons.

Overall, passive sampling is a great technique that can be used to detect and quantify multiple viruses at near-source monitoring. However more work needs conducting on passive sampling to explore the techniques full capabilities.

## 4.2 Future Work

The use of passive sampling has wider applications then just the ones talked about in this thesis. Much more research is required into the use of passive samplers not only for detection of human pathogens within wastewater but also different applications to wider environmental health issues. This thesis brings up multiple questions surrounding the precise mechanisms of passive sampling for the detection of viruses, the specifics of which are about to be discussed.

The second chapter which investigated the best method and material for passive sampling within wastewater. This chapter also touched on the ragging rates of passive samplers when deployed on a longer-term basis (over 24 h). More work needs to be done to reduce the rates of ragging and the difficulties this presents when conducting laboratory work. Such as investigating the best housing devices for different samplers and the best way to ‘clean’ the samplers of unwanted debris (i.e. sediments, cosmetic items). Further work could also be done exploring the

effectiveness of passive sampling over longer time periods (>24 hrs), this work could also explore the differences between automatic sampling devices and passive sampling further.

The third chapter addresses the fact that viral structure affects passive sampling viral recovery rates, therefore future work needs to address the effects of pH, temperature and disinfectants on viral recovery, as well as to understand the wastewater matrices and how that impacts viral recoveries. There is also the likelihood that different samplers will have greater viral recoveries for different viruses, this needs further exploration within the human pathogen and wastewater field. This could be done by using passive samplers such as tampons for the detection of a wider panel of human enteric viruses that have a wider range of viral structures, including enveloped and unenveloped, more rod like viruses and a variety of size classifications. This would further investigate the different properties of viruses that allow for their recovery from passive sampler materials.

The scope of passive sampling for use in the wider environmental health is far reaching, with the option to explore the possibilities of using this technique not only just within the wastewater matrices (such as exploring effluent sampling) but also within other water matrices like seawater and freshwater. The exploration of human virus levels within effluent wastewater would be of particular interest, especially with more attention being paid to effluent wastewater entering both fresh and seawater recently. This could also lead to the testing of environmental waters for different human and animal pathogens, such as *Vibrio* bacteria within coastal waters or HSV within waterways.