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Minireview

Monitoring COVID-19 through SARS-CoV-2 quantification in wastewater: progress, challenges and prospects

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Summary

Wastewater-Based Epidemiology (WBE) is widely used to monitor the progression of the current SARS-CoV-2 pandemic at local levels. In this review, we address the different approaches to the steps needed for this surveillance: sampling wastewaters (WWs), concentrating the virus from the samples and quantifying them by qPCR, focusing on the main limitations of the methodologies used. Factors that can influence SARS-CoV-2 monitoring in WWs include: (i) physical parameters as temperature that can hamper the detection in warm seasons and tropical regions, (ii) sampling methodologies and timetables, being composite samples and Moore swabs the less variable and more sensitive approaches, (iii) virus concentration methodologies that need to be feasible and practicable in simpler laboratories and (iv) detection methodologies that should tend to use faster and cost-effective procedures. The efficiency of WW treatments and the use of WWs for SARS-CoV-2 variants detection are also addressed. Furthermore, we discuss the need for the development of common standardized protocols, although these must be versatile enough to comprise variations among target communities. WBE screening of risk populations will allow for the prediction of future outbreaks, thus alerting authorities to implement early action measurements.

On December 31, 2019, several cases of pneumonia of unidentified aetiology were reported to the World Health Organization (WHO) from Wuhan city, Hubei Province of China. Declared the outbreak a Public Health Emergency of International concern, the causative agent has spread rapidly worldwide. By November 2021, SARS-CoV-2 has caused over 250 million infections and more than 5 million deaths (Table 1). To efficiently battle this and future similar pandemics, we must find quick and efficient methodologies to predict and/or monitor the extent of the infections. The importance of readiness and early detection of cases was soon emphasized by the WHO (Table 1), and it is vital to decrease the risk of transmission (Eftekhari *et al.*, 2021). Infected individuals can excrete coronaviruses by vomit, sputum and mostly by faeces. Several studies have reported the detection of viral RNA in faeces even after 25 days of the infection (Amirian, 2020; Panchal *et al.*, 2021). Therefore, to date, probably the most promising methodology to monitor SARS-CoV-2 infections is wastewater-based epidemiology (WBE). In this case, WBE aims at the detection and quantification of SARS-CoV-2 in wastewater (WW) to estimate the number of infected subjects in a population from a certain area by using a relatively fast, cheap and easy process. Furthermore, WBE can overcome clinical surveillance limitations related to the selection of the tested individuals, particularly for asymptomatic infected people and their real representation of the global population. The first study showing the positive presence of SARS-CoV-2 in sewage was reported on March 4/5 in the Netherlands (Medema *et al.*, 2020). Since then, monitoring based on WBE has been implemented in many

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Table 1. Temporal evolution of COVID-19 as reported by the World Health Organization (Source: WHO´s home page, https://www.who.int/).

31 Dec 2019	Several cases of pneumonia of unidentified aetiology reported to the WHO from Wuhan (China)
9 Jan 2020	The causative agent was characterized (by whole genome sequencing of RNA) and named novel coronavirus (2019-nCoV)
13 Jan 2020	WHO publishes first protocol for a RT-PCR assay to diagnose the novel coronavirus
30-31 Jan 2020	WHO declared the outbreak a Public Health Emergency of International concern Importance of readiness and early detection of cases was emphasized
4 Feb 2020	The possibility arises that there may be individuals who are asymptomatic that shed virus
11 Feb 2020	The International Committee officially designated the virus as SARS-CoV-2 due to its genetic resemblance with sever acute respiratory syndrome coronavirus (SARS-CoV) WHO named the disease COVID-19 (coronavirus disease 2019)
11 Mar 2020	COVID-19 was declared a global pandemic by the WHO
21 Mar 2020	WHO published laboratory testing strategy recommendations for COVID-19
2 Apr 2020	WHO reported on evidence of transmission from symptomatic, pre-symptomatic and asymptomatic people infected with COVID-19, noting that transmission from a pre-symptomatic case can occur before symptom onset
4 Apr 2020	WHO confirmed over 1 million cases of COVID-19 worldwide
11 Apr 2020	WHO published a draft landscape of COVID-19 candidate vaccines
22 Sep 2020	WHO issued the first Emergency Use Listing for a quality antigen based rapid diagnostic test for detecting the SARS-CoV-2 virus
14 Dec 2020	United Kingdom authorities reported a SARS-CoV-2 variant (B.1.1.7; Alpha) of concern to WHO
18 Dec 2020	South Africa authorities reported a new variant (B.1.351; Beta) of SARS-CoV-2 rapidly spreading
31 Dec 2020	WHO issued its first emergency use validation for a COVID-19 vaccine and emphasized the need for equitable global access
8 Jan 2021	WHO published guidance for laboratories on maximizing the impact of SARS-CoV-2 sequencing now and other emerging pathogens in the future
9 Jan 2021	WHO was notified by Japanese authorities of a SARS-CoV-2 variant, which was identified when whole-genome sequencing was conducted on samples from travellers from Brazil
12 Jan 2021	WHO moves to expand its scientific collaboration and monitoring of emerging variants of SARS-CoV-2 Increasing sequencing capacity across the world is a priority research area for WHO
29 Jan 2021	WHO published its new Essential Diagnostics List, including recommended COVID-19 tests (PCR and Antigen)
2 Feb 2021	Nomenclature groups held their first meeting to explore a mechanism to develop a standardized nomenclature for variants
26 Nov 2021	WHO designated new variant B.1.1.529 (named Omicron), first reported from South Africa, a variant of concern
29 Nov 2021	A total of 7.772.799.316 vaccine doses have been administered
30 Nov 2021	There have been 261.435.768 confirmed cases of COVID-19, including 5.207.634 deaths, reported to WHO

other countries, for example Australia, China, France, Italy, the United States and Spain (reviewed in Adeel *et al*. (2021) and Ali *et al*. (2021)). But is all that glitters gold? SARS-CoV-2 sewage-based epidemiology still has major challenges that need to be addressed (Fig. 1).

Stability of SARS-CoV-2 RNA in untreated wastewater

WW is a complex matrix that undergoes a multitude of physical and chemical changes both in space and time. Enveloped viruses such as coronaviruses are more sensitive to these changes than non-enveloped ones, for example enteric viruses (Corpuz *et al*., 2020) and, thus, less stable in WW. Temperature is one of the most critical parameters for enveloped virus inactivation. SARS-CoV-2 has proven to be very sensitive to high temperature as it can be efficiently inactivated at 70°C in just 5 min but is highly stable at 4°C. Nevertheless, SARS-CoV-2 has also proven to be more persistent than expected in untreated WW and can survive for several days (Ahmed, *et al*., 2020; Chin *et al*., 2020). Therefore, the persistence of SARS-CoV-2 in WW in the warm seasons or in tropical countries may be highly reduced, hampering the detection of the virus. However, fragments of the virus have been detected in WW even for weeks (Panchal *et al*., 2021), and this limitation can be overcome with the use of several probes for its detection.

Although thoroughly washing our hands with common soaps is very effective for the inactivation of SARS-CoV2, the use of sanitizers has expanded quickly during the pandemic. Also, the initial thought that the main transmission route was by contact with contaminated fomites provoked an excessive use of standard disinfection products such as cleaners and detergents that may end in WW. The indiscriminate and abusive use of disinfectants can rapidly compromise the lipidic viral envelope or the surface proteins (Ji *et al*., 2021), particularly in the vicinity of highly exposed areas as hospitals, and thus, artificially diminish the virus load in WW and alter the epidemiology data in WW surveillance.

Susceptibility of SARS-CoV-2 to pH changes is not so clear. On the one hand, Chan and coworkers reported that moderate changes to acid and basic pH during up to 6 days diminish SARS-CoV-2 stability while more dramatic changes to extreme pH values can completely inactivate the virus in less than 24 h (Chan *et al*., 2020). On the other hand, Chin and coworkers concluded that SARS-CoV-2 was extremely stable in a wide pH range at room temperature (Chin *et al*., 2020). It should be highlighted that pH alterations in WW are often associated with the presence of toxic compounds such as chlorine derivatives that can also have additional effects, for example oxidization and denaturing.

Overall, it can be concluded that the stability of coronaviruses in WW can be variable, being high temperature and the presence of disinfectants the main facts that can artificially diminish the detection rate in WBE.

Effects of WW treatments on SARS-CoV-2

The most common WW viruses are not only enteric viruses (e.g. hepatitis A) but also noroviruses, rotaviruses, adenoviruses and astroviruses (Chahal *et al*., 2016). SARS-CoV-2 can also be found in high concentrations in WWs, particularly in those coming from hospitals, because it is shed into the faeces similarly to enteric viruses (Gonçalves *et al*., 2021).

WW treatments usually combine chemical and biological degradation (Vo *et al*., 2019) completed with other disinfection methods such as advanced oxidation with ozone, active carbon, UV-C radiation and chlorination, although some of these processes may be harmful to the receptor environment (e.g. formation of organochlorines such as trihalomethanes) (Verlicchi *et al*., 2015; Moussavi *et al*., 2019) (Table 2).

In general, wastewater treatment plants (WWTPs) have proven to be very efficient in the elimination of SARS-CoV-2 (Sherchan *et al*., 2020) (Table 2). Regarding the biological processes, the extensive facilities

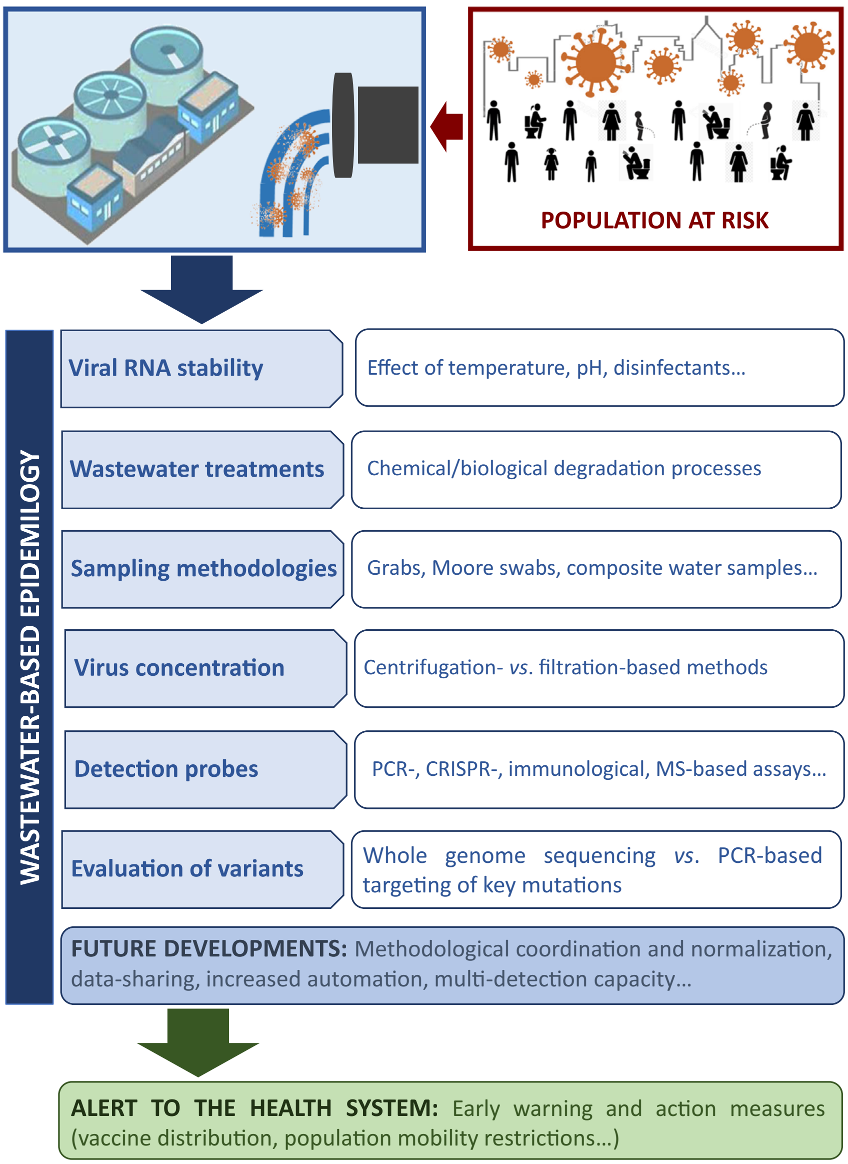

Fig. 1. Synoptic view showing the different steps of SARS-CoV-2 monitoring in wastewater.

Table 2. Efficiency of the treatments used in wastewater treatment plants (WWTP) for the removal of SARS-CoV-2.

Treatment level	Process	Results	References
Primary treatment (eradication of fixed and volatile suspended solids)	Flocculent precipitation, adsorption and gravity precipitation	50% of the effluents from the settle down in the primary stage of wastewater treatment contains SARS-CoV-2 RNA.	(Saawarn and Hait, 2020; Balboa *et al*., 2021)
Secondary treatment (elimination of biodegradable organic compounds)	Biological methods: activated sludge process, membrane bioreactor, sequencing batch reactor, pond system, moving bed biofilm reactor, upflow anaerobic sludge blanket and membrane treatment.	Biological stage removes almost 90–99% range of pathogens. Secondary treatment does not remove rotaviruses as effectively as enteroviruses. pH, HRT, BSRT and temperature affect the efficacy of the treatment stage.	(Gerba *et al*., 1981; Wigginton *et al*., 2015; Bogler *et al*., 2020; Haramoto *et al*., 2020; Saawarn and Hait, 2020; Thakur *et al*., 2021)
	Activated sludge process	Negative presence of SARS-CoV-2 RNA in 100% of wastewater samples.	(Randazzo *et al*., 2020)
	Pond system	Average reduction of pathogens by 1 log10 (14.5–20.9 days HRT).	(Feachem *et al*., 1983)
Tertiary treatment (removal of turbidity, and multiple inorganic compounds: phosphorous, nitrogen and metals)	Membrane technology	Adherence during the disinfection process in WWTP with membrane technology is the key to successful inactivation of SARS-CoV-2 in wastewater. Reverse osmosis, nanofiltration and ultrafiltration membranes should be able to remove SARS-CoV-2.	(Goswami and Pugazhenthi, 2020; Cervantes-Avilés *et al*., 2021)
	Ultraviolet radiation	Sustainable disinfection and elimination of SARS-CoV-2 from wastewater and water treatment. The interaction with sunlight reduces the number of SARS-CoV-2 in wastewater.	(Lesimple *et al*., 2020; Venugopal *et al*., 2020; Raeiszadeh and Taghipour, 2021)
	Chlorine disinfection	Effective in SARS-CoV-2 removal. Negative effect: Production of chloramines.	(Collivignarelli *et al*., 2020; Zhang *et al*., 2020; Lundy *et al*., 2021)
	Ozonation disinfection	Ozone destroys the composition of SARS-CoV-2. Ozonated full-scale effluent from activated sludge-WWTP gets SARS-CoV-2 removal.	(Tizaoui *et al*., 2020; Westhaus *et al*., 2021)

BSRT, biological solids retention time; HRT, hydraulic retention time.

based on conventional activated treatments have proven to be more efficient than those based on absorbed organic pollutants technologies using uptake of polluting organic matter through roots (*e.g*. *Canna indica*). Nevertheless, in general, the preceding adsorption-coagulation step turned out to be the most efficient step for the virus removal (Sherchan *et al*., 2020; Kumar *et al*., 2021). In this step, SARS-COV-2 co-precipitates with the particulate organic matter. Actually, there is a positive correlation between SARS-CoV-2 levels in WW and their *in situ* water quality parameters (electric conductivity, total dissolved solid, salinity and pH), probably due to the effectiveness of floc formation (Mazari and Abdessemed, 2020; Kumar *et al*., 2021). Nevertheless, WWTP effluents are not always SARS-CoV-2 free. A study carried out in 16 plants detected SARS-CoV-2 after the secondary process in 23.3% of the WWTP, which disappeared after treatment with membrane bioreactors and chlorination (Serra-Compte *et al*., 2021). In fact, the use of membrane bioreactors has proven to be an efficient process for the elimination of different types of viruses including adenovirus, norovirus and F+ phages (Amoah *et al*., 2020).

Effect of sampling methodologies and SARS-CoV-2 fluctuations

The sampling method and time of sampling are essential parameters for the application of WBE since data interpretation and potential comparisons across studies may depend on them. Most studies published to date have focused on water samples, both small or large grabs (Ahmed, *et al*., 2020; Peccia *et al*., 2020; Randazzo *et al*., 2020; Sherchan *et al*., 2020), and time or flow proportional composite samples (Nemudryi *et al*., 2020; Sherchan *et al*., 2020; Wu *et al*., 2020; Palmer *et al*., 2021; Westhaus *et al*., 2021). However, other studies have included other methods like the Moore swabs, a gauze pad that is suspended in flowing WW and then processed (Liu *et al*., 2020; Rafiee *et al*., 2021). Each method may provide different information that will depend on the volume of sample gathered (Kitajima *et al*., 2020), the time of the day (Kopperi *et al*., 2021) if the sample is not an equalized composite, as well as the point of sampling within the WW treatment train (Balboa *et al*., 2021; Palmer *et al*., 2021). Grabs can potentially have lower costs and be easier to perform than composite samples but could also have a higher degree of variability. This variability mainly depends on: (1) the volumes used, (2) the time of the day chosen, given the fluctuations in both the water usage and the source strength, which is linked to toilet habits (Heaton *et al*., 1992) and (3) the sewer distance to the WWTP as SARS-CoV-2 decay over time (Ahmed, *et al*., 2020; Bivins *et al*., 2020). However, depending on the study design, grab samples may provide stronger signals than composite samples due to the potential lesser dilution. A recent study compared the influence of the sampling strategy (grab *vs*. composite samples) over the SARS-CoV-2 detection and quantification on WW catchment basins with a range of flowrates and concluded that composite samples were superior to grab samples, specially under lower flowrates (George *et al*., 2021). Also, Liu and coworkers compared grab samples and Moore swabs in a low-flow setting and concluded Moore swabs were more sensitive (Liu *et al*., 2020). There may not be a ‘one-size-fits-all’ approach since the intrinsic characteristics of the communities such as the prevalence of the disease, the catching basins and flowrates, the WW treatments and their economic resources may determine the best local approach. Nevertheless, researchers should strive for representative samples that can capture efficiently the variability in the SARS-CoV-2 signal for early detection of spikes in contagion.

SARS-CoV-2 virus concentration for WBE

Virus detection in WW is not a new field although not too old either. One of the first problems that needed to be tackled was the concentration of the virus particles from the usually very complex WW (Calgua *et al*., 2008; Hellmér *et al*., 2014; Ahmed *et al*., 2015).

The partial degradation of the SARS-CoV-2 viruses in the sewage network leads to the formation of virus particles in colloidal suspension, close to being considered dissolved material due to their size. Therefore, the concentration methods that lead to the agglomeration and uptake by adsorption of the particulate organic matter, through coagulation-flocculation, forming micelles and flocs within the WW, increases the virus recovery, but also that of the dissolved polluted organic matter (Bogler *et al*., 2020). However, the presence of many other organic and inorganic pollutants in these waters can interfere with the later stages in SARS-CoV-2 quantification. Therefore, several modifications of these methods have been proposed to eliminate big particulate organic matter by pre-centrifugation or pre-filtration. Even though inhibition of molecular techniques can be avoided, sensibility may also decrease (Ikner *et al*., 2011; Sherchan *et al*., 2020).

Two concentrating approaches are being mainly used for SARS-CoV-2 isolation: (1) by centrifugation after coagulation-flocculation with aluminium and pH modification, that can be further supplemented with beef extract (Randazzo, *et al*., 2020) or with polyethylene glycol (PEG) (Torii *et al*., 2021), and, (2) by filtration based on adsorption-elution using electronegative membranes (Sherchan *et al*., 2020; Calderón-Franco *et al*., 2022). Both methodologies have their pros and cons. Centrifugation-based methods often need the use of dangerous chemicals and expensive instruments. On the other hand, disadvantages of the filtration methods include the requirement of washing and cleaning expensive filtration units as clogging may occur due to high turbidity.

One of the most common methods uses PEG for the virus precipitation as some authors have described that this chemical allows the removal of materials inhibiting RT-qPCR for SARS-CoV-2 gene detection from the WW samples, and at the same time produces constant and lower threshold cycle values for the quantification of MS2 control phage (Kumar *et al*., 2021). Nevertheless, PEG needs long incubation times at low temperatures and high-speed centrifugation for virus precipitation. Thus, PEG is being substituted for polyaluminium chloride (PAC) followed by low-speed centrifugation, which is not so time-consuming and requires simpler equipment commonly used in WWTP laboratories. PAC concentrated samples are stable for one week at 4°C, and the elimination of PCR inhibitors is similar than in PEG concentrates (Wehrendt *et al*., 2021).

Finally, several labs are also including a heat pretreatment to reduce SARS-CoV-2 virulence in the samples without compromising its quantification, in order to avoid the need for hard safety measures to prevent lab workers infections (Batéjat *et al*., 2020; La Rosa *et al*., 2020; Pastorino *et al*., 2020; Palmer *et al*., 2021).

SARS-CoV-2 detection probes and evaluation of virus variants

From the beginning, the PCR-based approach for the detection and quantification of the viral RNA has been the reference method to detect the SARS-Cov-2 virus in

WW. Quantitative reverse transcription PCR (RT-qPCR) shows high sensitivity and selectivity, and even today, it is the ‘gold standard’ for the detection and quantification of viral particles at low concentrations in complex matrices (Patel *et al*., 2020; Hamouda *et al*., 2021). PCR-based assays target different regions of the SARS-CoV-2 genome, including the open reading frame (ORF1a and ORF1b) regions, the nucleocapsid (N), the envelope (E) and the spike (S) protein or the RNA-dependent RNA polymerase (RdRP) genes. Of them, N (N1, N2 and/or N3) has been the most used primer-probes set during detection assays in WW. Use of proper controls during the quantification is highly recommended to minimize analytical uncertainty, for example use of positive and negative controls, multiple primer sets, biological/technical replicates, a proper surrogate virus as internal standard and indicators of PCR inhibition (Ahmed *et al*., 2020; Michael-Kordatou *et al*., 2020; Hamouda *et al*., 2021; Li *et al*., 2021). Droplet digital PCR (ddPCR) has recently emerged as an alternative that shows much lower detection limit and higher sensitivity, allows absolute quantification without requiring the use of a standard curve since it uses external calibration and is less affected by PCR inhibitors. However, it is more costly than quantitative PCR (Alygizakis *et al*., 2021; Buonerba *et al*., 2021; Hamouda *et al*., 2021; Li *et al*., 2021; Patel *et al*., 2021). While it has only been used for clinical applications, the recently developed CRISPR-based assays can be advantageous for WW-based epidemiological studies due to its high sensitivity and rapidity (Broughton *et al*., 2020). Although most of the studies detect and quantify viral RNA, the analysis of proteins has been also proposed to study the presence of SAR-CoV-2 in WW. Thus, the potential application of immunological and mass spectrometry (MS)-based methods has been recently reviewed (Buonerba *et al*., 2021).

To improve the feasibility and practicability of WBE, research efforts are also being focused on developing faster and cost-effective methods such as biosensors, which have the potential to be miniaturized, can be easily operated by non-experts, are portable and disposable and can allow on-site measurements (Bhalla *et al*., 2020; Mao *et al*., 2020; Lu, 2021). In this sense, paper-based devices have been strongly recommended for in situ analysis of SARS-CoV-2 in water environments, although its practical application in WW has to be validated (Mao *et al*., 2020; Tran *et al*., 2021).

Despite the accelerated and unprecedent effort of many research groups around the world, there are no standardized protocols for sampling and SARS-CoV-2 analysis in WW yet (Michael-Kordatou *et al*., 2020; Patel *et al*., 2021; Zhou *et al*., 2021). Methodological challenges at the different steps (*i.e*. virus shedding, sampling and storage, transportation, concentration, extraction, quality control and analysis methods) highlight the need for further improvement on analytical approaches to minimize uncertainties (Li *et al*., 2021). Within this context, Bivins and coworkers called for a global collaborative effort to coordinate methodologies and data sharing (Bivins *et al*., 2020). Furthermore, in April 2020, the NORMAN SCORE ‘SARS-CoV-2 in sewage’ database emerged as a voluntary joint initiative to provide a platform for the exchange of information and harmonization of protocols ((Lundy *et al*., 2021); http://www.normandata.eu/?q=node/361).

With the spread and long-lasting transmission of the virus, new SARS-CoV-2 mutations are constantly emerging, increasing the concern over the appearance and community spread of new variants with increased infectivity, virulence or ability to escape from the host immune response. The rapid emergence of variants worldwide highlights the importance of genetic surveillance of the SARS-CoV-2 pandemic. Thus, WHO and the European Centre for Disease Prevention and Control (ECDC) recommend increasing the routine sequencing of SARS-CoV-2 virus isolates to identify cases of new variants in a timely manner (La Rosa *et al*., 2021) (Table 1). In this sense, WBE is recognized as a valuable tool to assess the appearance and spread of virus variants at a community level and to identify new outbreaks even before they are clinically detected (Martín *et al*., 2011; Crits-Christoph *et al*., 2021). High-throughput sequencing of whole SARS-CoV-2 genomes from WW samples has allowed for the monitoring of the diversity of the circulating viruses, paying special attention to determining the frequency of viral variants of concern, including B.1.1.7 (UK) and B.1.351 (South Africa), which showed rapid spread, increased transmissibility and the uncertainty of vaccines effectiveness (Bar-Or *et al*., 2021). Most recently, on 24th November, a new variant (B.1.1.529), with a large number of mutations, has been reported from South Africa to WHO. Preliminary evidence suggests an increased risk of reinfection with this variant, as compared to other variants of concern (Table 1). Since whole genome sequencing is time-consuming, costly and requires specialized computational infrastructure and technical skills, a long PCR-nested RT-PCR assay targeting key mutations of the spike protein has been recently developed and used for variant detection in clinical samples and in highly challenging matrices such as WW. This has been proposed as a rapid and cost-effective approach for the screening of SARS-CoV-2 variants in sewage for WBE (La Rosa *et al*., 2021). Since both symptomatic and asymptomatic individuals contribute to WW inputs, WBE can provide a more comprehensive picture of the SARS-Cov-2 genomic diversity circulating in a community than clinical testing (Fontenele *et al*., 2021).

In our view, future trends should be oriented towards increasing automation capacity through the design of well-established platforms consisting of programmed autosamplers coupled with sensitive and selective biosensors that allow real-time monitoring. Platforms should be designed with multi-detection capacity, not only of SARS-CoV-2 but also of other viruses and pathogens. This will allow for continuous screening to anticipate and report future outbreaks, which in turn can alert authorities, allowing for the implementation of early action measures: vaccine distribution, population mobility restrictions etc. As suggested, 'WBE may transform the wastewater infrastructure into a public health observatory' (Michael-Kordatou *et al*., 2020).

In summary, WBE is probably our best option to monitor the current pandemic at the community level, but we must be aware of its limitations. Variables such as temperature, sampling methods, virus mutations or different hygienic habits can substantially alter the viral loads in WW and the subsequently estimated infection rates. Nevertheless, these challenges can be overcome using composite samples, several PCR probes or even new automatic molecular devices for the virus detection. In addition to that, this approach may not be feasible in many areas of the world, such as low-income countries due to the open defecation rates in many areas, or zones without WW sanitation networks. Finally, we need more efforts for standardizing the protocols in a way both effective and flexible so the WBE outcomes can be compared among different communities.

Conflict of interest

The authors have no conflict of interest to declare.

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