

REVIEW

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SARS-CoV-2 wastewater genomic surveillance: approaches, challenges, and opportunities

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Abstract

Wastewater-based genomic surveillance (WWGS) has proven effective for monitoring SARS-CoV-2 and other viruses within communities. It enables rapid detection of known and emerging mutations and provides insights into circulating lineages. Despite its advantages, WWGS faces challenges in sample processing and computational analysis, particularly in distinguishing similar lineages and identifying novel ones. Recent methods for wastewater sequencing (WWS) analysis remain largely untested amid declining clinical surveillance and ongoing viral evolution. This review examines opportunities and limitations of WWGS, focusing on sample preparation, sequencing technologies, and bioinformatics approaches, and highlights its potential to strengthen public health monitoring systems.

Introduction

Early during the COVID-19 pandemic, the presence of SARS-CoV-2 RNA in the feces of infected individuals, including those who were asymptomatic or recovered from respiratory symptoms [1–5], prompted researchers to explore the use of wastewater networks for community-wide surveillance of SARS-CoV-2 trends. From April to July 2020, several teams submitted proof-of-concept findings to peer-reviewed publications outlining the use and potential of wastewater-based genomic surveillance (WWGS) for SARS-CoV-2 [6–16]. The remarkably rapid dissemination of methods and results during that period facilitated the widespread adoption of WWGS as a



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valuable tool for tracking the pandemic in municipal settings worldwide [6, 10, 11, 17, 18]. These accomplishments have emphasized the potential of wastewater surveillance as a method to estimate disease trends within the community [12] and demonstrated that WWGS for SARS-CoV-2 can detect emerging lineages at an earlier stage compared to clinical monitoring [13, 19]. This approach has also demonstrated its feasibility in monitoring variants of concern (VOC), as designated by the World Health Organization [20–23], and can serve as a valuable warning system for detecting regional surges in VOC frequencies [24–26]. Additionally, it allows for the detection of novel cryptic variants, including those resistant to naturally acquired or vaccine-induced immunity, and those rarely observed in clinical samples [27]. Furthermore, wastewater analysis allows the development of community-level profiles for SARS-CoV-2 viral loads and variants, circumventing testing accessibility and availability issues, and incorporating asymptomatic individuals. In turn, this has assisted in developing new laboratory and bioinformatics methods to obtain and analyze wastewater sequencing (WWS) data. Although many laboratory methods and bioinformatics tools have been rapidly developed in response to the COVID-19 pandemic, ongoing efforts persist in advancing and standardizing WWGS methodology. Furthermore, it is noteworthy to mention the demonstrated generalizability of wastewater surveillance methods to other respiratory viruses, including human influenza, metapneumovirus, parainfluenza, respiratory syncytial virus, rhinovirus, and seasonal coronavirus [28–32], non-respiratory viruses [33], and additional microbes [34].

Wastewater-based epidemiology (WBE) is a population-scale surveillance framework that leverages sewage analysis to monitor public health dynamics, enabling real-time assessment of epidemiological trends, emerging health threats, and environmental exposures [35–37]. By detecting pathogen signatures, profiling community-level drug consumption, and quantifying a broad spectrum of biochemical and genetic biomarkers, this approach facilitates rapid response strategies and strengthens public health preparedness. Beyond pathogen surveillance, wastewater epidemiology has broad applications spanning infectious disease monitoring, environmental exposure assessment, and forensic analysis. A key application of this field, wastewater-based surveillance (WBS), systematically detects and quantifies pathogens in sewage, providing high-resolution insights into community infection dynamics and enabling the early detection of emerging disease threats [35]. Wastewater-based genomic surveillance (WWGS) further extends this capability by extracting and analyzing genetic material from wastewater to characterize pathogen evolution, identify pathogens mutations, and estimate the prevalence of circulating variants at the population level [38, 39].

WBE of SARS-CoV-2 has proven effective in tracking SARS-CoV-2 infection dynamics across numerous countries around the globe [6, 10, 11, 16, 27]. WWGS plays a crucial role in both early detection and large-scale monitoring. It can help identify isolated peak events—such as potential outbreaks—and guide resource allocation in small and medium-sized populations (e.g., monitored retirement communities, university campuses, elementary schools). At the same time, it enables broad surveillance in large populations at centralized water reclamation facilities (WRFs) [40]. Building on recent laboratory and analytical advances to identify the diverse pathogens present in wastewater is essential for improving disease risk assessment and expanding pathogen

surveillance [41]. Importantly, wastewater-based surveillance (WBS) can provide viral trends at different spatial scales, from individual buildings to large centralized WRFs, and does not require healthcare-seeking behavior [42–45].

Despite the numerous advantages of WWGS in tracking and understanding the spread and ongoing evolution of SARS-CoV-2, challenges remain that may affect its accuracy and effectiveness. One challenge is the degradation of viral RNA concentrations by components of wastewater that do not contain human fluids (e.g., gray water), industrial wastewater, and stormwater infiltration. These factors contribute to variability in viral load measurements, making direct comparisons difficult and potentially masking infection trends in the community [46, 47]. In addition, the introduction of genetic material from animal hosts into the wastewater system can complicate the interpretation of WWGS data, necessitating the distinction between human and non-human sources of SARS-CoV-2 and other pathogens [48]. Further concerns include sample degradation, data protection, and privacy issues, as wastewater data collection and analysis must be conducted in a manner that safeguards community privacy [49, 50].

Typical WWGS comprises multiple steps (Fig. 1) after the initial assay design, including: wastewater sampling, viral particle concentration, RNA extraction, RNA quality testing, library preparation, sequencing, bioinformatics analysis, data sharing, and investigation of emerging mutations and lineages hinting towards potential outbreaks. WWGS involves a multitude of experimental and computational methods, offering researchers a wide array of options. Ultimately, the data generated are shared through public repositories, enhancing the utility of SARS-CoV-2 genome sequence data and supporting collaborative research efforts. However, this versatility is constrained by the inherent complexities of wastewater samples, including wastewater physicochemical properties, low viral loads, fragmented RNA, multiple genotypes, and high background genomic noise. Despite current advancements, WWGS faces critical limitations, primarily due to potential experimental biases and the rigorous demands of computational analyses and interpretations.

Here, we present a comprehensive overview that delves into best practices, challenges, and opportunities surrounding WWGS for SARS-CoV-2 by examining the current status of WWGS, and by evaluating the obstacles and prospects with both experimental and bioinformatics methodologies. Available options and common challenges are addressed at each WWGS step. Our review covers the role of WWGS in monitoring viral evolution, identifying novel mutations, tracing emerging lineages, estimating infection rates, and detecting indicators of potential outbreaks. Additionally, we examine how WWGS can assess the effectiveness of vaccinations, providing a crucial tool in public health decision-making. However, our review also acknowledges the challenges in WWGS, highlighting the current gaps and technical and logistical hurdles that must be overcome to maximize its utility. The ultimate goal of this review is to drive further advances in the field of WWGS, which has the significant potential to complement other surveillance systems and help guide public health strategies.

To unlock the potential of WWGS data, robust and accurate bioinformatics algorithms and analytical pipelines are needed. Additionally, comprehensive methodologies must be established to efficiently access SARS-CoV-2 viral genomic material, optimize adaptive sampling strategies, recover viral particles, and select appropriate sequencing

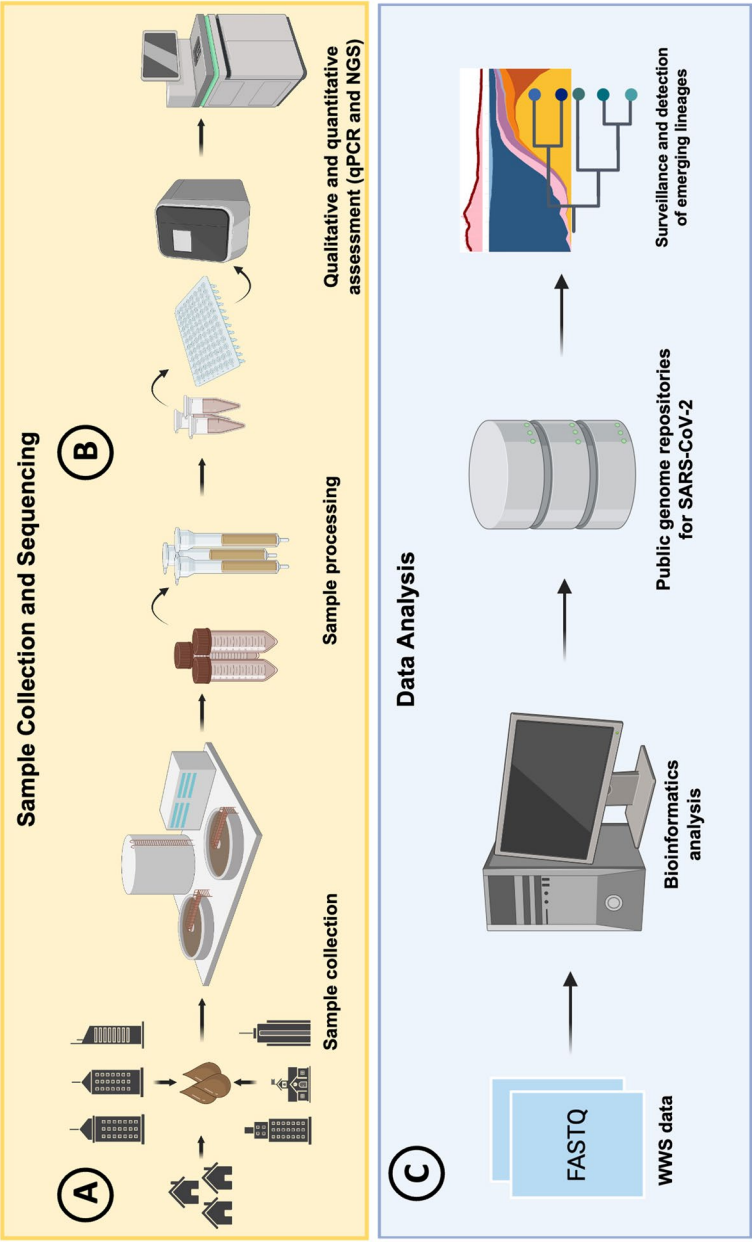


Fig. 1 Overview of wastewater-based genomic surveillance (WWGS) for SARS-CoV-2. **A** Workflow of sample collection and preparation for sequencing. Wastewater samples are collected from water reclamation facilities (WRFs), followed by subsequent concentration and extraction of viral RNA. **B** SARS-CoV-2 quantification is based on real-time PCR using primers specific for viral target genes (such as N1, N2, and E-gene) to assess the molecular concentration of the virus. Positive samples then proceed through library preparation and next-generation sequencing (NGS) technologies, usually via amplicon or capture-based enrichment and sequencing methods. **C** The data analysis pipeline for wastewater sequencing (WWS) data typically employs a reference-based bioinformatics workflow. The raw WWS data and assembled sequences are stored in public repositories for broader accessibility. The subsequent analysis aids in the identification and surveillance of mutations, as well as estimation of lineage frequencies which, in turn, enables the detection of emerging lineages and assists in monitoring hotspots for evolutionary changes

technologies. Establishing such efforts is critical for the widespread adoption of WWGS as an all-encompassing approach for monitoring SARS-CoV-2 variant prevalence and detecting novel cryptic lineages. Overall, the true power of real-time SARS-CoV-2 tracking through WWGS comes from combining the two methodologies, PCR and sequencing and even various sequencing techniques for cross-validation or increased resolution. By including sequencing approaches, samples can be explored for novel mutations and emerging lineages. When a concerning mutation profile or a new potential lineage are discovered, primers and probes can be adjusted for these new lineages to provide rapid turnaround monitoring via PCR assays.

Experimental approaches for effective wastewater genomic surveillance of viral RNA

As WWGS expanded, the laboratory workflows to collect, quantify, and identify SARS-CoV-2 from wastewater rapidly increased (Table 1). With the variety of workflows available to researchers, steps should be taken toward developing standard methods for cross-study comparability. Standards should include environmental microbiology minimum information (EMMI) [76], minimum information for publication of quantitative digital PCR experiments (dMIQE) [77], and minimum information for publication of quantitative real-time PCR experiments (MIQE) [78]. In the context of NGS, minimum required metadata should be provided for each wastewater sample to ensure comparability of data between different studies and sites, similar to what the Public Health Alliance for Genomic Epidemiology (PHA4GE) has defined for SARS-CoV-2 sampling and sequencing in general [79].

Wastewater Sampling

Access to SARS-CoV-2 viral genomic material in wastewater infrastructure is provided through a highly variable and complex wastewater collection system rather than direct access to individual clinical specimens. Ambient conditions within the wastewater collection system are harsh to viral material because of changing biophysicochemical conditions of this specific environment. Specifically, ambient conditions include non-ideal and fluctuating temperatures, variable pH, water quality parameters (e.g., presence of DNases and RNases) that promote the degradation of the viral capsid and nucleic acids, during the time between release from the human host and sampling at water reclamation facilities (WRFs) [80, 81]. Given that human viruses cannot replicate outside of the host cell, the viral load discharged into the wastewater collection system can be monitored to reflect infection dynamics within a sewershed [82], but can also be biased by external factors, such as heavy rainfall [46, 47]. As a result, viral genetic material can be severely degraded and fragmented prior to sample collection. In a similar way, large-scale events that significantly impact social life or human population movements in a monitored area, such as school holidays, international sports events or congresses, also play a crucial role as influencing factors. Contamination from animal hosts poses another challenge, as it can introduce pathogens from less or non-relevant sources that complicate the analysis and interpretation of surveillance data. The question of how sampling can be carried out to mitigate the impact of such events on the data is a central and still highly debated topic in the field of wastewater monitoring [47].

Table 1 Collection of laboratory methods used for SARS-CoV-2 wastewater-based genomic surveillance (WWGS)

Method	Available options
Sampling Sources and Scales	WRF: Raw Sewage [11, 20, 21, 51–64] WRF and Hospital: Raw Sewage [65] Manholes: Raw Sewage [23] WRF and Manholes: Raw Sewage [66] WRF: Sludge [67] WRF: Raw Sewage and Treated Effluent [68] Building and WRF: Raw Sewage [19, 39, 67]
Sampling Type and Frequency	Grab [54, 56, 67, 69] Grab: 1x, 2 x a week [57] 3-h composite [66] 24-h composite [11, 23, 51, 53, 55, 63–66, 69–71] 24-h composite: 1x [20, 60, 62], 2x [21, 59], 3x [19, 66], 5x [19] a week Grab and 24-h composite [39, 52, 68]
Concentration Technique	Ultrafiltration [11, 21, 23, 52, 58–60, 65] Ultracentrifugation [56, 66] PEG precipitation [53, 55, 57, 61, 62, 66] Electronegative Membrane Filtration [20, 39, 63, 71, 72] Centrifugal Ultrafiltration [51, 64, 68, 69] Al(OH) ₃ precipitation [68] Affinity Capture Magnetic Hydrogel [68]
Extraction Method	QIAamp Viral RNA mini kit [52, 57, 61, 64, 66, 69] RNeasy mini kit [11, 23, 65] NucliSens Kit [56, 58, 60] RNeasy PowerMicrobiome Kit [59] MagMAX-96 Viral RNA isolation kit [21] Viral RNA/DNA Concentration and Extraction Kit from Wastewater (Promega) [53] Wizard® Enviro Total Nucleic Acid Kit (Promega) [73] Direct-zol 96 MagBead RNA kit [53, 62] Chemagic Prime Viral DNA/RNA 300 Kit H96 [20] Qiagen AllPrep DNA/RNA minikit [71] NucliSENS easyMAG [63] EZ1 Virus Mini Kit [51] NucliSENS MiniMag Nucleic Acid Purification System [68] Maxwell RSC Pure Food GMO and authentication kit [54] Zymo QuickRNA-Viral Kit [39] Magnetic-bead based with MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit [39] Zymo Quick-RNA Fecal/Soil Microbe Microprep Kit [67]
Quantification Method	RT-qPCR [11, 19, 21, 23, 39, 51, 53–66, 68, 71] ddPCR [53, 59, 60, 67] dPCR [23] V2GqPCR [72, 74]

Table 1 (continued)

Method	Available options
Sequencing Panel	ARTIC v1, v3 [11, 20, 21, 39, 51–54, 57, 63, 67, 68], v4 [23, 66] and v4.1 [23] panel Swift Nomalase Amplicon SARS CoV-2 Panel (SNAP) [19, 55, 62, 65] Tiled amplicon approach [58, 59] EasySeq™ RC-PCR SARS CoV-2 WGS kit v3.0 [56] VarSkip 1a panel [53] NEBNext Fast DNA Library Prep Set for Ion Torrent and Ion Xpress Barcode Adapters [61] NEBNext VarSkip Short SARS-CoV-2 Primer [20] Illumina Respiratory Virus Oligo Panel [71] CleanPlex SARS-CoV-2 FLEX Pane [69]
Sequencing platform	Illumina [19–21, 23, 39, 51–58, 62–65, 67–69, 71] Oxford Nanopore [11, 58, 59] Ion Torrent [71, 75] Sanger [11]

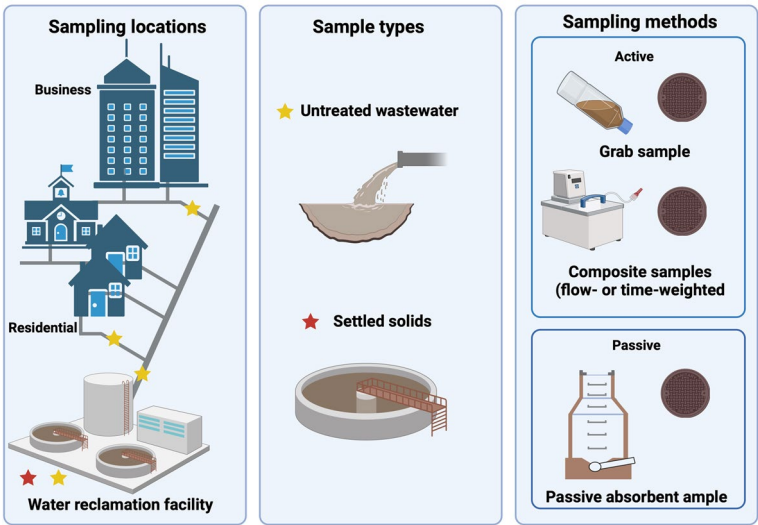


Fig. 2 An outline of different sampling types and locations for WWGS. Sampling for WWGS can be conducted across various population scales, from smaller settings such as retirement homes, to medium-sized communities, like small towns, and up to larger areas, like counties. The interpretation of the results must be done with respect to these varying scales and contextualized appropriately

Throughout numerous studies, sampling techniques varied (Fig. 2). The wastewater network includes a series of pipes through which wastewater flows as it travels towards a centralized wastewater reclamation facility. Samples can be collected anywhere within this wastewater network using active (through composite or grab liquid samples) or passive absorbent sampling techniques. Once the wastewater is at the reclamation facility, it usually undergoes treatment through the settling of solids. These solids can also be sampled for viral material. Advantages of sampling within the wastewater network is that these samples can be obtained from any access point within the sewershed, allowing

for the isolation of subsets of communities which may be experiencing an outbreak. For example, during the COVID-19 pandemic many Universities collected wastewater from individual residence hall buildings, in efforts to identify infected populations thereby mitigating disease spread [83–86]. The advantage of sampling of settled solids at the wastewater reclamation facility is that these samples provide longer term integration of wastewater viral quality and tend to concentrate viruses that tend to adhere to the particulate phase [87]. However, primary settled solids do not possess the same predictive capabilities as untreated wastewater, providing a much shorter lead-time to clinical diagnosis [12], while SARS-CoV-2 concentrations in untreated wastewater can precede clinical data by 4–10 days [88].

One of the difficulties associated with sampling the upstream wastewater network is the high variability in wastewater quality [89]. This variability is associated with the hour-to-hour and day-to-day changes in water uses by the contributing populations. To address this variability, passive absorbent samplers or active composite wastewater samples can be collected [7, 90–92]. Passive absorbent samplers, which are placed within the blowing wastewater network, are inexpensive, simple and provide integration of the viral signal over time [93] and are generally ideal for low resourced settings [94]. Their main disadvantage is the limited knowledge about the kinetics of viral uptake [95]. They cannot be used to quantify the concentration of virus in the wastewater, thereby making it difficult to develop relationships with disease prevalence within a community due to the inability to establish viral loads (total amount of virus released per population). Composite samples can be either flow- or time-weighted. Flow-weighted samples provide accurate measurements of total amount of virus release per population through the product of the flow of wastewater and the concentration of virus measured in the sample. However, flow-weighted samples require the measurement of flow which adds to the expense and complexity of sample collection. More common is time-weighted samples, with equal volume samples collected hourly over a 24-h period. Although not as accurate as flow-weighted, time-weighted samples serve to integrate the hour-to-hour variability of wastewater characteristics providing for a sample that represents wastewater over an entire day. However, even time-weighted samples are instrumentation intensive which requires considerable financial investment and maintenance. In areas that are not suitable for composite sampling due to equipment costs and complexity, grab samples may be the most convenient option, but they represent wastewater at only one point of time. If grab samples are to be used, they should be collected at a consistent time point (i.e., same time of day and same days of the week) to decrease the effect of hour-to-hour and day-to-day variability [7, 83, 96].

Additional variance adds to the challenge of establishing relationships between SARS-CoV-2 wastewater trends and clinical data. Data alignment necessitates aggregation so that both wastewater and clinical data are on the same time scale. In addition, the development of accurate models will also require an understanding of the progression of the disease and viral shedding for infected individuals. Viral shedding kinetics, including the duration and concentration of shedding, vary across individuals and over the course of infection, which affects the interpretation of wastewater data [97, 98]. Studies suggest that SARS-CoV-2 can be shed in feces before symptoms develop, making wastewater surveillance a potential early indicator of infection prevalence. However, the time

lag between peaks in wastewater viral loads and reported clinical cases can range from approximately 8 to 11 days, highlighting the need for careful temporal alignment in data analysis. There remains a paucity of information on shedding rates across infections of SARS-CoV-2 and other diseases. Moving averages (e.g., 7 days to 3 weeks) are typically utilized to evaluate overall trends to reduce the short-term variability inherent in wastewater measurements and clinical case counts [82]. Here, it is important to note that the size of the surveyed population when collecting untreated wastewater at a WRF is dictated by the sewershed service area. Large sewersheds that are typical of centralized WRFs in many urban areas, can make public health interventions challenging. Sub-sewershed sampling (e.g., from a manhole within the sewer network) or building-scale sampling allows for a more targeted spatio-temporal analysis of SARS-CoV-2 in a community, and thus can help better identify the source of emerging pathogens or VOCs.

Virus concentration and RNA extraction methods

Due to the complexity of wastewater matrices, concentrating and extracting viral RNA can be a challenging step in the laboratory workflow. Without an effective recovery protocol, downstream quantification may significantly underestimate SARS-CoV-2 concentrations and affect sequencing outcomes. There are numerous methods to concentrate viral particles from wastewater (Table 1); however, the most frequently used are polyethylene glycol (PEG) precipitation, electronegative membrane filtration (EMF), ultrafiltration, ultracentrifugation, and bead-based assays [7, 88, 90, 91, 96, 99, 100] (Fig. 3). PEG precipitation has been a part of WBE workflows for decades and has helped achieve low viral concentrations of numerous disease agents, including polio [101]. PEG precipitation requires the amendment of wastewater samples with a solution of salt and PEG, resulting in a supernatant that contains concentrated SARS-CoV-2 particles. This method provides a reliable and inexpensive option for viral particle concentration, achieving SARS-CoV-2 recovery rates up to 62.2% [102–104]. However, it can be a severe bottleneck in the wastewater analysis workflow. PEG precipitation takes 2 to 6 h for initial mixing, overnight incubation, and a lengthy centrifugation step. A rapid PEG

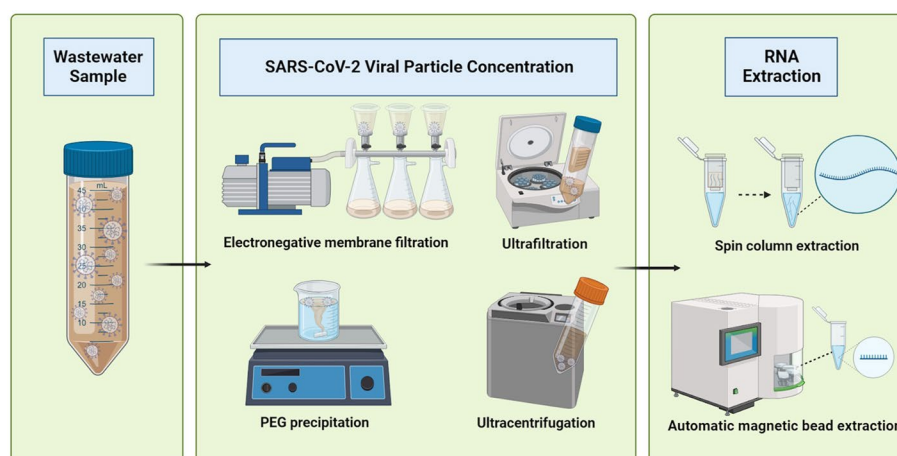


Fig. 3 Common methods for viral particle concentration and RNA extraction in WWGS (for detailed description see Table 1)

approach, without an overnight incubation step, yields drastically lower recovery efficiencies between 18.8% and 35% [102].

Another long-standing viral particle concentration method is utilizing EMFs originally used to increase the recovery of enteroviruses [105]. When applied for WWGS workflows, EMFs in conjunction with a cation conditioning solution (e.g., NaCl or MgCl₂) provide a simple, high-speed method to concentrate SARS-CoV-2 viral particles. Typically, the pore diameter of electronegative membranes is between 0.22 and 0.8 µm, thereby accumulating larger particles on the membrane surface, while the cation conditioning solution forms salt bridges within the negatively charged membrane, promoting the adsorption of free floating SARS-CoV-2 virus particles that are significantly smaller than the membrane pore size. This method boasts a high recovery efficiency of SARS-CoV-2, up to 65.7% [106, 107]. However, the attractive forces of EMFs can pose issues in wastewater with high turbidity, which may lead to organic matter clogging the pores [108]. This organic matter may lead to inhibition when utilizing qPCR for quantification [109].

As an alternative to EMFs and PEG precipitation, ultrafiltration is a direct virus concentration method without conditioning treatment or a lengthy precipitation process. This method differs from electronegative membranes as it concentrates SARS-CoV-2 particles based on size exclusion rather than electrostatic forces, maintaining pore sizes ranging from 5 nm to 0.1 µm down to 3 kDa. While this does seem promising, the efficiencies in recovering viral particles are lower than those observed with other methods (28–56%) [107]. This method can only process small volumes of wastewater and is prone to clogging. The complexity of wastewater matrices necessitates multiple ultrafiltration units to overcome this, but the equipment and cartridges are expensive and concentrate potential PCR inhibitors alongside SARS-CoV-2 virus particles [107].

Ultracentrifugation is a long-standing method of concentrating viral material by centrifuging the wastewater sample at upwards of 100,000 g to create a pellet [107, 110, 111]. The high centrifugal force allows for viruses to be concentrated from both sludge and wastewater. Although this method provides a quick concentration of viral particles, it co-concentrates inhibitors and relies on larger sample volumes to achieve a large pellet to extract RNA [110]. Further, ultracentrifugation results in consistently low recovery rates of SARS-CoV-2, as low as 19% [107, 110].

Bead-based concentration techniques have emerged as an efficient approach for SARS-CoV-2 concentration from wastewater. Magnetic particles can bind to viral particles through several mechanisms, such as antibody-coated beads that bind to viral surface proteins, charged beads that attract oppositely charged viral surfaces, and beads with hydrophobic surfaces that interact with viral envelope proteins [112–115]. For example, magnetic hydrogel particles (Ceres Nanosciences Inc.) use reactive dye baits to capture SARS-CoV-2 through electrostatic and hydrophobic interactions with viral surface proteins. Several benchmarking studies have compared magnetic bead-based concentration with commonly used concentration techniques by using SARS-CoV-2 spiked wastewater and real wastewater samples. Antkiewicz et al. [115] demonstrated that Nanotrap[®] particles (Ceres Nanosciences Inc.), provided 2.8 × higher SARS-CoV-2 concentrations compared to EMFs; however, Ahmed et al. [116] and Babler et al. [72] find that magnetic bead-based concentration methods provide similar results to EMFs.

Following sample concentration, it is necessary to lyse the concentrate via mechanical or chemical methods. Mechanical lysis is typically needed for targets with cell walls, but is not recommended for virus detection because it leads to the release of nucleic acids from cells also present in the sample, potentially interfering with analyses of viral targets. Chemical lysis using commercially available products (such as Zymo's DNA/RNA Shield or guanidinium thiocyanate formulations) generally suffices for lysing the outer protein coat of viruses, releasing the viral genomic material while reducing interference from cellular genomic material. Once the samples are lysed, they can be stored without considerable degradation. After lysis, samples undergo extraction to purify RNA. Several commercially available kits exist, including New England Biolabs Monarch RNA Mini-Prep, Qiagen PowerViral DNA/RNA kit, and Zymo Environ Water RNA Kit. The indicated kits yield >70% extraction efficiency when using spiked concentrations of BCoV as a surrogate in wastewater [111]. However, column-based extraction kits require manual extraction, increasing turn-around time and potentially introducing human error. Conversely, automated RNA extraction reduces risk of user error and drastically increases throughput. Instruments such as the Maxwell RSC, MagMAX, and KingFisher Flex system offer magnetic bead RNA extraction. Both magnetic bead and column-based extractions have demonstrated equitable numbers of usable sequencing reads [117].

Few studies have investigated the effects different virus concentration methods have on SARS-CoV-2 sequencing [118–120], and each study evaluated a unique set of concentration techniques and sequencing kits. However, in each experiment, the researchers demonstrated that the wastewater viral concentration method is a critical step in workflows due to their substantial impact on sequencing coverage. Further research is needed to optimize workflows that achieve high sensitivity and coverage for sequencing.

PCR-based quantification methods for wastewater genomic surveillance

At the start of the pandemic, RT-qPCR assays were predominantly utilized to qualitatively confirm clinical diagnosis of COVID-19 and quantitatively describe wastewater viral concentrations. With the addition of a fluorescent dye, a qPCR instrument can measure the fluorescence as the thermal cycler progresses and provide a real-time amplification curve with each cycle. This analysis compares the quantification cycle (C_q) value of a sample with an unknown concentration to a standard curve of known concentrations, allowing for the extrapolation of SARS-CoV-2 virus copy numbers; however, this provides an inherent quantification bias as this method is dependent on the accuracy of the standard curve. Further, due to the complexity of wastewater matrices, amplification and quantification can be affected by inhibitors [121, 122].

RT-ddPCR emerged as a robust alternative to RT-qPCR. Instead of comparing to a standard curve, this technique applies Poisson statistics to determine the absolute concentration of the target [123]. Each PCR reaction consists of an oil–water emulsion that partitions each sample into tens of thousands of droplets. Each droplet will generate a positive or negative partition, and the reader will distinguish the partitions based on their fluorescent signal. For wastewater, RT-ddPCR has demonstrated a stronger resilience to inhibitors and a higher sensitivity compared to RT-qPCR [121, 124–126]. With newer instruments, up to 6 different fluorescent dyes can be detected with ddPCR, enabling an amplitude multiplex of up to 12 targets.

A variation of RT-qPCR was developed during the pandemic for detecting SARS-CoV-2 called Volcano 2nd Generation (V2G)-qPCR [74, 127]. The V2G-qPCR method uses a novel polymerase capable of reading both RNA and DNA templates and, therefore, it does not require a separate cDNA synthesis step. Results from V2G-qPCR and RT-qPCR measures are statistically equivalent [128]. Another employed methodology is proteomic quantification detection. Proteomics can provide insight into proteins and their role specific to the target [129]. SARS-CoV-2 RNA genome encodes for at least 29 proteins and can be identified using several types of mass spectrometry analyses [130, 131]. While mass spectrometry per sample assay cost may be less expensive, and can provide shorter and cheaper runs than RT-qPCR [130, 132, 133], RT-qPCR has displayed better sensitivity and specificity [134].

In addition to RT-qPCR methods, loop-mediated isothermal amplification (LAMP) is a rapid, sensitive, and cost-effective nucleic acid amplification method that operates under isothermal conditions using a set of six target-specific primers and a DNA polymerase with strand displacement activity [135, 136]. This technique allows for continuous DNA amplification, enabling various molecular diagnostic applications, including pathogen detection and genomic surveillance [137]. Given these advantages, LAMP has been successfully integrated into WWGS for SARS-CoV-2 monitoring [138, 139]. An advancement of LAMP-based approaches is LAMP-Seq, which couples high-throughput barcoded LAMP amplification with NGS for scalable pathogen surveillance [140]. This method has been demonstrated as an efficient and cost-effective alternative to qPCR for large-scale testing and has potential applicability in wastewater surveillance by enabling multiplexed detection of viral targets across multiple samples, making it particularly useful for mass-scale epidemiological studies and WWGS of pathogens. LAMPore sequencing integrates Oxford Nanopore sequencing with LAMP amplification allowing for real-time genomic surveillance and for the simultaneous detection of multiple SARS-CoV-2 variants in wastewater samples [139]. Due to its portability and scalability, LAMPore is particularly advantageous in resource-limited settings where conventional sequencing infrastructure may be unavailable. Additionally, colorimetric RT-LAMP has been evaluated as a cost-effective alternative for WWGS [138]. While less sensitive than RT-PCR, it offers advantages such as faster results and operational simplicity, making it well suited for rapid, decentralized wastewater surveillance. LAMP-positive samples may be prioritized for sequencing, optimizing analytical efficiency by focusing resources on high-confidence detections [138].

Although PCR-based approaches for SARS-CoV-2 can reveal temporal changes in virus concentrations in a population [141–143], the nature of PCR restricts its ability to detect mutations that can be associated with already defined SARS-CoV-2 lineages and estimate their prevalence in the population. PCR-based methods are relatively inexpensive, well-established, and allow for the direct quantification of SARS-CoV-2 in wastewater samples, presenting the following advantages: an ability to probe a sample site at high frequency to generate near real-time information; ease of implementation by any lab running standard PCR assays; short turn-around time and low costs of reagents [144]. The number of genomic sequences targeted by PCR assays are also limited by available fluorophores and the detection instrument [145]. But most critically, these PCR assays may lag in the capability to discover the emergence of new virus variants because they

require a specific primer–probe design according to the details of the genomic information of new variants [146], usually derived from sequencing and analyzing patient samples. Thus, PCR assays may lose effectiveness for detecting new virus variants as mutations arise. Regardless of this, PCR-based techniques provide efficient, cost- and resource-saving approaches to track and quantify known variants circulating in communities [145].

Several primer–probe sets are available to identify SARS-CoV-2, typically in the most conserved regions, such as the *N* gene [147]. Despite being a relatively conserved region, the *N* gene is not immune from mutations [147]. As VOCs emerge, primer/probe sets may become less specific and have a degrading ability to detect positive SARS-CoV-2 samples [14]. Compared to the Index reference sequence from Wuhan strain [148], over 1000 *N* gene nucleotide mutations have been detected, and more than 300 of them are in commonly used primer sets [149]. Deletions in target genes, such as the *N* gene in Omicron lineages, can hinder the ability to detect SARS-CoV-2 accurately [147, 150]. Therefore, updating primer sets is an ongoing need to adapt to VOCs.

High-throughput sequencing can be employed to overcome the limitation of pre-defined sequence targets and to identify emerging viral lineages [51, 145]. The use of sequencing technologies coupled with advanced bioinformatics methods for analyzing WWS data has provided improved detail in assessing wastewater samples. Sequence data collected at sufficient depth can be deconvoluted to estimate lineage and sublineage proportions. Thus, sequencing overcomes some of the limitations of PCR-based technologies, allowing for the comprehensive detection of SARS-CoV-2 mutation profiles present in wastewater samples, including those of novel mutations. However, the tiled amplicon sequencing methods primarily used in SARS-CoV-2 surveillance are still vulnerable to changes in primer binding sequences as new lineages emerge (see next section). Including high-throughput sequencing with appropriate bioinformatics methods is the foundation of fundamental transformations of environmental genomic surveillance and virology to complement epidemiological data analysis and thus contribute to outbreak early detection and prevention [83, 90–92, 96].

Advancements in genomic sequencing technologies for wastewater analysis

Genomic sequencing approaches have proven effective in detecting mutations and deconvoluting this information to estimate SARS-CoV-2 lineage and sublineage frequencies for WWGS [52, 53, 151]. Wastewater-derived RNA can be processed using different sequencing methodologies, each with distinct advantages and limitations. The sequencing process typically involves RNA extraction, reverse transcription into complementary DNA (cDNA), and sequencing using either short- or long-read technologies [152]. The effectiveness of wastewater-based epidemiology heavily depends on the sequencing approach chosen, as it impacts genome recovery and analytical accuracy.

Metagenomics sequencing allows the direct recovery of SARS-CoV-2 RNA genome fragments from wastewater samples without any further enrichment or depletion of potentially contaminating material from other sources, thus capturing a broad representation of the microbial community. The RNA is reverse-transcribed into cDNA and then shotgun-sequenced. This approach is particularly powerful for characterizing diverse microbial populations [153]; however, it faces significant challenges in detecting

low-abundance SARS-CoV-2 RNA. One major limitation is that much of the sequencing capacity is consumed by non-target RNA (such as ribosomal and human RNAs), reducing the proportion of viral reads. Previous wastewater metagenomics studies showed that genetic material derived from bacteria was more abundant despite additional depletion efforts via size exclusion [153].

To enhance the detection of SARS-CoV-2, targeted sequencing methods such as capture-based and amplicon-based sequencing utilize enrichment strategies to selectively isolate or amplify specific genomic regions [152]. These enrichment approaches significantly boost viral signal strength, improving detection sensitivity and sequencing efficiency. Capture-based sequencing uses specific oligonucleotide probes (baits) that are complementary to the target regions of interest, such as the whole SARS-CoV-2 genome or specific genes. These capture probes selectively bind and isolate viral RNA fragments from a complex wastewater sample. Following capture, the enriched RNA is converted to cDNA, processed into sequencing libraries, and sequenced. This method provides a comprehensive genome recovery but may struggle to detect novel mutations if the probes fail to hybridize effectively to emerging viral strains.

Amplicon-based sequencing employs polymerase chain reaction (PCR) amplification to selectively target specific genomic regions. Primer pairs are designed to amplify predefined sections of the SARS-CoV-2 genome, facilitating sequencing. This method is widely used for clinical genomic surveillance and has been adapted for wastewater analysis due to its ability to enrich low-abundance viral material. Tiled amplicon-based approaches, including open-source primer schemes such as the ARTIC Network [11, 154], “Midnight” SARS-CoV-2 [155] and VarSkip Primer Schemes [156, 157], have become standard in WWGS. However, amplicon-based sequencing is highly sensitive to primer design. Mutations in primer-binding regions can result in amplification failure, leading to incomplete genome coverage. This phenomenon, known as amplicon drop-out, can obscure critical genomic regions, affecting lineage classification and variant identification. Additionally, RNA degradation in wastewater samples can exacerbate amplification inefficiencies, leading to missing genomic information. The continuous evolution of SARS-CoV-2 requires frequent primer updates, often guided by clinical genomic surveillance data, but declining availability of clinical genomes may hinder accurate redesigns. With declining SARS-CoV-2 genomic surveillance in clinical settings, the accuracy of primer designs may decrease, increasing the risk of under-sequenced regions (USRs) and false negatives.

Several challenges impact the efficacy of sequencing technologies in WWGS. Primer mismatches and amplicon drop-out can cause sequencing failures, leading to gaps in genome recovery. Primer schemes, such as ARCTIC [158] and Midnight [159] have reported such biases. Coverage bias can result from primer-primer interactions, leading to uneven amplification and distorting the representation of different genomic regions [158–160]. These biases not only affect genome completeness but can also misrepresent variant frequency, leading to erroneous epidemiological conclusions. Such biases can hinder the accurate identification of SARS-CoV-2 mutations and lineage determination. Under-sequenced regions introduce blind spots in variant-calling algorithms, potentially leading to the omission of critical variants. Additionally, deconvolution-based quantification methods may yield

suboptimal results, and classification-based approaches risk generating higher false positives by misassigning reads. As new SARS-CoV-2 lineages continue to emerge, timely updates to primer panels will be necessary to maintain sequencing reliability. Spike-in primers can serve as temporary solutions while major redesigns are implemented.

Each sequencing approach presents distinct advantages and challenges. While metagenomics provides an unbiased representation of wastewater microbial communities, its low sensitivity limits SARS-CoV-2 detection. Capture-based sequencing offers enhanced specificity but depends on well-designed hybridization probes. Amplicon-based sequencing remains the most widely used method in WWGS due to its high sensitivity but requires constant primer updates to counteract emerging mutations. Moving forward, advancements in sequencing methodologies and computational tools will be essential to improving wastewater-based genomic surveillance, ensuring accurate detection of viral diversity and evolution.

Sequencing technologies used in WWGS

Several sequencing technologies can be used to sequence SARS-CoV-2 RNA from wastewater, each with its own set of advantages and disadvantages. Illumina sequencing is the most widely used sequencing technology for genomic surveillance of SARS-CoV-2 in general [161] and WWGS in particular [54, 68]. Short reads produced by Illumina sequencing have high accuracy, and the platform can generate a large number of reads in a single run. In situations where consensus genomes are reconstructed de novo, or large structural variations need to be detected, the major drawback is the limited read length. An alternative short-read sequencing technology, more rarely used but also applied in WWGS, is IonTorrent sequencing [75, 151, 162].

As alternatives, single-molecule real-time sequencing (SMRT) technologies, such as those provided by Oxford Nanopore Technologies (ONT), can produce longer amplicon reads, e.g., approximately 400 bp reads based on ARTIC Network Protocols [11, 154], or even up to 1200 bp amplicons from the adapted ARCTIC “Midnight” protocol [155], which can be useful for resolving complex regions of the SARS-CoV-2 genome. In the specific context of WWGS, longer reads can help infer synteny information about mutations that belong to the same viral variant because they are detected on the same read. However, it is challenging to derive long RNA fragments from wastewater samples, and the amplicon approach limits maximum achievable read lengths. In clinical SARS-CoV-2 genomic surveillance, ONT is placed second among the most used sequencing technologies [161] due to its lower initial and subsequent costs. It is the putative option to sequence longer amplicons [163] and has potential future applications regarding real-time and on-site sequencing. In addition, ONT can also sequence RNA natively without the need for cDNA transcription, but in such a setup all the crucial advantages of enrichment methods are lost. SMRT technologies, particularly ONT sequencing, had higher error rates than other technologies, which may affect accurate variant detection. However, recent improvements, such as the R10.4.1 flow cell with dual reader heads that enhance signal resolution, and the Dorado [164] basecaller, which employs

GPU-accelerated neural networks, have significantly improved accuracy, positioning nanopore sequencing as an increasingly reliable option for high-resolution variant calling [165].

Bioinformatics analysis for wastewater sequencing data

In response to the COVID-19 pandemic, the scientific community rapidly developed and adapted bioinformatics tools to address the large amount of SARS-CoV-2 sequencing data, including the data from WWGS initiatives (Table 2). Figure 4 outlines a typical workflow diagram used in WWGS data analysis. Given the complexities, fragmentation, and noise level inherent to wastewater samples, the analysis of WWGS data poses unique challenges for bioinformatics methods. While several tools have been developed or adapted for SARS-CoV-2 WWGS (Table 2), many lack validation specifically for WWGS data. Therefore, there is a pressing need for rigorous benchmarking of these tools and potentially crafting novel computational methods attuned to wastewater intricacies [191].

Data quality control and error correction tools

The initial stages of a bioinformatics analysis for WWS data usually includes quality control, error correction, followed by trimming of adapters and primers (Fig. 4). FastQC [192] and MultiQC [193] are the most popular and efficient tools for quality control in sequencing data analysis and, thus, also applicable to WWS. Ensuring high sequence data quality is crucial for WWS data analysis, with trimming and filtering being important steps to refine raw reads. For WWS data analysis, tools such as BBDuk [194], Trimmomatic [195], and Trim Galore [196] are largely used for read trimming. iVar [197] is predominantly used for read filtering, while PRINSEQ-lite [198] and fastp [199] are used for managing both trimming and filtering processes based on Phred quality and read length for short reads, and Filtlong [200] for filtering long reads by size. Error correction by conventional tools can be quite challenging when dealing with WWS data, as these tools have been primarily optimized for human genome reads and may not be able to recognize the subtle variations among viral lineages or sublineages [201]. Several error correction methods tailored for viral sequencing have been proposed to address this issue, such as KEC [202], ET [202], MultiRes [203] or ShoRAH [204].

Adapter-contaminated reads can disrupt downstream bioinformatics analysis, including assembly and variant calling. They might induce misalignment or yield incorrect assembly sequences. Therefore, it is imperative to eliminate or trim these adapter sequences from the reads prior to subsequent analysis. Tools commonly used for this purpose, akin to those for primer trimming, include BBDuk [194], cutadapt [205], Trimmomatic [195], fastp [199], and Trim Galore [196]. In amplicon-based enrichment, reference-derived primer sequences are incorporated into the sequenced reads, potentially diluting and masking actual variant sites. Removing primer sequences from raw reads (primer trimming) is therefore a mandatory step of an amplicon-based WWS pipeline. Tools like iVar [197], BBDuk [194], cutadapt [205], and Primerclip [206] can perform primer trimming (among other tasks)

Table 2 Collection of bioinformatics methods used for SARS-CoV-2 wastewater sequencing data (WWS data) analysis. This overview comprises tools that were developed for the general analysis of sequencing data, adapted for WWS data, and specifically developed for WWS. The overview is not exhaustive but provides a starting point for most representative and popular bioinformatics tools for WWS data analytics

	Software	Source
Data QC, primer trimming trimming and adapter removal, paired-end reads merging	BBMap (BBDuk) [20, 27, 53, 61, 90, 166]	https://github.com/BioInfoTools/BBMap
	FastQC [64, 167, 168]	https://github.com/s-andrews/FastQC
	MultiQC [31, 63, 169, 170]	https://github.com/MultiQC/MultiQC
	iVAR [19, 20, 39, 56, 57, 65, 67, 90, 167, 169–173]	https://github.com/andersen-lab/ivar
	fastp [31, 169–171, 174]	https://github.com/OpenGene/fastp
	PRINSEQ-lite [175]	https://github.com/uwb-linux/prinseq
	Trim Galore [56, 61]	https://github.com/FelixKrueger/TrimGalore
	Trimmomatic [48, 55, 62–64, 67, 176]	https://github.com/usadellab/Trimmomatic
	cutadapt [27, 54, 58, 167, 176–178]	https://github.com/marcelm/cutadapt
	Primerclip [62, 166]	https://github.com/swiftbiosciences/primerclip
	BBTools [20, 27, 53, 54, 90, 166, 167]	https://github.com/kbaseapps/BBTools
	BAMClipper [179]	https://github.com/tommyau/bamclipper
	BWA-MEM [20, 53, 55, 57, 58, 61, 62, 64, 65, 67, 68, 90, 166, 167, 169, 173, 174]	https://github.com/lh3/bwa
	BWA-MEM2	https://github.com/bwa-mem2/bwa-mem2
Read mapping	Minimap2 [19, 27, 31, 58, 154, 172, 178, 180]	https://github.com/lh3/minimap2
	Bowtie2 [48, 71, 171, 177, 181]	https://github.com/BenLangmead/bowtie2
	iVAR [19, 20, 39, 57, 58, 65, 69, 171, 178, 182]	https://github.com/andersen-lab/ivar
	LoFreq [20, 39, 53, 61, 64, 90, 169, 173]	https://github.com/CSB5/lofreq
SNV calling	FreeBayes [62, 172]	https://github.com/freebayes/freebayes
	BCFTools [53, 55, 62, 64]	https://github.com/samtools/bcftools
	V-PIPE [27, 61]	https://github.com/cbg-ethz/V-pipe
	Nanopolish [11]	https://github.com/jts/nanopolish
	VarScan [56, 68, 170, 183]	https://github.com/dkoboldt/varscan
	GATK [166]	https://github.com/broadinstitute/gatk
	SAMtools [54, 56, 61, 183]	https://github.com/samtools/
	Medaka [184]	https://github.com/nanoporetech/medaka

Table 2 (continued)

	Software	Source
Relative abundance estimation (LRA), allele frequency estimation (AF), lineage detection (LD)	Freyja (LRA) [19]	https://github.com/andersen-lab/Freyja
	QualID (LD) [20]	https://gitlab.com/treangenlab/qualid
	SAM Refiner (AF) [27, 61, 177]	https://github.com/degregory/SAM_Refiner
	Kallisto-VLQ (LRA) [67]	https://github.com/baymlab/waste_water_analysis
	iVAR (AF) [57, 182]	https://github.com/andersen-lab/ivar
	VaQuERo (LRA) [53]	https://github.com/fabou-uobaf/VaQuERo
	Cojac (LD) [52, 56]	https://github.com/cbg-ethz/cojac
	Lineagespot (AF) [172]	https://github.com/BiodataAnalysisGroup/lineagespot
De novo assembly	LCS (LRA) [185]	https://github.com/rvalieris/LCS
	SOAPdenovo	https://github.com/aquaskyline/SOAPdenovo2
	Velvet	https://github.com/dzerbino/velvet
	ABYSS	https://github.com/bcgsc/abyss
	IBDA	https://github.com/loneknightpy/idba
	SPAdes [186]	https://github.com/ablab/spades
	Edena	https://github.com/ddhz/edena
	Vicuna	https://www.broadinstitute.org/viral-genomics/vicuna
Lineage and clade assignment, Phylogeny	Haploflow	https://github.com/hzi-bifo/Haploflow
	Flye	https://github.com/fenderglass/Flye
	IQ-Tree2 [11, 19, 178]	https://github.com/Cibiv/IQ-TREE
	Pangolin [53, 65, 171, 175, 187–189]	https://github.com/stevenlovegrove/Pangolin
	NextClade [58, 168, 173, 188]	https://github.com/nextstrain/nextclade
Pipelines	USHER [170, 173]	https://github.com/yatisht/usher
	COVID-19 Viral Epidemiology Workflow (C-VIEW) [19]	https://github.com/ucsd-ccbb/C-VIEW
	CFSAN Wastewater Analysis Pipeline (C-WAP) [190]	https://github.com/CFSAN-Biostatistics/C-WAP
	PiGx SARS-CoV-2 Wastewater Sequencing Pipeline [169]	https://github.com/BIMSBbioinfo/pigx_sars-cov-2
	VLQ-n [67]	https://github.com/rki-mf1/vlq-nf

ensuring that the sequencing data is not biased by the synthetic primer sequences. The primer sequences can be removed directly from the raw reads (FASTQ) with the above-mentioned tools or after mapping to a reference genome (SAM/BAM), for example, using BAMClipper [207] see Fig. 4. A caveat of removing trimming primers from raw reads before mapping is the potential introduction of edge effects that may obscure deletions near the primer sites [179]. Therefore, it is advisable to remove primer sequences after mapping.

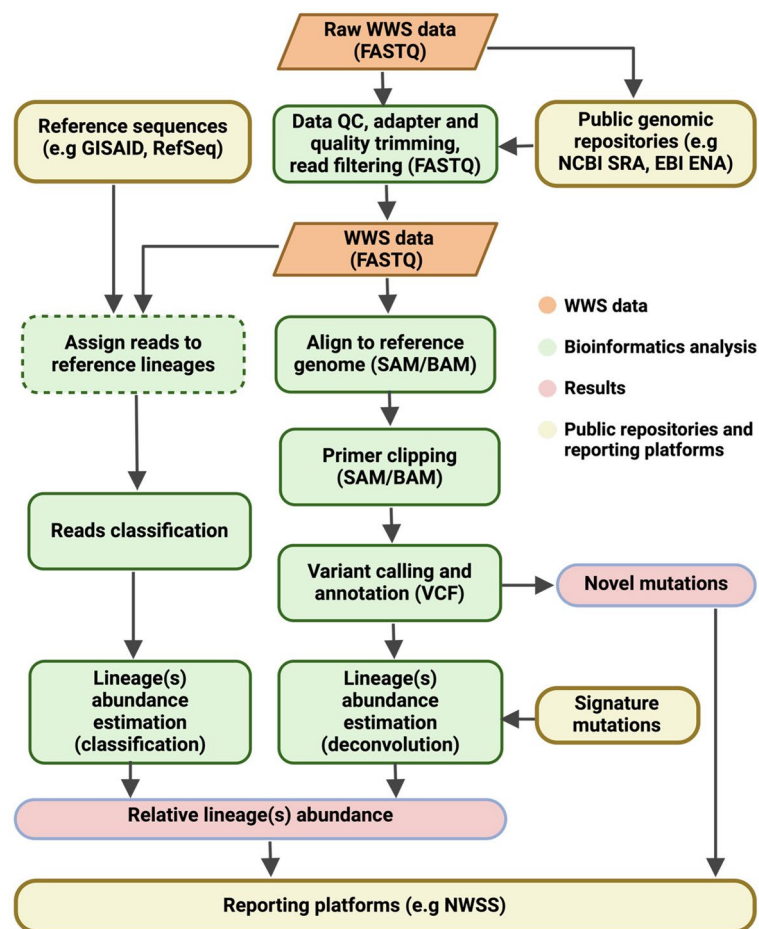


Fig. 4 Overview of a typical reference-based bioinformatics workflow used in amplicon-based wastewater sequencing (WWS) data analysis. Here, 'variant' specifically means genomic mutation

Read mapping

Read mapping is a common technique for bioinformatics analysis pipeline, enabling the accurate identification and subsequent detailed characterization of the viral mutations present in the sample (Fig. 4). Read mapping is reference-based, which makes it particularly well-suited for target genomes with limited variability, such as those of SARS-CoV-2. It is a critical step in identifying and tracking SARS-CoV-2 (sub)lineages and VOCs. After the preprocessing steps (see section before), reads are aligned to a reference genome of SARS-CoV-2. This step precedes the identification of viral mutations via the variant calling tools, and subsequent viral lineage classification. If the data was generated using paired-end reads, they can also be merged prior to alignment using tools like BBTools [208]. This merging process utilizes the overlapping regions between paired reads for correcting sequencing errors, potentially resulting in sequences of higher quality. Commonly used scalable bioinformatics tools for read mapping include BWA MEM [209], Bowtie [210], which are preferred for short-reads and minimap2 [211], which is preferred for long read technologies. While it is also possible to remove artificial primers before mapping, it is important to clip them off after mapping to avoid edge effects, such

as missing important deletions due to incorrect soft-clipping of the mapping tool near amplicon ends [179] (Fig. 4).

Given the dynamic nature of viral genomes and the potential for new variants, regular updates to reference genomes and robust practices are vital to ensure accurate representation and understanding of the SARS-CoV-2 populations in wastewater samples. Incomplete or outdated reference sequences may lead to inaccurate identification of target variants, reduced sensitivity to detect new or existing variants, and finally, misinterpretation of data and compromised tracking of viral evolution. It is also crucial that the scientific community relies on a harmonized reference genome for variant calling to share comparable results. At the time of this writing, the community-recognized reference sequence for SARS-CoV-2 is the index consensus sequence obtained from a patient in Wuhan with GenBank accession number MN908947.3 and RefSeq accession number NC_045512.2.

Single nucleotide variants (SNVs) calling

In WWS data, the information about how mutations are organized into distinct haplotypes is lost. This is due to fragmentation of the genetic material in the sample, amplification protocols amplifying genomic regions in separate amplicons, and the length of sequencing reads being much shorter than the genome length. Viral diversity within a patient is often ignored in genomic epidemiology based on human infection samples, as only a single consensus sequence is reconstructed, representing the dominant inferred lineage. Still, mutations and their frequencies are commonly calculated when constructing the genome. However, this information is often not further used, and only the final consensus sequence is shared for downstream analysis. While this simplistic assumption that a patient is infected with only one (dominant) virus variant is often sufficient for genomic surveillance, it can also lead to the overlooking of minority virus variants in the within-patient virus population. The situation is usually much more complex in wastewater samples because many more different virus variants from different excretors come together. Thus, reconstructing a single consensus is usually unsuitable for complex environmental samples, as wastewater likely contains multiple lineages from individuals infected with different variants. This sample heterogeneity must be considered and is further complicated because these lineages often share the majority of genetic information and even signature mutations because of phylogenetic relationships between lineages and sublineages or due to convergent evolution (Fig. 5 A). Thus, accurate variant calling from mixed wastewater populations remains a challenging task. Mutation or variant calling can be performed by a variety of tools also depending on the used sequencing technology, such as iVar [197], SAMtools [212], ShoRAH [213], LoFreq [214], GATK [215], FreeBayes [216], BCFTools [212], Medaka [217], or custom scripts [75]. Variant callers designed expressly for genetically diverse samples such as ShoRAH expect a number of quasispecies which do not have to be estimated beforehand, and allow for identifying local haplotypes in addition to individual SNVs. All these variant calling tools have different parameters for filtering according to metrics such as sequencing depth, quality, and allele frequency, impacting the final mutation calls. Comparative performance of some of these tools when applied to SARS-CoV-2 wastewater surveillance data has been the subject of published studies [218]. A recent benchmarking analysis that

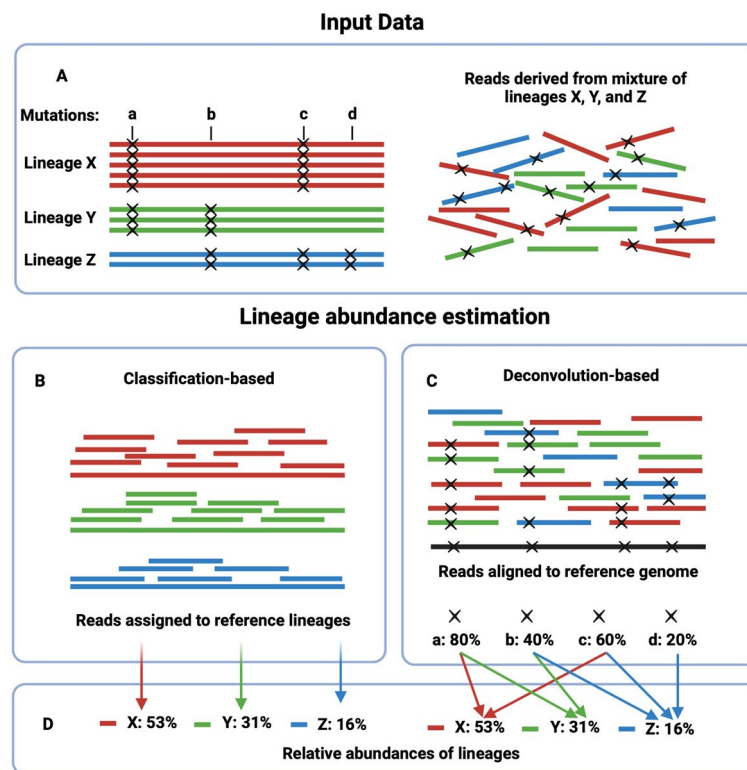


Fig. 5 Estimating the relative abundances of SARS-CoV-2 lineages from WWS data. **A** The lineages X, Y and Z each have unique but partially overlapping mutation profiles, situated on loci a, b, c, and d. **B** In a classification approach, each read is assigned to the variant that most likely generated it. The counts are then aggregated to estimate the relative abundance of variants in the sample. **C** In the deconvolution approach, the reads from a wastewater sequencing experiment are aligned to the reference genome, and mutations are called. The proportions of mutated reads at each variable locus are decomposed into the individual contribution of each variant. **D** Relative abundance estimates for lineages X, Y and Z

evaluated variant calling algorithms specifically for WWGS showed that tools like Var-Scan [219], BCFTools, and Freebayes are generally preferable, particularly when mutations are unknown, due to their higher specificity and sensitivity [218]. However, when specific mutations are known and expected in the output, iVar performed best based on lineage-defining lists of mutations [218]. Accurate variant calling, including insertions and deletions (INDELs), relies on a combination of optimized laboratory protocols and specialized bioinformatics pipelines that can account for the unique characteristics of wastewater-derived nucleic acids. These include high levels of fragmentation, chemical degradation, and the presence of diverse microbial and host backgrounds. To ensure reliability, comprehensive benchmarking of variant calling tools is essential—using wastewater-specific datasets that reflect the variability in sequencing platforms, read lengths, coverage depth, and target enrichment strategies [191]. Such evaluations are necessary not only to assess accuracy and reproducibility but also to guide tool selection and parameter tuning. Addressing these technical and analytical challenges is critical for enhancing the sensitivity, specificity, and overall confidence in variant detection in wastewater-based surveillance systems [191].

Relative abundance estimation and lineage detection

Despite the global haplotype information being lost in WWS data as detailed above, the relative abundances of known SARS-CoV-2 lineages can still be estimated from the observed individual mutations. Various computational tools have been developed to accurately estimate the relative prevalence of SARS-CoV-2 variants in a population through WWGS. These tools are based either on a classification approach, such as COJAC [52], VLQ pipeline [67], and Pipes [220] or based on a deconvolution approach, such as LCS [185], VaQuERo [53], Alcov [221], PiGx [169], Freyja [19], LolliPop [222]. The classification approach works at the reads level and assigns each read (probabilistically or deterministically) to the different reference lineages by comparing observed mutations (on the read) with signature mutations (on the reference). Aggregating the counts of reads assigned to different virus variants provides an estimation of their relative abundances (Fig. 5 B). By contrast, the deconvolution approach takes the individual mutation frequencies computed from the alignment and mutation calling as input. In a mixed sample, the expected proportion of mutated reads at a given genomic position can be assumed to equal the sum of the relative abundances of variants harboring this particular mutation. Using a reference set of variants, their relative contributions to the observed distribution of mutation frequencies are then estimated by a constrained regression method (Fig. 5 C). Some of these methods also allow for considering time dependency in the data by employing different nonparametric smoothing approaches [19, 53, 222]. Some methods additionally provide confidence intervals for the estimates of variant relative abundances, which is done using bootstrap methods [19, 185, 222] or closed-form expressions [222]. Different methods for estimating the relative abundances of SARS-CoV-2 variants in WWS have been subjected together on a simulated data benchmark [223, 224].

All of these methods are reference-based and rely on precise definitions of the variants, which can be generated from clinical sequences generated since the beginning of the pandemic. The reference set can be prepared based on two general approaches to estimate variant frequency—mutation-based (the deconvolution approach that uses a set of marker mutations of lineages) and sequence-based (the classification approach that uses full genome sequence information). It is crucial to acknowledge that both approaches have their limitations. A recent study shows [225] that using sequence-based reference sets can generate higher false positives than the mutation-based approach. On the other hand, the mutation-based approach creates a challenge in selecting sublineage-defining marker mutations that provide robust assignment in the context of increasing diversity of the lineages and ongoing (convergent) evolution. Regardless of the approaches, reference sets of variant genomes may be constructed from existing databases, such as GISAID [226], CoV-Spectrum [227], UshER [228], or NextClade [229]. The selection of appropriate reference datasets is not trivial, and the results of some deconvolution methods may vary significantly depending on the reference dataset or classification scheme used [225].

Therefore it is important to account for the temporal and geographical range of the references while constructing a reference set [225]. VLQ [67] selects reference lineages based on the spatio-temporal context of the wastewater sample and samples a specific number of genomic sequences for every lineage according to a predefined

threshold for the genomic variation that should be captured [67]. Freyja reconstructs a set of characteristic lineage mutations based on the UShER phylogenetic tree [19], while other SNV-based tools like wastewaterSPAdes [186] and SAMRefiner [177] rely on a rule based selection of characteristic sets of mutations considering lineage-differentiating power. Because of the rapid evolutionary changes of the virus, reference data need to be re-evaluated for every sample and pandemic timeframe. Specifically, convergent evolution and novel lineages challenge the current strategies for reference reconstruction: depending on the circulating lineages of interest, it becomes more challenging to represent genomic variation and still guarantee sufficient differentiation power between sub-lineages. Furthermore, most currently applied tools rely on a large amount of clinical sequence data to reconstruct their reference data sets. Decreased clinical sampling poses a challenge for bioinformatic WWGS and should be considered for further research in method development, especially in terms of identifying and quantifying unknown lineages.

Early identification of unknown viral variants based on novel genomic signals represents one desired benefit and also a great challenge for WWGS. Currently, novel variant detection is mostly conducted retrospectively, while real-time cryptic variant detection represents an ongoing bioinformatics research topic where the first approaches are slowly published. Previously, CryKey was developed as one of the first tools for non-retrospective cryptic variant detection [230]. CryKey identifies cryptic variants based on sets of mutations that co-occur on the same reads but have not been observed to co-occur before in clinical sequence data. The tool addresses bias and artifacts in WSD by rule-based filtering of mutations and reconstructs a reference table mapping SNP information and lineage assignments from clinical sequence data. Overall, biases of WWS data and their epidemic context should be continuously monitored and considered during bioinformatic methods development.

In computational methods for estimating relative abundances, the number of reads that map to a particular (sub)lineage reference or the number of reads containing a specific variant associated with certain (sub)lineages are used as indicators of the relative frequency of these (sub)lineages within the sample. However, there are two main reasons why this assumption is not always correct. First, most library preparation methods include polymerase chain reaction (PCR), which is known to amplify some fragments more efficiently than others, depending on their GC content and length [231, 232]. Second, in Illumina technologies, there is a specific type of duplicate reads called optical duplicates that arise from miscalling a single cluster as two separate clusters, or there is a probability that one molecule of the library initiates two independent clusters [232]. These factors can lead to biases in the number of reads, resulting in an inaccurate representation of the initial abundance of RNA molecules. These biases are particularly critical for the highest and the lowest abundant RNA types. As of the writing of this paper, no studies have been found that investigate the impact of deduplication methods on the relative quantification of (sub)lineages in wastewater samples. However, research in the field of RNA-seq quantification for gene expression has revealed that for single-end reads, using molecular indices for deduplication is essential for accurately identifying differentially expressed genes. Furthermore, the usage of pair end reads and deduplication based on two indices can markedly increase

the sensitivity and accuracy of quantification analysis. Future benchmarking projects should prioritize addressing challenges like amplicon dropout and optimizing read deduplication strategies in bioinformatics methods [191]. These areas are essential for enhancing the accuracy and reliability of WWGS for SARS-CoV-2.

Automated bioinformatics pipelines for WWS data analysis

Bioinformatics pipelines consist of sequences of automated processes designed to analyze biological data, and they have been extensively used for WW data analysis in different reported studies [11, 19, 27, 169, 171]. Predesigned workflows facilitate the complex tasks of organizing, interpreting, and extracting meaningful information from complex WWS data. Some of the most used bioinformatics pipelines include COVID-19 Viral Epidemiology Workflow (C-VIEW) [19], CFSAN Wastewater Analysis Pipeline (C-WAP) [190], PiGx SARS-CoV-2 Wastewater Sequencing Pipeline [169], viralrecon pipeline [233], and V-Pipe [27, 234]. Additionally, many researchers resort to custom-developed or modified pipelines tailored to their unique research requirements [55, 56, 187]. The widespread adoption of comprehensive bioinformatics pipelines is driven by the necessity to systematically assess a large volume of samples, by ensuring the accuracy and robustness in detecting specific SARS-CoV-2 lineages, determining their prevalence in the population, and estimating their relative abundance.

Applications of wastewater genomic surveillance

When implemented carefully and integrated with other surveillance systems, WWGS can effectively contribute to understanding and monitoring the spread of infectious diseases and pathogen evolution. By tracking the evolution of viruses, detecting novel mutations, and identifying emerging lineages, WWGS can help identify hotspots and elevated infection rates in communities, especially in small to medium-sized populations (Fig. 6). If locally limited, the latter might signal potential outbreak events. In this context, it is crucial to recognize that the reliable detection of pathogen transmission fundamentally depends on individual-level information, which is missing in WWGS, necessitating the use of traditional epidemiological tools. In contrast to what may be implied in other publications, it's thus important to highlight that wastewater surveillance primarily aids in monitoring the relative abundances of pathogen indicators and cannot establish epidemiological links confirming an outbreak. Regardless, by providing critical insights into pathogen evolution and incidence, WWGS can additionally assist in assessing the impact of vaccination programs, thereby shaping and informing public health strategies and interventions (Fig. 6). Understanding the benefits and limitations makes WWGS a robust and powerful public health tool that complements other surveillance systems [235].

Tracing emerging lineages and monitoring infection rates

WBE can be used as an early warning tool for viral spread in the community, identifying emerging lineages, and monitoring infection trends, proactively potentially informing public health actions and policy decisions. While routine WWGS can

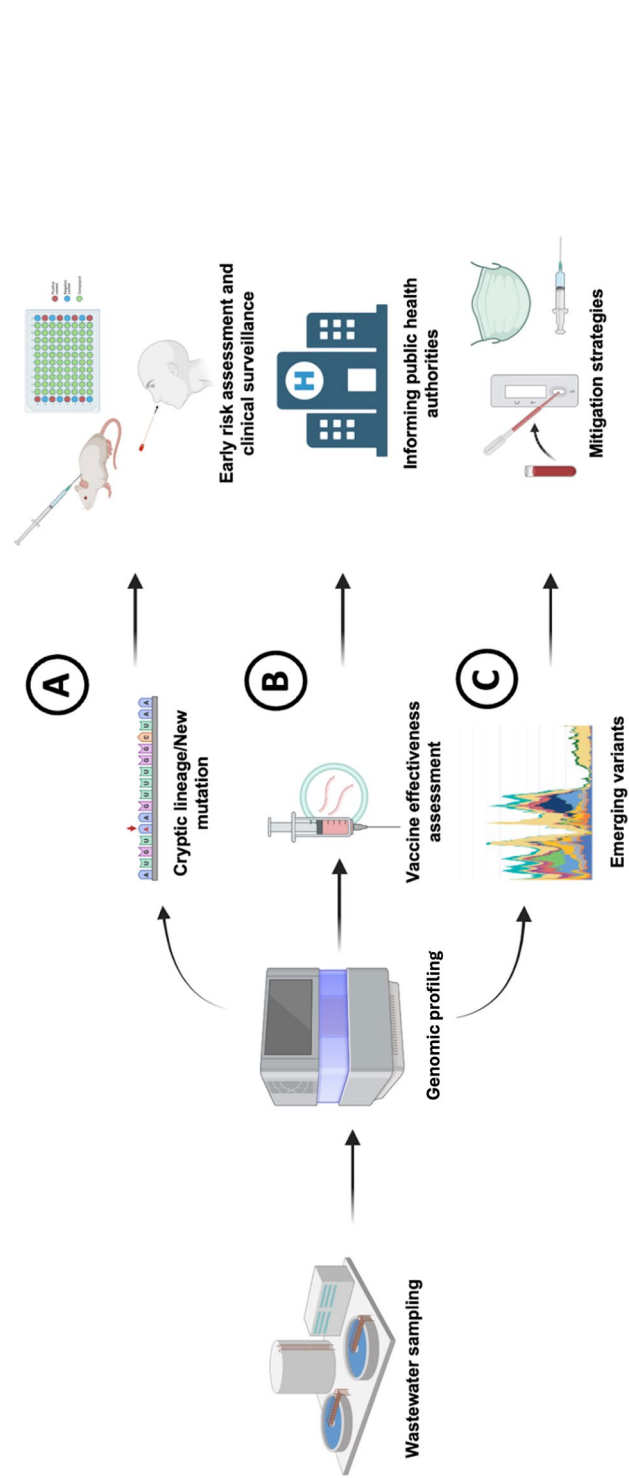


Fig. 6 Key applications of wastewater genomic surveillance (WWGS). WWGS acts as an effective early warning system, enabling the continuous tracking of SARS-CoV-2 lineages and detecting increasing trends, particularly viral when clinical testing and genomic surveillance of patient samples are scaled down. **A** WWGS facilitates the discovery of new mutations, aiding clinical genomic surveillance, for instance, by refining variant-specific PCR assays. **B** Additionally, WWGS can provide insights into vaccine effectiveness, offering valuable information to public health officials regarding health strategies. **C** The identification of emerging variants through WWGS could/should play a crucial role in enhancing health authorities' mitigation policies and tools

never fully replace other surveillance instruments, it can become a primary and central source of information on trends of SARS-CoV-2 circulation in various communities. A recent study from the USA showed that forecasting models derived from WBE data can accurately predict the weekly new hospital admissions due to COVID-19, providing a 1–4 weeks window for introducing mitigation measures [236].

The qualitative assessment offers additional advantages in this regard. Tracking the dynamics of the contribution of particular (sub)lineages in wastewater is a powerful early warning tool to understand viral shifts that occur at the community level on different scales. Their spatial and temporal spread can be tracked, in real-time or retrospectively, by integrating data derived from various catchment areas, allowing for the identification of hot spots of specific viral (sub)lineages [55, 56, 237, 238]. Foremost, qualitative WWGS can detect them much earlier than clinical testing, ahead by weeks or even months [19, 57, 171, 239, 240], enabling expedition of an effective containment response and by guiding, as a complementary surveillance strategy, public health policies regarding face masking, booster vaccinations, and/or decreased social mobility. Ultimately, WWGS, coupled with (sub)lineage-oriented risk assessments, can become an effective and complementary tool to decrease infection rates, long-term consequences of COVID-19, hospital admissions, and mortality.

Moreover, WWGS has the potential to screen cross-border SARS-CoV-2 spread. Applied to aircraft wastewater samples, it can effectively monitor viral (sub)lineages potentially carried by onboard passengers and enrich data on viral diversity in departure areas. In the past, selected SARS-CoV-2 variants were detected in clinical samples from returning overseas travelers [162, 241]. Therefore, establishing a global aircraft-based WWGS network is postulated with use in the context of COVID-19 and future viral threats [242]. Such a network could compensate for limited genomic surveillance in various world regions, particularly low- and middle-income countries, which is essential to counter the threat of future viral variants [243].

Outbreak investigation

While an outbreak is defined as an uncontrolled transmission of a pathogen among individuals [244], WWGS cannot provide evidence of this transmission since it lacks detailed information about individual cases, which is crucial for establishing an epidemiological link between those (see above). However, WWGS can estimate the incidence of disease cases within a specified location and timeframe. Should this estimated count significantly surpass expected levels, it can serve as an indicator of uncontrolled dissemination. This is becoming increasingly applicable as wastewater samples for genomic monitoring can be collected and analyzed at different population levels, from small facilities such as nursing homes to medium-sized environments such as small towns to large areas such as counties. However, traditional clustering and phylogenetic methods, which are instrumental for identifying evolutionary relationships and tracing pathogen transmission, usually require the analysis of complete genomes or at least substantial parts of them obtained from sequencing data of diagnostic samples. This ensures the high resolution required for reliable confirmation of clonality and, thus, detection of potential outbreaks. Consequently, integrating genomic data from diagnostic samples with

epidemiological information is crucial for the accurate detection and delineation of outbreaks also in WWGS [245].

The analysis and forecasting of evolutionary dynamics of SARS-CoV-2 from WWS data have been carried out using various methods, from stochastic to machine learning [246–252]. Previous models designed to predict and monitor the dynamics of SARS-CoV-2 using WWS data typically incorporate additional epidemiological data, such as case numbers and hospital admissions specific to particular locations [247, 249–251]. When relying solely on WWS data, these models primarily forecast epidemiological measurements like hospital occupancies or the number of cases within a community [248]. To enable accurate estimates of disease trajectory for specific or all variants, multiple studies report growth models fitted on WWS data to infer SARS-CoV-2 variant fitness advantage [52, 253–255]. Nonetheless, this model as well as most of such forecasting models primarily concentrates on predicting the emergence of individual mutations. The non-additive phenotypic effects of combinations of SARS-CoV-2 mutations have been suggested to be responsible for the non-linearity of SARS-CoV-2 evolution that significantly complicates its dynamics and, therefore, its forecasting [256–258].

While there has been extensive research into variant and mutation detection and forecasting using clinical SARS-CoV-2 data, such exploration of wastewater data remains relatively limited in current studies and methods. Extending such models to be applied to wastewater data would hold significant importance as a supplementary resource to more traditional surveillance methods, contributing to more comprehensive public health monitoring efforts and gaining insights into the virus' spreading paths and evolutionary trajectory prior to the availability of clinical data in the beginning of an outbreak event.

Monitoring of viral evolution and detecting novel mutations

WWGS offers an additional, independent, non-invasive resource for tracking SARS-CoV-2 evolution, which is crucial for long-term adaptation to co-existence with this pathogen and its continuous control to decrease the COVID-19 health burden in the post-acute pandemic period [259]. This is of particular value during the phase of reduced clinical surveillance, lifted restrictions, and increasing genomic diversity, with different viral sublineages in side-by-side circulation and higher odds for co-infections and recombination events [260]. WWGS can detect the emergence or introduction of known (sub)lineages in particular regions weeks prior to their identification in clinical samples, subsequent monitoring of their contribution to SARS-CoV-2 infections at the population level, prediction of the reproductive advantage, and further accumulation of novel mutations and putative novel so-called cryptic lineages [19, 54, 171]. A cryptic lineage of SARS-CoV-2 is a set of co-occurring mutations that have never been reported or rarely observed (prevalence less than 0.0001) in publically available assembled genomes [230]. This allows viral trees that have evolved over time and among various regions to be recognized and compared. Identifying novel mutation signals and potential (sub)variants through WWGS may even prompt their increased and targeted clinical surveillance [261], indicating that both approaches are complementary and can strengthen the viral monitoring network.

Earlier characterization of amino acid substitutions in Spike protein and other viral proteins through WWGS offers a more swift initiation of experimental studies on immune escape mutations and drug resistance, pivotal in vaccine-adaptation efforts and predicting the efficiency of authorized direct-acting antivirals. Using WWGS to detect more severe viral variants, e.g., harboring mutations enhancing fusogenicity, would allow for more targeted and rapid public health responses (e.g., providing data for authorities recommending vaccine antigen composition, recommending face masking, promoting vaccinations), translating into decreased morbidity and mortality. Furthermore, WWGS could be employed to track mutational signatures from exposure to mutagenic antivirals (i.e., molnupiravir authorized in selected world regions) [262], essential to explore the impacts of such treatments on the trajectory of (sub)lineage generation and onward transmission. Moreover, WWGS is a tool to track the cryptic circulation of SARS-CoV-2 variants that may appear entirely de-escalated using clinical surveillance but may otherwise re-emerge or lead to the generation of new lineage, e.g., through recombination events [263].

The presence of such cryptic lineages, are thought to result from unobserved infections, including prolonged shedding from immunocompromised individuals or potential zoonotic reservoirs contributing to cryptic viral persistence [19, 264]. WWGS offers a powerful approach for detecting viral RNA from zoonotic reservoirs in urban environments, enabling the identification of SARS-CoV-2 sequences with mutations indicative of adaptation to non-human reservoir that SARS-CoV-2 has already established (e.g., free-ranging white tailed deer) [27, 265, 266]. Cryptic SARS-CoV-2 lineages in wastewater exhibit mutations expanding receptor tropism to human, mouse and rat *ACE2*, supporting potential non-human hosts adaptation [27, 267]. Metagenomic sequencing has detected rRNA from cats, dogs, and rats in wastewater, suggesting possible zoonotic contributions. However, the overwhelming dominance of human RNA complicates direct attribution of cryptic lineages to animal reservoirs. Variability in detection across sewersheds further suggests that no single species alone sustains these lineages [27]. Future studies integrating wastewater genomics with ecological and serological data are essential to clarify the role of non-human reservoirs in SARS-CoV-2 evolution. The clinical consequences of such retransmission to the human population are challenging to predict since mutation-driven adaptations to a new host may lead to decreased adaptation to the human environment but also to improved evasion of acquired immunity, including cellular response, and thus higher susceptibility to severe disease [268, 269]. Therefore, detecting such events presents a significant computational challenge, stemming from the difficulty in distinguishing the source of the viral RNA. However, some approaches could be undertaken to overcome this issue, including a comparison of SARS-CoV-2 genomes from human cases with those found in animals to reveal potential animal reservoirs or employment of advanced computational tools, such as machine learning models, to analyze viral sequences to predict potential animal hosts [270, 271]. Recent methods have shown promising in bridging this gap. For example, machine learning frameworks like DeepHoF and RNAVirHost can predict host range based on viral genome features by training on large scale host-virus associated data, effectively classifying host types at various taxonomic levels [272, 273]. Additionally, VIDHOP tool

utilizes neural network-based classifiers trained on a broad range of viral sequences to predict with high accuracy host taxa from viral genomes, including coronaviruses [274]. These tools, while limited by reference data and taxonomic resolution, represent important steps toward inferring potential non-human sources contributing to observed variant diversity in environmental samples. Moreover, identifying host-specific marker mutations, particularly those derived from animal-origin SARS-CoV-2 genomes, could further support host attribution efforts.

Additionally to zoonotic reservoirs, chronic carriers may serve as continuous sources of viral shedding, introducing SARS-CoV-2 RNA into wastewater systems even in the absence of active outbreaks, thereby enabling active detection of viral RNA in wastewater [38, 275]. WWGS may uncover genomic cryptic viral circulation, including genetically distinct SARS-CoV-2 lineages and recombinant variants that had not been previously identified through clinical testing [38]. These findings highlight the utility of WWGS in capturing transmission dynamics that may be overlooked by clinical surveillance, particularly in areas with limited testing or asymptomatic infections [65].

Assessing the effectiveness of vaccinations

In the post-acute pandemic era, COVID-19 vaccination remains an essential and primary public health intervention to decrease SARS-CoV-2 morbidity and mortality. Omicron sublineages are clinically milder compared to previous viral variants, but their infections still can lead to severe outcomes in selected patient groups, causing health and economic burdens, management of which requires appropriate preparedness [276, 277]. However, vaccine-induced humoral immunity is short-lived, while the virus accumulates immune escape mutations, justifying booster dose recommendations and vaccine updates. At least one booster dose seems reasonable annually, particularly for the elderly, patients with comorbidities and immune deficiencies, and healthcare workers [278, 279].

Data obtained from WWGS can be used to evaluate the efficacy of vaccination by showing a decrease in SARS-CoV-2 RNA positivity in response to immunization, as was successfully demonstrated in the initial phase of mass COVID-19 vaccination [280, 281]. However, as with all analyses of wastewater data, careful consideration must be given to other potential confounding factors that may affect RNA concentrations in addition to vaccination. Nevertheless, the possibilities arising from such analyses have yet to be fully exploited in the context of vaccination. Similar studies following subsequent booster administration, integrating data on vaccination coverage in particular areas, could reinforce confidence in COVID-19 vaccinations, especially when resources for real-time tracking of vaccine effectiveness are available to the public. Such an approach could also be employed in specific settings, e.g., hospitals or nursing homes, before, during, and after booster vaccination campaigns, enabling a better understanding of the effect of immunization on virus spread in the community. One important limitation here is that once the COVID-19 vaccination transitioned from mandatory to recommended, the wastewater samples will be representative of individuals vaccinated with different vaccine types and doses in different time intervals as well as those who remain unvaccinated.

A high-throughput assay has been developed to define the epitopes of neutralizing antibodies accurately, aiming to study the effects of any mutation in the spike protein, even those not yet observed, to facilitate vaccine development [282, 283]. Additionally, a machine-learning-guided technology has been created to explore a vast sequence space of combinatorial mutations, representing billions of RBD variants, and predict their impact on ACE2 binding and antibody escape accurately [284]. Such additional data could also be used to screen for such potentially dangerous mutations in wastewater. Using WWGS, such data could be obtained earlier than through clinical surveillance and epidemiological analyses but, again, needs to be interpreted carefully and in light of the population scales of wastewater sampling. However, this can be of particular use if one considers that even with an mRNA platform, the time needed to develop and authorize an updated vaccine may be enough for SARS-CoV-2 to generate progenitors that diverge from the selected antigen. Furthermore, WWGS can potentially provide a more accurate assessment of vaccine effectiveness on the population level than analyses based on cases of breakthrough infections with presenting clinical symptoms. Lastly, since SARS-CoV-2 eradication is highly unlikely with currently available vaccines, WWGS could generate data on which mutational sites are positively selected under increased immunization levels.

Most importantly, data generated through WWGS can be integrated into the system of continued monitoring of the evolution of SARS-CoV-2, which is pivotal in guiding antigen selection for updated COVID-19 vaccines. Of note, none of the authorized COVID-19 vaccines is based on attenuated live SARS-CoV-2; thus, shedding of the vaccine-derived virus will not confound WWGS with false positive signals [285], although such a possibility needs to be considered if replication-competent vaccines would become available. With the increasing integration of wastewater surveillance into public health surveillance, its potential as a rapid method to assess the effectiveness of vaccines and booster vaccinations in community virus circulation is also increasing, indicating either silent spread or a decrease in transmission post-vaccine uptake.

Discussion

Genomic sequencing of wastewater samples has emerged as an effective tool for infectious disease monitoring due to key aspects such as community-wide sampling, early detection of emerging threats, cost-effective surveillance, its non-invasive and unobtrusive nature, and complementing other well-established surveillance systems such as syndromic surveillance and genomic sequencing of clinical samples. Optimized sequencing protocols and tailored bioinformatics methods to address wastewater-specific genomic data should be developed to make this feasible [191]. Many tools have already been developed for similar problems in genomics, but it is imperative to perform comprehensive benchmarking before they can be applied to genome-based wastewater sequencing. In addition, and especially during the COVID-19 pandemic, customized tools for analyzing wastewater sequencing data of SARS-CoV-2 were rapidly developed and implemented. Continuous benchmarking will allow not only an understanding of the quality of state-of-the-art methods but also help determine the future direction for methods development and their current feasibility.

Genome-based wastewater surveillance is an excellent supplement to clinical or epidemiological monitoring of pathogens' spread. Despite its promise, wastewater surveillance is not yet mainstream, only 70 out of 194 countries currently use it for monitoring health threats surveillance [286]. In many developing countries, limited resources make it difficult to sequence large numbers of individual samples to track emerging SARS-CoV-2 variants [287]. An appealing alternative to that can be collecting and sequencing viral samples from wastewater, which is significantly more cost-effective and expands the coverage of a surveilled population. However, conventional WWGS predominantly relies on centralized sewer systems, which can lead to the exclusion of populations in unsewered areas. To mitigate this gap, alternative sampling strategies have been developed to extend WWGS to decentralized settings. For areas with open defecation or where formal wastewater collection systems are absent, surface water sampling has emerged as an effective method for detecting SARS-CoV-2 RNA in regions where wastewater is directly discharged into rivers, streams, or drainage channels [288, 289]. This approach has proven particularly useful in areas with open defecation or where formal wastewater collection systems are absent. Additionally, passive sampling using absorbent materials placed in open drains, latrines, or standing water bodies have shown promise in capturing viral RNA over time, making surveillance feasible in decentralized and low-resources settings [290]. Additionally, septic tanks and pumping stations can provide viable sampling opportunities for targeted surveillance [291]. Portable sequencing devices, such as Oxford Nanopore MinION may facilitate mobile wastewater surveillance, enabling near real-time genomic surveillance of SARS-CoV-2 and emerging pathogens [292, 293]. When coupled with community-level wastewater sampling from public sanitation facilities, markets, or schools, this approach allows for targeted epidemiological assessments in unsewered areas, enabling early detection and response strategies [292].

A typical COVID-19 wastewater surveillance program is a powerful epidemiological tool that provides quantification of SARS-CoV-2 and acts as an early warning system for community infections [35, 250, 287]. While wastewater genomic surveillance can never fully replace other typical surveillance systems, it provides similar or complementary assurances while also generating sequencing data, which can be used for novel mutation or cryptic VOC detection. To make it more cost-effective, pooled sequencing and advanced algorithmic processing can be used. Pooling will increase the number of samples sequenced in a single run. It should be noted that computational methods for inference of heterogeneous viral populations from pooling data exist [294, 295], but should be benchmarked and adjusted to the specifics of wastewater surveillance data. Novel bioinformatics pipelines specific to wastewater surveillance can be developed to detect potentially novel lineages and their abundances. Currently, universal standards are not established to collect wastewater samples, concentrate viral particles, extract RNA, and quantify viral loads. Standard operating procedures (SOP) should be defined, and data can be shared on public repositories. That data can be used to detect putative novel variants before they appear in a large population, and to implement preventive measures. Wastewater data can help identify the relative abundance of existing lineages and VOC and potentially assemble novel mutation profiles, hinting toward emerging lineages. Additionally, wastewater can be

used to monitor other viruses without a significant increase in the cost of monitoring, including Influenza A and B, Mpox, and norovirus [32, 70, 296–303] and shotgun DNA and RNA sequencing are comprehensive methods for simultaneous mapping of bacteria, viruses, fungi, and eukaryotes [265–268]. However, careful benchmarking and adjustments of sequencing protocols and bioinformatic pipelines are necessary. Nevertheless, all the current initiatives exploring WBS possibilities indicate its tremendous potential for reliable viral surveillance. WWGS can be a powerful supplement or even a main methodology for cost-efficient and reliable surveillance of current and future viral pandemics.

Supplementary Information

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Additional file 1: Review history.

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Review history

The review history is available as Additional File 1.

Peer review information

Veronique van den Berghe was the primary editor of this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

Authors' contributions

S.M., A.S., M.H., V.M. and M.A.S. led the project. S.M. and V.M. conceived of the presented idea. Introduction (S.M., S.M., A.S., M.H., V.M., M.A.S., W.O.O., C.G., K.A.C. and P.R.). Experimental approaches (S.M., S.M., A.S., M.H., V.M., M.A.S., J.O.O., V.G., J.M.S., A.L., M.C., C.E.M., B.T.T., A.G.L. and H.S.G.). Bioinformatics analysis (S.M., S.M., A.S., M.H., V.M., M.A.S., J.F., D.C., V.B., V.G., N.D., J.M.S., N.K., N.K.S., S.K., E.A., C.E.M., B.T.T., A.G.L., C.G., K.A.C., P.S., H.S.G., B.N. and A.Z.). Applications (S.M., S.M., A.S., M.H., V.M., M.A.S., F.M., J.S., N.C.W., R.A.O., C.G., P.R., P.S., K.A.C. and H.S.G.). Discussion (S.M., S.M., A.S., M.H., V.M., M.A.S., E.A. and K.A.C.). All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Ethical approval was not needed for the study.

Consent for publication

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Competing interests

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