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Wastewater-based SARS-CoV-2 environmental monitoring for Piedmont, Italy

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ABSTRACT

The experience gained over the last hundred years clearly indicates that two groups of viruses represent the main risk for the development of highly transmissible epidemics and pandemics in the human species: influenza viruses and coronaviruses (CoV). Although the search for viruses with pandemic potential in the environment may have an important predictive and monitoring role, it is still based on empirical methodologies, mostly resulting from the clinic and not fully validated for environmental matrices.

As far as the SARS-CoV-2 pandemic, currently underway, is concerned, environmental monitoring activities aiming at checking the presence of SARS-CoV-2 in wastewater can be extremely useful to predict and check the diffusion of the disease.

For this reason, the present study aims at evaluating the SARS-CoV-2 diffusion by means of a wastewater-based environmental monitoring developed in Piedmont, N–W Italy, during the second and third pandemic waves.

Wastewater sampling strategies, sampling points sample pre-treatments and analytical methods, data processing and standardization, have been developed and discussed to give representative and reliable results. The following outcomes has been highlighted by the present study: i) a strong correlation between SARS-CoV-2 concentration in untreated wastewater and epidemic evolution in the considered areas can be observed as well as a predictive potential that could provide decision-makers with indications to implement effective policies, to mitigate the effects of the ongoing pandemic and to prepare response plans for future pandemics that could certainly arise in the decades to come; ii) moreover, the data at disposal from our monitoring campaign (almost 500 samples analysed in 11 months) confirm that SARS-CoV-2 concentrations in wastewater are strongly variable and site-specific across the region: the highest SARS-CoV-2 concentration values have been found in sewer networks serving the most populated areas of the region; iii) normalization of viral concentrations in wastewater through Pepper Mild Mottle Virus (a specific faecal marker) has been carried out and commented; iv) the study highlights the potential of wastewater treatment plants to degrade the genetic material referable to SARS-CoV-2 as well.

In conclusion, the preliminary data reported in the present paper, although they need to be complemented by further studies considering also other geographical regions, are very promising.

1. Introduction

The current COVID-19 pandemic, by the SARS-CoV-2 coronavirus, represents an unprecedented worldwide public health emergency. SARS-CoV-2 is a highly contagious respiratory virus, whose death toll has overcome 4.2 million people in July 2021. SARS-CoV-2 infection is transmitted through different routes, directly (1), through respiratory droplets emitted by infected people, while sneezing, coughing or talking, even if they are asymptomatic (Morawska and Cao, 2020), indirectly (2), through contact of a susceptible person with a contaminated object or surface (fomite transmission, Marques and Domingo, 2021), and through airborne
Transmission (3), defined as the spread of an infectious agent caused by the dissemination of aerosols that remain infectious when suspended in air over long distances and time and could be inhaled (WHO, 2020).

Recently, the World Health Organization (WHO, 2021) and the Center for Disease Control and Prevention of the USA (CDC, 2021a) clearly reported the predominance of airborne transmission, whereas the risk of SARS-CoV-2 infection via fomite transmission route has been assessed to be low, generally less than 1 in 10,000 (CDC, 2021b).

Until now, the faecal–oral transmission route has not been established yet. Most studies focused on SARS-CoV-2 clinical symptoms in the respiratory tract, but the angiotensin converting enzyme 2 (ACE2) receptor is also expressed in the intestine. Many patients with COVID-19 have gastrointestinal symptoms and a study developed in Hong Kong reported that 48.1% of faecal samples from patients with COVID-19 have tested positive for viral RNA (Cheung et al., 2020). Sometimes, faecal samples remained positive even after swab samples turned out to be negative (Xu et al., 2020); a meta-analysis by van Doorn et al. (2020) reported that 51.8% (95% CI 43.8–59.7%) of patients have their stool samples tested positive while another systematic review by Gupta et al. (2020) reported a similar percentage (53.9%). Moreover, infective virus particles were isolated in faecal samples (Xiao et al., 2020) and an in vitro study reported 1–2 survival days of SARS-CoV-2 in faeces (Guo et al., 2021). Some direct evidence of a potential faecal–oral transmission of SARS-CoV-2 was also found in ferrets (Kim et al., 2020). Thus, the dissemination of aerosols that remain infectious when suspended in air over long distances and time and could be inhaled (WHO, 2020).

SARS-CoV-2 infection via fomite transmission route has been assessed to be low, generally less than 1 in 10,000 (CDC, 2021b).
around potential, limiting factors, current knowledge, and research needs for WBE.

Sampling and analysis of untreated wastewater, purified discharges and surface water that may be subject to untreated discharges represent the key to understanding the interactions between SARS-CoV-2 and the environment. The relevant data obtained by means of the described approach support crucial choices by health authorities.

2. Materials and methods

2.1. Wastewater sampling plan

Piedmont is a region placed in North Western Italy, with more than 4.3 million of inhabitants. In Piedmont there are 171 wastewater treatment plants (WWTP) over 2000 population equivalents (PE), treating 93% of the overall regional pollutant load, and more than 2600 settlements under 2000 PE (Regione Piemonte, 2020). The length of the regional sewer network is 21,665 km (5 m per inhabitants), the wastewater volume treated by WWTPs is around 314 million m$^3$/y.

Concerning wastewater samplings aiming at SARS-CoV-2 surveillance, some essential aspects must be addressed:

1. Sampling points should be chosen in order to be representative of the population distributed on the territory;
2. Sampling strategy should be decided with consideration to grab samples which could give only a partial information of wastewater composition whereas composite samples, in particular flow-composite, can be more representative, as highlighted by previous studies on different human markers in wastewater (Ort et al., 2010);
3. The frequency of wastewater collection should be as high as possible (bimonthly, weekly or even higher);
4. The volume of sampled wastewater should be in the range 50–200 mL;
5. Transport temperature of samples should be 4 °C. Medema et al. (2020) demonstrated that SARS-CoV-2 RNA is stable in wastewater when stored at 5 °C;
6. A systematic collection of wastewater samples should be coupled with the precise knowledge of sewer networks to better identify the onset of an outbreak of the disease. Fig. 1 reports an example of the sewer network for the city of Torino, Piedmont.

Since the month of September 2020, aiming at supporting regional epidemiological surveillance, Arpa Piemonte started to sample wastewaters entering the 13 largest urban WWTPs of the region. Samples were taken firstly on a bimonthly basis and then, from April 2021, weekly or more frequently. All considered wastewater treatment plants have tertiary treatment, that is a disinfection process, through chlorination and/or ultraviolet irradiation, before discharging in rivers.

To ensure maximum significance to the environmental monitoring and to standardize the WBE approach, a few strategies were followed. First, the WWTPs have been chosen in order to cover all 8 piedmontese provinces according to criteria aimed at guaranteeing the accuracy of the results and the maximization of the underlying population. The wastewaters treated by the selected WWTPs account for much more than a half of the population living in Piedmont. Based on the same rationale aiming at representativeness, both instantaneous samples and 24 h flow-composite samples were carried out: as already mentioned, composite samples could be preferable because of the complexity and variability of wastewater and SARS-CoV-2 shedding. Nevertheless, when the catchment area is particularly large and big equalization basins are present, SARS-CoV-2 concentrations in grab and composite samples could be almost equivalent. Third, we tried to sample wastewaters always in the same day of the week, at the same hour.

All wastewater samples that couldn’t be analysed within a couple of days were stored at a temperature of –20 °C. Up to now, almost 500 wastewater samples has been analysed.

The studied WWTPs are reported by Table 1. In the present report, we focused on 4 WWTPs in particular:

1) Castiglione Torinese (N–W of the region), collecting wastewaters from 44 towns (among them, Torino) and almost 1.5 million people,
2) Cuneo (S–W of the region), collecting wastewaters from 17 towns and around 102,000 people,
3) Asti (S–E of the region), serving 83,000 people,
4) Govone (S–W of the region), collecting wastewater from 27 towns (Alba among them) and almost 78,000 people.

Fig. 2 reports the position of the plants.

Other regional WWTP inlets (5 plants around Alessandria, S–E of the region) have been sampled with different frequencies following a control criterion inspired by greater territorial capillarity. The
corresponding results will not be considered in the present paper.

It is worth reporting that the European Commission RECOMMEN-
DATION (EU) 2021/472 sets out guidance on the design and manage-
ment of SARS-CoV-2 wastewater surveillance systems, also promoting
minimum requirements for efficient wastewater surveillance strategies
and the use of common methods for sampling, testing and data analysis.

The following EC recommendations have been already implemented
by the wastewater monitoring campaign run by Arpa Piemonte: i) the
surveillance system should cover a significant part of the Member State’s
population, the monitoring system should include at least wastewaters
from large cities with over 150,000 inhabitants; ii) “the samples should
be taken at inlets to wastewater treatment plants or, where relevant,
upstream at the wastewater collecting networks. The presence of SARS-
CoV-2 virus and its variants should be regularly analysed, ideally twice a
month”; iii) “the samples are taken over a period of 24 h, using a flow or
time composite sampler”; iv) “for early warning surveillance purposes,
the results for each sample should be recorded as soon as possible and
preferably no later than 48 h following sample collection”; v) “analyses
are carried out in laboratories operating appropriate RT-PCR methods
under standard quality management conditions”; vi) “all samples should
be run at least in duplicates to avoid false positive or false negative
results”; vii) “threshold cycle value of real-time polymerase chain reaction
(RT-qPCR) should be below 40 to report a sample as positive”; viii) “the
analytical procedure of real-time polymerase chain reaction used should
include adequate controls to assess at least the efficiency of the concentra-
tion/extraction steps and the absence of significant reaction inhib-
tion”; ix) “viral number of gene copies should be normalized by the
population number served by the sewer system and using the waste-
water flow for better comparability of measurements between different
locations. Additional normalization controls using cross-assemblage phage
or Pepper Mild Mottle Virus are recommended for this purpose”. Rec-
ommendations from v) to ix) will be commented in the next chapter.

2.2. Wastewater sample pre-treatment and analysis

In December 2020, the experimental tests were completed for the
development and validation of a method for the detection of SARS-CoV-
2 in wastewater. The analytical protocol developed includes a phase of
concentration/extraction of genetic material and a subsequent amplifi-
cation phase by RT-Realtime PCR (three specific targets for the SARS-
CoV-2 virus and two targets used as controls of the analytical process).

Total Nucleic Acid (TNA) extraction is performed using 40 mL of
wastewater collected either from a 500 mL or 1 L collection bottles from
a wastewater system at specific points. Both grab samples and 24-h
composite samples have been collected. The samples have been frozen
at −20 °C if not analysed immediately.

At the time of analysis, the samples have been completely thawed
using a 37 °C water bath. 40 mL of water have been collected in a Class II
biosafety cabinet. TNA extraction has been performed using the
Wastewater Large Volume Total Nucleic Acid Capture Kit AX9550
(Promega Corporation, USA). Briefly, the extraction consists of 2 steps:

- in the first one the TNA is captured on a GFA/silica column and then
  eluted in 1 mL; in the second step, the TNA is purified and concentrated
  in 40 μl.

The SARS-CoV-2 RT-qPCR detection KIT for Wastewater (Promega
Corporation, USA) is used for the amplification phase. The detected
regions are N1 and N2 (regions of the Nucleocapsid gene) and E (region
of the Envelope gene).

The applied kit can also evaluate a series of very important analytical
process controls to normalize obtained data and avoid the presence of
false negatives:

- a set of primers for the detection of an “internal process control”
target (Pepper Mild Mottle Virus-PMMoV, a RNA virus commonly
found in faeces) that allows to monitor the entire analytical process
(extraction phase, reverse transcription, absence of inhibitors); this
kind of internal control is not influenced by seasonal fluctuations or
social aspects (D’Aoust et al., 2020);

- an internal amplification control (IAC) that allows to check that there
are no inhibitors during the amplification phase.

The PCR Master MIX, necessary for amplification to take place, is
designed in order to avoid that different compounds (e.g. humic acids),
normally present in wastewater samples, can act as PCR inhibitors.

PCR samples are analysed in duplicate for each of the targets (N1, N2,
E, PMMoV, IAC).
The results are expressed with the CT values (Cycle Threshold) for each detected target. In case of absence of amplification (absence of the desired target) the result is reported as CT > 40. The theoretical LoD is 3 genomic copies per reaction.

The specificity of the RT-PCR kit has been further investigated using RNA from HCoV-OC43 (Human Coronavirus OC43) and from HCoV-229E (Human Coronavirus 229E). HCoV-OC43 belongs to the Betacoronavirus genus like SARS-CoV-2, whereas HCoV-229E belongs to the Alphacoronavirus genus; both are endemic in the human population.

### 2.3. Data analysis

Wastewater-based epidemiology relies on the assumption that there is a relation between prevalence or incidence of COVID-19 in the population, and the concentration of SARS-CoV-2 RNA in wastewater, expressed as RNA copies per mL of wastewater or simply as Cycle Threshold (CT) resulting from Real Time PCR. Wastewater analysis results are then associated with clinical test results involving the population living in the sewer catchment area, on a time scale of several months. Two different approaches have been used to connect SARS-CoV-2 concentrations in wastewater with the COVID-19 prevalence data. The first looks for temporal correlations between the two datasets. The second aims to build a reliable correlation between the databases in order to have at disposal a prediction tool based on wastewater analysis. Where appropriate, regression analyses are performed to assess a correlation between SARS-CoV-2 detected CT and the circulation of COVID-19 in the population. In order to build a reliable epidemiological tool, regression analyses could also take into account the expected delay between SARS-CoV-2 signal in wastewaters and COVID-19 test reports, or rather, statistical analysis could provide a quantification of the advance due to a proper use of WBE on clinical outbreaks.

Therefore, SARS-CoV-2 found in untreated wastewater can be seen as a biomarker and the measured concentration can then be used to infer the size of the shedding population and therefore provide community-level health information (as described also by Xagoraraki and O’Brien, 2020).

A stochastic approach, aiming at calculating absolute numbers of infected people, has not been proposed as the current knowledge is still far from enough to support successful WBE application in absolute calculations.

All these aspects and the corresponding pros and cons will be discussed in the following chapters, as well as some reasonings about underreporting and the effects of vaccination and new emerging variants.

### 3. Results

Based on the preliminary tests carried out with a SARS CoV-2 RNA standard/reference material (LGC, UK), the recovery efficiency of the concentration/extraction/purification phase applied by Arpa Piemonte turned out to be around 50%. These results would be pretty good if compared with the recovery efficiencies reported by Ahmed et al. (2020b), who tested the efficiency of seven wastewater virus concentration methods (adsorption-extraction with three different pre-treatment options, centrifugation, polyethylene glycol (PEG 8000) precipitation, and ultracentrifugation) on murine hepatitis virus (MHV), finding mean recoveries ranging from 26.7 to 65.7%. Torri et al. (2020) carried out concentration experiments on Pseudomonas phage ø6, using a method combining polyethylene glycol (PEG) precipitation and acid guanidinium thiocyanate-phenolchloroform extraction; a mean recovery efficiency of 29.8–49.8% was obtained, confirming the good performance of the method applied by our research group.

Taking into account the recovery efficiency attributed to the virus concentration/extraction/purification phase, the overall analytical LoD for the applied protocol is about 1200 genetic copies per litre of raw wastewater, that is very close to the detection limit of 10^3 copies/L estimated by Wurtzer et al. (2020).

According to European Commission recommendations, the specificity of the applied RT-PCR kit for SARS-CoV-2 has been demonstrated: no amplification and no aspecific signals have been detected in any of the two samples containing HCoV-OC43 and HCoV-229E.

In the analysed wastewater samples, the presence of the 3 different virus-specific target sequences (E, N1 and N2) was mostly detected, indicating the presence of SARS-CoV-2 RNA in the samples undergoing analysis.

The data reported in Figs. 3–6 show temporal correlation between concentrations in wastewater, expressed as CT values of SARS-CoV-2 E gene, and COVID-19 prevalence data, that is the reported number of active cases in the population living within the catchment area. Prevalence data are available for the studied WWTPs catchment basins from the September 1, 2020 to March 15, 2021.

Here we can observe that SARS-CoV-2 concentrations in wastewater clearly anticipates the trend of disease for all considered sites. Data illustrated by Fig. 3 are particularly important because the population served by the sewer network in this case is very large, almost 1.5 million people, that means 35% of the regional population. The calculated peak values are well below CT 30 in all the reported cases, corresponding to concentrations ranging from 400 to 600 SARS-CoV-2 genomic copies per mL of raw wastewater.

Fig. 7 reports the CT values of the 3 different targets (N1, N2 and E genes) obtained for wastewater collected by WWTP placed in Novara, N-E part of the region, showing a good overall agreement as far as viral gene detection is concerned.

Provided that the wastewater monitoring campaign is still going on, and almost 500 samples have been analysed throughout all the region, it is possible to show all analytical results together in Fig. 8. Here, the second and third infection waves are clearly visible even though the variability of SARS-CoV-2 concentrations for the same period is quite large. The figure highlights the results coming from the WWTPs of Castiglione Torinese, Asti, Govone and Cuneo, that are also reported by Figs. 3–6.

As already mentioned, besides using composite wastewater samplings to minimize the influence of the uneven diurnal wastewater flowrates and the timing of toilet flushing, wastewater analysis results should be normalized using specific population or faecal markers because every WWTP could show different dilution factors of human dejections according to the served activities. In this regard, we used the detection of Pepper Mild Mottle Virus (PMMoV) target to compare results coming from different wastewater treatment plants. Over almost 500 samples, PMMoV average Cycle Threshold was 22.31, with a 95% CI between 22.16 and 22.46. This result is quite important since it means that human excrements are diluted similarly in all considered sewer networks and dilution does not show seasonal variations from September to July.

Nonetheless, SARS-CoV-2 analyses coming from the 4 WWTPs the study focuses on have been normalized using PMMoV: since we used Cycle Thresholds, both for SARS-CoV-2 and PMMoV, the normalized data are obtained dividing PMMoV CT by SARS-CoV-2 E gene CT. The results have been grouped according to sampling periods, with a duration of 15 days each: for each period, from 2 to 11 data are at disposal. Fig. 9 reports the calculated normalized signal with average values and 95% confidence intervals for each sampling period from September 1, 2020 to July 14, 2021.

Finally, a regression analyses is proposed to assess a correlation between wastewater detected SARS-CoV-2 concentrations and COVID-19 prevalence in the population: the attempt was carried out for Castiglione Torinese treatment plant because of the amount of analyses at disposal, higher than the other sites, and the significance of served population. The best correlation was found between SARS-CoV-2 concentrations in wastewater and the average COVID-19 active cases reported in the period going from the 7th to the 21st day after wastewater sampling. The described correlation, valid for the period September
4. Discussion

On the basis of the results reported by Figs. 3–6, the first aspect to observe is that the peak values, corresponding to concentrations ranging from 400 to 600 SARS-CoV-2 genomic copies per mL of raw wastewater, perfectly fall within the range reported by Medema et al. (2020) regarding an EU-wide feasibility study based on tests developed in 52 WWTPs serving cities of 17 countries (the concentrations turned out to be 1–946 gene (N2) copies per mL).

Then, comparisons can be made with the COVID-19 reported number of active cases in the WWTP catchment area, showing a strong correlation between SARS-CoV-2 concentration in sewers and diagnostic tests results for all considered sites, perfectly in line with the indications of the literature studies. The same correlation (data not shown) could be found between SARS-CoV-2 concentration and incidence of new cases of COVID-19 in the catchment area.

Moreover, Figs. 3 and 6 show a lower SARS-CoV-2 concentration peak around Christmas time that was not detected by official testing because of the reduced activity during the festivity. Therefore, wastewater monitoring could be considered a complementary tool for the pandemic management.

The preliminary results obtained by our research group clearly pointed out all potentiality of wastewater monitoring in order to provide authorities with helpful data to manage SARS-CoV-2 pandemic outbreaks.

The systematic application of the described monitoring approach to a
large number of WWTPs over a vast territory, within the regional environmental monitoring plan carried out by Arpa Piemonte, could be the basis of a new dynamic knowledge of the spread mechanisms of the disease.

Given that the concentration trends are crucial, it is possible to strengthen the environmental monitoring going upstream from the initial sampling points, analysing the wastewater in the various branches of the network in search of the primary outbreak. As a matter of facts, wastewater samplings could be done also through sewer pumping stations or via manholes in the street (Weidhaas et al., 2020) or the sewer pipes leaving a single building or structure.

The analysis of wastewater concentration trends, associated with demographic, social and geographic statistics, could help identify a few key monitoring points at a regional level serving as an early warning network to monitor fluctuations of SARS-CoV-2 circulation in communities.

Moreover, the monitoring activities carried out in the last 11 months have pointed out that, even though the wastewater entering the WWTP could be strongly positive for SARS-CoV-2, genetic materials related to the virus are not detectable in the discharged waters, after treatment. This statement has been tested both in Castiglione Torinese and Govone WWTPs, confirming similar results reported by other studies (ISS, 2020).

Indeed, the current wastewater purification practices are effective to degrade the virus, given the retention times combined with hostile environmental conditions (temperature, solar irradiation, high pH levels, indigenous microbial populations). The final disinfection optimizes the conditions for the complete removal of the virus before the purified water is reintroduced into the environment.
With regard to all data collected since the wastewater monitoring campaign started in September 2020, as reported by Fig. 8, a strong variability between SARS-CoV-2 concentrations found in different wastewater treatment plants can be observed, as expected. It is worth mentioning that data coming from the only big city of the region, Torino, served by Castiglione Torinese plant, are regularly placed in the highest part of the concentration range (that is, the lowest CTs). This behaviour would confirm that SARS-CoV-2 spreads more where people live concentrated in smaller areas compared to different demographic patterns, such cities with less than 100,000 inhabitants, towns and villages.

The observed variability reported by Fig. 8 is also confirmed by Fig. 9 where normalized data coming from the 4 studied WWTPs are aggregated according to a bimonthly frequency. Here the disease regional trend, made by the second and the third COVID-19 waves, is still evident. Nevertheless, provided that reported confidence intervals are relevant, it would be not advisable to extend the validity of a wastewater monitoring campaign results from a place to another, even though distances between the considered cities are not big. As a matter of fact, it is not correct to perform aggregated analysis across a regional context.

Concerning the quantification of COVID-19 prevalence in the population from wastewater surveillance, Medema et al. (2020) reported a comparison between theory and practice of quantitative relation between SARS-CoV-2 circulation in the population and SARS-CoV-2 RNA in wastewater.

Theory is based on the following simplified formula:

\[ C_{\text{sewage}} = \frac{N \cdot F \cdot C_{\text{faeces}}}{Q} \quad (1) \]

where \( C_{\text{sewage}} \) is wastewater SARS-CoV-2 concentration, \( N \) is the number of people shedding, \( F \) is the faecal load (g/person/day), \( C_{\text{faeces}} \) is the concentration of RNA in the faeces of infected people and \( Q \) is the total flow to sewer per day.
Based on literature reported by the cited author, SARS-CoV-2 concentration in the faeces can vary from $10^2$ to $10^{10}$ genetic copies per gram, according to time of infection (some studies report that pre-symptomatic time represents the peak of shedding), and person-to-person variability (not all stool samples from infected patients are positive for SARS-CoV-2 RNA). Medema reports that the large uncertainty observed in faeces SARS-CoV-2 concentration is interpreted as an erratic behaviour by some author (Walsh et al., 2020). After all, great variability of virus shedding potential is expected as observed SARS-CoV-2 concentration ranges from $10^2$ to $10^{11}$ RNA copies/mL of mucus in swab samples in asymptomatic persons as well (Buonanno et al., 2020). Another author (Zhu et al., 2021) defines faecal shedding as long-tailed as the shedding of SARS-CoV-2 in faecal specimens can outlast that in respiratory: this behaviour can mask new outbreaks, in particular when an infection peak has recently ended and the shedding population remains large.

The uncertainty budget relating to the term $C_{\text{faeces}}$ is much higher than the other factors of the reported formula, strongly influencing the overall uncertainty of the quantification approach. Medema reports the results of a Monte Carlo simulation where a concentration of 1000 SARS-CoV-2 genomic copies per mL of sewage corresponds to a prevalence in the range 70–3000 infected persons per 100,000 inhabitants. Similar results were pointed out by Ahmed et al. (2020a) who report: 'given the uncertainty and variation in the input parameters, the model (Monte Carlo simulation) estimated a median range of 171 to 1090 infected persons in the catchment'.

Stochastic SARS-CoV-2 prevalence estimates based on wastewater surveillance are still generally highly uncertain, also because of the need for reliable and complete data on virus RNA shedding by asymptomatic and presymptomatic people, as well as on the effects of vaccination and new virus variants on shedding. Indeed, vaccination of a large part of population could increase the number of asymptomatic people and decrease viral loads in patient, whereas new variants could enhance viral loads, also for breakthrough infections occurring in fully vaccinated people (CDC, 2021c). The development of epidemiological models and serological data could therefore help future progress and a heavy research work is still to be done to reduce the uncertainty in the faecal viral shedding data. Nevertheless, as suggested by some authors (Zhu et al., 2021), if the shedding profile is eventually too unpredictable to be modelled, absolute calculations of COVID-19 prevalence in population will not be as reliable as we need.

Another possible quantitative approach could involve the statistical analysis of the correlation between SARS-CoV-2 concentrations in wastewater and registered COVID-19 prevalence in the population. Prevalence predictions could be based on a mathematical function with a good correlation coefficient, as highlighted by Fig. 10. In this case, data collected from September 2020 and February 2021 in the metropolitan area of Torino have been elaborated and wastewater analyses seem to represent a very good predictor of average prevalence, with a few days advance. Nonetheless, it should be remembered that the clinical testing normally underestimates real prevalence of the disease in the population, therefore predictions based on the correlation function will undervalue the active cases in the population by the same percentage, unless a reporting correction factor is applied to estimates. Moreover, the correlation function is strictly site-specific, as already commented in the previous chapters, then predictions should not be exported to other areas. Finally, a correlation function between SARS-CoV-2 wastewater concentrations and COVID-19 prevalence could be valid as long as new factors, such as new virus variants or a large-scale vaccination
Concentrations of human dejections in wastewater treated by the WWTPs considered in the present study turned out to be very similar, as suggested by the statistical analysis carried out on Pepper Mild Mottle Virus. Nevertheless, normalization of viral concentrations in wastewater through the population number served by the sewer system, composite samplings, and internal control such as PPMoV, is recommended.

In the context of the ongoing massive vaccination campaign SARS-CoV-2 wastewater surveillance could contribute to answer the question whether vaccines will reduce not only the incidence of the COVID-19 disease but also SARS-CoV-2 circulation among the population.

The data obtainable with the described approach can therefore represent a valid support for subsequent processing and elaborations, including epidemiological ones, by Health Authorities, thus realizing the regulatory provisions aiming at linking the functions of environmental protection and health prevention.

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