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CHAPTER 6

# Route of SARS-CoV-2 in sewerage and wastewater treatment plants: dilution, decay, removal, and environmental transmission

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## 6.1 Introduction

Although the most common clinical presentation of COVID-19, the disease caused by SARS-CoV-2,[1] is fever and respiratory tract symptoms, this viral infection may cause other extrarespiratory manifestations, including gastrointestinal problems—such as diarrhea, nausea, vomiting, and abdominal pain—that often represent an early symptom of the disease.[2–4] However, SARS-CoV-2 positivity can be seen also in the stools of people without gastrointestinal symptoms[5] or with asymptomatic or mild disease.[6]

To detect SARS-CoV-2 in fecal samples, the gold standard is the quantitative reverse-transcription polymerase chain reaction (RT-PCR) that is based on the amplification of specific genomic regions that are specific for SARS-CoV-2[7–12] and the simultaneous monitoring of the amplification process through sequence-specific oligonucleotides labeled with fluorescent probes, which allow the quantification of the viral load. The viral load is then expressed as the number of SARS-CoV-2 RNA copies in a known volume and the units are genomic units (GU) per L or mL.

The viral load measured in the stool of COVID-19 patients depends on the day of sampling after infection initiation and can vary largely in the range of $10^3$–$10^7$ GU/g feces.[13,14] The highest concentrations of viral RNA in stool samples were found during the first week of symptoms, while it gradually decreases over time. However, patients may remain RNA-positive

*Environmental Management of COVID-19*
ISBN 978-0-323-85780-2
https://doi.org/10.1016/B978-0-323-85780-2.00005-6

in stool over three weeks[14] and even for up to 50 days or more,[6] despite the complete resolution of symptoms.[11,12,14] In general, the presence of SARS-CoV-2 in the stool may be due to: (1) swallowing of respiratory secretions, (2) residues of infected cells, or (3) replication of the virus in the intestine.[15,16] The recent demonstration of the active replication of SARS-CoV-2 in human intestinal organoids suggests that the human intestinal tract might be a transmission route of SARS-CoV-2.[17]

Although urine is frequently found negative for SARS-CoV-2[10,18] the literature confirming the presence of SARS-CoV-2 in the urine of patients affected by COVID-19 is continuously increasing.[5,19,20] However, when detected, the SARS-CoV-2 load in urine is low[20] and thus the contribution of urine to the transmission route appears less relevant than that originated from feces.

The detection of SARS-CoV-2 RNA in the feces of infected patients raises the question of potential fecal-oral transmission.[21] Theoretically, the presence of RNA fragments does not imply a priori the presence of infectious virions, but recent studies report the presence of infectious virions isolated from feces,[10,16,22,23] supporting thus the possibility of fecal-oral or fecal-respiratory transmission through aerosolized feces.[16] This latter aspect may occur for example in toilets or in the case of faulty sewage pipelines, as happened during the 2003 SARS-CoV pandemic, when hundreds of residents of a building in Hong Kong were infected due to the aerosolization of contaminated feces.[24]

Jeong et al.[25] demonstrated that viable SARS-CoV-2 can be shed through stool and urine of COVID-19 patients. Using the feces and urine from COVID-19 patients, the authors did not observe viable virus using cell culture isolation (Vero cells), despite some naso/oropharyngeal swabs and saliva specimens appeared positive for virus isolation. Conversely, the presence of infectious virus in fecal and urine specimens was identified after the inoculation in an animal model highly susceptible to SARS-CoV-2. These findings suggested that SARS-CoV-2 may be potentially transmitted via routes such as fecal-oral contact and urine.[25]

When released in infected urine/feces, the path of spread of SARS-CoV-2 RNA begins with the virus being transported from the toilets to the sewer network, where a relevant dilution of feces occurs, and then to the subsequent wastewater treatment plants (WWTPs), where the concentration of SARS-CoV-2 can be significantly reduced before the discharge of the treated effluents into the receiving water bodies.

This chapter focuses on the fate of the viral load of SARS-CoV-2 from feces of positive persons to the sewer network and further in WWTPs and treated effluents. In particular, the progressive drop of the load of SARS-CoV-2 along this route was evaluated on the basis of dilution, decay, removal in WWTPs, and experimental data available in raw wastewater or treated effluents.

## 6.2 Dilution of SARS-CoV-2 from the feces to the sewerage

When the feces of COVID-19 infected persons are discharged into the sewerage, they undergo a relevant dilution in water and the virus is subjected to several transformations due to the presence of suspended solids, organic substances, micropollutants, and bacteria, in particular, enteric.

The dilution of feces in the sewerage ($D$) can be estimated by the ratio between the volume of feces per day ($V$) and the wastewater flow rate ($Q$), both expressed per capita (*cap*) and per day ($d$), according to Eq. (6.1).

$$D = \frac{V[\mathrm{L\ cap^{-1}d^{-1}}]}{Q[\mathrm{L\ cap^{-1}d^{-1}}]} \tag{6.1}$$

$V$ can be calculated as the ratio between the wet mass of feces ($M$) and the density of feces ($\rho$). M assumes a typical value of 128 g $\mathrm{cap^{-1}\ d^{-1}}$ in high-income countries, but can double in low-income countries where the fiber intake of the population is more abundant.[26] The typical density of feces is in the range from 1.06 to 1.09 $\mathrm{g/cm^3}$ or g/mL.[27]

$Q$ is calculated taking into account the average daily water consumption per capita ($w$, expressed as liters per person per day) and assuming the sewage discharge as 0.8 times the water supplied (using the coefficient $\alpha$ of 0.8). For example, in the case of households in Europe, $w$ is 144 L $\mathrm{cap^{-1}\ d^{-1}}$,[28] while the value is 50–100 L $\mathrm{cap^{-1}\ d^{-1}}$ or less in developing countries. According to these assumptions, Eq. (6.1) can be rewritten according to Eq. (6.2).

$$D = \frac{\dfrac{M[\mathrm{g\ cap^{-1}d^{-1}}]}{\rho[\mathrm{g/mL}]}}{\alpha[-]\cdot w[\mathrm{L\ cap^{-1}d^{-1}}]} \tag{6.2}$$

Using the values indicated above, the result of the calculation in Eq. (6.3) indicates a dilution factor of $10^{-3}$.

$$D = \frac{\dfrac{128\ \text{g cap}^{-1}\text{d}^{-1}}{1.09\text{g/mL}}}{0.8 \cdot 144\text{L cap}^{-1}\text{d}^{-1} \cdot 1000\ \text{mL/L}} = 0.0010 \tag{6.3}$$

Eq. (6.3) indicates that the SARS-CoV-2 load in the feces of a COVID-19 infected person undergoes an important dilution in the sewer network, and it is reduced by 1000 times passing from feces to wastewater.

## 6.3 SARS-CoV-2 load of in raw wastewater

The sewer network collects municipal and industrial wastewater and a variable amount of rainwater, the amount of which depends on the presence of a separate or combined wastewater system. The sewerage flow rate may be further increased by the infiltration of parasitic waters (originated by wrong sewer connections or groundwater infiltration).

Therefore the sewerage is a complex network where the SARS-CoV-2 load in the stool of COVID-19 patients that may vary in the range from $5 \cdot 10^3$ to more than $10^7$ GU/mL,[14,29,30] is hugely reduced by the following factors:

1. The dilution of feces of COVID-19 positive persons by $10^3$ in wastewater, according to the calculation in Section 6.2.
2. Only a fraction of the entire population connected to the sewerage is positive against COVID-19 and has a significant presence of SARS-CoV-2 in the feces. For example, considering 5% of positive cases in a population, a further reduction of the viral load by 20 times occurs in the sewerage.
3. The presence of stormwater and infiltrations of parasitic waters that increases the amount of water in the sewerage and leads to a further reduction of the viral load.

Taking into account all these factors simultaneously, the theoretical estimation shows that the SARS-CoV-2 load in the sewerage can be reduced by four orders of magnitude or more with respect to the load in stool. The result is a concentration of SARS-CoV-2 around $10^{-1}$–$10^3$ GU/mL wastewater. This estimation is obtained from an approximative theoretical calculation, based on simplified assumptions and uncertain data, but permits to understand why the concentrations of SARS-CoV-2 in the sewerage are so low with respect to the values found in the feces.

Table 6.1 presents a comparison among the studies that investigated the viral load in sewerage up to now. The large variation of the results reported in the literature is associated with different dilution in the sewerage, but also

**Table 6.1** Comparison among the studies which detected SARS-CoV-2 in raw wastewater entering the WWTPs. When quantified, the viral load is indicated in terms of genomic units per unit of volume (GU/L or GU/mL).

| References | Location, date of sampling | Plants and type of sampling | Details on the study | SARS-CoV-2 load in raw wastewater |
|---|---|---|---|---|
| Ahmed et al.[31] | South East Queensland, Australia. March 27 and April 1, 2020. | • 2 WWTPs and a pumping station.<br>• Automated samplers or grab sampling. | • 22.2% (2/9) of samples tested positive. | Range: $1.9 \times 10^1$ $-1.2 \times 10^2$ GU/L |
| Ampuero et al.[32] | Santiago, Chile From March to June 2020. | • 2 WWTPs.<br>• 24-h composite samples taken monthly. | • All samples were negative in March and April 2020 due to the few cases in Santiago.<br>• GU increased progressively from May to June and correlated with the increasing number of COVID-19 confirmed cases. | n.a. |
| Arora et al.[33] | Jaipur, Rajasthan, India. From May 3 to June 14, 2020. | • 6 WWTPs.<br>• 2 hospitals for treatment of COVID-19 patients. | • 2/6 sites showed two or more target genes in raw wastewater.<br>• Areas with positive results showed a continuous increase in the COVID-19 confirmed cases.<br>• Positive results were detected at ambient temperature very high (45°C). | n.a. |

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**Table 6.1** Comparison among the studies which detected SARS-CoV-2 in raw wastewater entering the WWTPs. When quantified, the viral load is indicated in terms of genomic units per unit of volume (GU/L or GU/mL).—cont'd

| References | Location, date of sampling | Plants and type of sampling | Details on the study | SARS-CoV-2 load in raw wastewater |
|---|---|---|---|---|
| Balboa et al.[34] | Ourense, Spain. From 6 to Apr 21, 2020. | • 24-h composite samples taken twice a week. | • SARS-CoV-2 RNA was systematically detected in the influent wastewater.<br>• The level of SARS-CoV-2 RNA was low in all the samples. | n.a. |
| Chavarria-Miró et al.[35] | Barcelona, Spain. From April 13 to May 25, 2020. | • 2 large WWTPs.<br>• 24-h composite samples taken weekly.<br>• For 1 WWTP, frozen archival samples from 2018 (Jan—Mar), 2019 (Jan, Mar, Sep—Dec) and 2020 (Jan—Mar) were assayed. | • From 15 Jan to Mar 4, 2020 the presence of SARS-CoV-2 increased, as demonstrated by the analysis of archival samples.<br>• Positive samples were found 41 days (15 Jan) before the 1st COVID-19 positive case (25 Feb).<br>• Despite negative samples in WWTPs around May 18—25, grab samples from urban sewers collected 8—9 AM were positive (as a consequence of different dilution and type of sampling). | 12-Mar:<br>Target IP2: $6.4 \times 10^2$ GU/L<br>Target IP4: $8.3 \times 10^2$ GU/L |

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|---|---|---|---|---|
| Fongaro et al.[36] | Florianopolis, Santa Catalina, Brazil. From October 30, 2019 to March 4, 2020. | • 6 independent sampling taken from a well of the sewerage (well for inspection/cleaning). | • positive samples were detected 97 d before the 1st COVID-19 confirmed case in Santa Catarina (Nov 27, 2020).<br>• the viral load increased by approximately 1 $log_{10}$ in correspondence of the 1st COVID-19 confirmed case (Mar 4, 2020). | 30-Nov: 5.5 $log_{10}$ GU/L<br>11-Dec: 5.8 $log_{10}$ GU/L<br>20-Feb: 5.6 $log_{10}$ GU/L<br>4-Mar: 6.7 $log_{10}$ GU/L |
| Gonzalez et al.[37] | Virginia, USA. From March 9 to July 28, 2020. | • 9 WWTPs.<br>• 24 h flow-weighted composite samples taken weekly in 3 WWTPs.<br>• Grab sampling in the other 3 WWTPs. | • 198 samples were analyzed: 98 positive for 3 assays, 22 positive for 2 assays, and 30 positive for only 1 assay.<br>• COVID-19 confirmed cases were 69 on 9 March and they increased to 1,180,000 on 28 July. | Range: $10^2$–$10^5$ GU/L |
| Green et al.[38] | Syracuse and Onondaga County, NY, USA. May 6 and 13, 2020. | • 24-h composite samples taken from 11 access points (i.e., WWTPs, pump stations or interceptor lines). | • 18/22 samples tested positive.<br>• 13/22 samples were in the quantifiable range. | Average: 42.7 GU/mL<br>Highest: 112 GU/mL |
| Haramoto et al.[39] | Yamanashi Prefecture, Japan. From March 17 to May 7, 2020. | • Grab sampling of raw and secondary-treated wastewater. | • Despite secondary-treated wastewater was positive, influent samples tested with the same procedure were negative.<br>• The discrepancy may be due to the different volumes used for concentration and the low presence of COVID-19 in the region. | n.a. |

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**Table 6.1** Comparison among the studies which detected SARS-CoV-2 in raw wastewater entering the WWTPs. When quantified, the viral load is indicated in terms of genomic units per unit of volume (GU/L or GU/mL).—cont'd

| References | Location, date of sampling | Plants and type of sampling | Details on the study | SARS-CoV-2 load in raw wastewater |
|---|---|---|---|---|
| Hata et al.[40] | Ishikawa and Toyama, Japan. From March 5 to Apr 23, 2020. | • 4 WWTPs.<br>• Grab sampling in the morning during the peak flow, weekly or biweekly. | • 7/27 positive for at least one assay.<br>• The detection frequency was 15% (3 positives out of 20 samples) when COVID-19 confirmed cases were below 10 in 100,000 people.<br>• The detection frequency was 57% (4/7) when cases were above 10 in 100,000 people. | Range: $1.2 \times 10^4$ $-4.4 \times 10^4$ GU/L |
| Hong et al.[41] | Jeddah, Saudi Arabia. From April 15 to Jul 9, 2020. | • Grab sampling at a frequency of 3–5 samples per week.<br>• Hospital wastewater. | • 75.4% (43/57) of samples tested positive for N genes (N1, N2, N3). | N1: 173.7 GU/L<br>N2: 772.1 GU/L<br>N3: 1327.4 GU/L |
| Kocamemi et al.[42] | Istanbul, Turkey. On April 21 and 25, 2020. | • 7 WWTPs.<br>• 24-h composite samples.<br>• Grab sampling in manholes near pandemic hospitals. | • 5/7 samples from WWTPs tested positive.<br>• All samples from manholes tested positive. | Range in positive WWTPs: $2.9 \times 10^3$ $-1.8 \times 10^4$ GU/L<br>In manholes: $4.5 \times 10^4$ GU/L<br>$9.3 \times 10^4$ GU/L |

| | | | | |
|---|---|---|---|---|
| Kumar et al.[43] | Gujarat, India. On May 8 and 27, 2020. | • Grab sampling.<br>• A composite sample made from 3 samples taken in each location. | • A 10-fold increase from 8 to 27 May, corresponding to more than double the number of COVID-19 patients (4912 and 10,674 cases on 8 and 27 May, respectively). | Range: $5.6 \times 10 - 3.5 \times 10^2$ GU/L |
| La Rosa et al.[44] | Milan and Rome, Italy. From February 3 to April 2, 2020. | • 24-h composite samples. | • 50% (6/12) of samples were positive.<br>• On 24 and 28 February, when the samples were positive in Milan, COVID-19 infections were still limited in Italy. | n.a. |
| La Rosa et al.[45] | Milan, Turin, Bologna, Italy. From October 9, 2019 to February 28, 2020. | • 5 WWTPs (2 in Milan, 2 in Turin and 1 in Bologna).<br>• 24-h composite samples. | • 88% (23/26) of samples were below the analytical LOQ ($5.9 \times 10^3$ GU/L). | Range: from <LOD to $5.6 \times 10^4$ GU/L |
| Lodder and de Roda Husman[46] | Amsterdam Airport Schiphol, Netherlands. From February 17, 2020. | • 24-h samples taken once a week. | Samples tested positive 4 days after the 1st (apex) COVID-19 confirmed case (identified in The Netherlands on Feb 27, 2020). | n.a. |
| Manupati et al.[47] | Hyderabad Metropolitan City, India. From July 7 to August 8, 2020. | • Sampling was performed in the day time, on the days with no report of rainfall during the last 24 h. | • All WWTPs were positive.<br>• In 1 WWTP samples were taken at different times to assess the dynamics of disease spread with time: highly fluctuating data were observed inside a few weeks. | 8-Jul: 219.540 GU/L<br>14-Jul: 30.818 GU/L<br>29-Jul: 266.360 GU/L |

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**Table 6.1** Comparison among the studies which detected SARS-CoV-2 in raw wastewater entering the WWTPs. When quantified, the viral load is indicated in terms of genomic units per unit of volume (GU/L or GU/mL).—cont'd

| References | Location, date of sampling | Plants and type of sampling | Details on the study | SARS-CoV-2 load in raw wastewater |
|---|---|---|---|---|
| Medema et al.[48] | The Netherlands. From February 5 to March 16, 2020. | • 24-h flow-dependent composite samples. | • 58% (14/24) of samples were positive.<br>• On 4/5 March (1 week into the epidemic) 4/6 WWTPs were positive, with only 38 and 82 COVID-19 cases confirmed through the health surveillance system. | Range: $2.6 \times 10^3$ $-2.2 \times 10^6$ GU/L. |
| Or et al.[49] | Tel Aviv and Israel. From March 10 to April 21, 2020. | • Automated samplers at targeted hot-spot areas for 24 h. | • The Ct value measured in rRT-PCR correlated with the number of COVID-19 positive individuals. | n.a. |
| Randazzo et al.[50] | Murcia, Spain. From March 12 to April 14, 2020. | • 6 WWTPs in the larger municipalities. | • 83% (35/42) of samples were positive for at least one target. | Average values: N1: $5.1 \pm 0.3$ $\log_{10}$ GU/L N2: $5.5 \pm 0.2$ $\log_{10}$ GU/L N3: $5.5 \pm 0.3$ $\log_{10}$ GU/L |

| | | | | |
|---|---|---|---|---|
| Rimoldi et al.[51] | Milan, Monza, Brianza, Italy. On April 14 and 22, 2020. | • 3 WWTPs.<br>• Grab sampling around 1.00 p.m. | • All WWTPs positive on Apr 14, 2020.<br>• Only 1 WWTP positive on 22 Apr, probably following the epidemiological trend in the area. | n.a. |
| Sherchan et al.[52] | Southern Louisiana, USA. From January to April 2020. | • 2 WWTPs.<br>• 24-h composite and grab samples collected monthly. | • 2/7 samples tested positive. | N1 assay: $7.5 \times 10^3$ GU/L<br>N2 assay: $3.1 \times 10^3$ $-4.3 \times 10^3$ GU/L |
| Trottier et al.[53] | Montpellier, Lattes, France. From May 7 to June 25, 2020. | • 24-h composite samples. | • Viral RNA in wastewater increased from April to 15–25 Jun despite the decrease in the number of COVID-19 patients hospitalized. | n.a. |
| Westhaus et al.[54] | North Rhine-Westphalia, Germany. April 8, 2020. | • 9 WWTPs.<br>• 24-h flow-weighted composite samples.<br>• Sampling during dry-weather. | | Range: 3–20 GU/mL |
| Wu et al.[55] | Massachusetts, USA. March 18–25, 2020. | • A major WWTP.<br>• 24-h composite samples. | • Samples collected before the 1st known US SARS-CoV-2 case were negative.<br>• Then, all samples taken from 18 to 25 Mar tested positive. | Range: ~10–100 GU/mL |

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**Table 6.1** Comparison among the studies which detected SARS-CoV-2 in raw wastewater entering the WWTPs. When quantified, the viral load is indicated in terms of genomic units per unit of volume (GU/L or GU/mL).—cont'd

| References | Location, date of sampling | Plants and type of sampling | Details on the study | SARS-CoV-2 load in raw wastewater |
|---|---|---|---|---|
| Wurtzer et al.[56] | Paris, France. From March 5 to Apr 23, 2020. | • Several major WWTPs of the Parisian area. | • All samples tested positive.<br>• The time-course monitoring displayed a 2-log increase. | 5-Mar: $5 \times 10^4$ GU/L Exponential increase from $5 \times 10^4$ GU/L to $3 \times 10^6$ GU/L. |
| Yaqub et al.[57] | Parts of Lahore, Pakistan. From July 13 to 25, 2020. | • Smart lockdown implemented in the area on Jul 9, 2020.<br>• 28 sites (lift stations and sewage lines).<br>• Grab sampling and 24-h sampling. | • 16/28 sample were positive on the 1st day of sampling (13 Jul) with variable load.<br>• Then viral load decreased toward the end of lockdown.<br>• Only a few sites did not follow a clear pattern (due to the complexities in sewage water based surveillance). | 13 Jul Positivity: 16/28 cases Range: 2.4 $\log_{10}$—4.55 $\log_{10}$ GU/mL 25 Jul Positivity: 9/28 cases Range: 0.6 $\log_{10}$—3.64 $\log_{10}$ GU/mL |

Legend: n.a. = not available.

with the fact that the number of COVID-19 positive persons may vary widely inside the population served by the sewerage network.

On the basis of the most recent literature, summarized in Table 6.1, the concentration of SARS-CoV-2 in wastewater was found to be largely variable, with the maximum values in the order of 4 $\log_{10}$ GU/mL which were found in severe situations or in presence of a lockdown in the urban area monitored. However, the viral loads revealed in wastewater are very low in comparison with those found in feces, which may reach an order of magnitude up to 7 $\log_{10}$ GU/mL.

This in turn highlights the effort needed and the difficulty to detect such low quantities of viral RNA in a complex matrix like wastewater. SARS-CoV-2 RNA is present at concentrations orders of magnitude lower, not only with respect to feces, but also when compared to the standard nasopharyngeal swabs used in COVID-19 diagnostics. This extreme difference in concentrations is the main reason for SARS-CoV-2 RNA detection methods that are extremely well standardized in nasopharyngeal swabs to still lag behind when it comes to detecting viral RNA in wastewater. Before any downstream analysis, it is therefore crucial to concentrate the initial wastewater sample. However, this procedure introduces further potentially problematic steps, including freezing and inactivation of the sample. Since concentration protocols are usually laborious and not scalable to many samples, wastewater is indeed commonly frozen and stored at −20°C (or seldom at −80°C) before processing,[44,58] which greatly impacts RNA integrity and consequently further lowers SARS-CoV-2 concentration.[59] The same is true for the thermal inactivation of the virus,[60,61] a necessary step in many protocols[44] to avoid exposure to aerosols that may generate during concentration in laboratories with limited biosafety measures (e.g., lack of BSL-2 cabinets and/or aerosol-tight centrifuge rotors). However, low viral concentrations and poor RNA integrity are not the only limiting factors for easy SARS-CoV-2 RNA detection from this matrix. Wastewater indeed contains a number of PCR-inhibitory substances like humic and fulvic acids that can prevent the correct amplification of viral RNA and even produce false-negative results.[62] Taken together, this set of limitations explains why the detection of SARS-CoV-2 in sewerage is still an open issue.

## 6.4 The approach for SARS-CoV-2 detection in sewerage is an open issue

The approach for the detection of SARS-CoV-2 in wastewater includes several steps that start with the sampling in the sewerage or in the WWTPs

and the transportation and conservation of the samples. Then, the subsequent analysis can be performed in a Biosafety Level 2 (BSL-2) laboratory, while virus isolation and cultivation require a BSL-3 laboratory.[63] At the moment, some steps in the procedure present critical aspects, not fully investigated yet, when applied to wastewater samples. Despite the quickly evolving situation and the continuous efforts in the research, a standard protocol is not available yet and the quantification of the viral load in wastewater remains an open issue because of the heterogeneity of the approaches proposed in the literature, which may lead to different and not always comparable results.

First, the analysis of SARS-CoV-2 in wastewater requires an appropriate sampling, transportation, and storage of the samples, according to the following steps.

**(1)** Sampling of wastewater: In order to obtain an accurate estimation of the viral content in the influent wastewater, the collection of composite samples with automated samplers monitors the strong fluctuations of flow rates and contaminants occurring over time. Composite samples[64] can be formed with: (1) aliquots of wastewater with equal volume taken at equal intervals during the day, or (2) 24-h flow proportional samples that are aliquots with volume proportional to the flow rate. Although the second alternative would be preferable,[64] it requires a flow measurement device installed in the inlet channel of a WWTP that is not always available. In some cases, to monitor load peaks during short time intervals, a grab sampling is performed. A comparison on the adoption of the various types of sampling is shown in Table 6.1.

**(2)** During collection and transportation to the lab, the samples must be maintained at low temperature, i.e., 4°C, with the aim of preserving the number and the viability of the virus.[65] In fact it was widely demonstrated that freeze-thaw cycles affect the integrity of viral RNA, and that storage at −80°C causes a reduction of 1−3 cycle threshold (Ct) in real time RT-PCR of the N1, N2, and N3 gene fragments,[59] and even more in case of storage at −20°C. Conversely, the viral RNA was stable after the storage at 5°C up to 15 days.[59] This led some authors[31,59] to suggest transporting the samples on ice and store them at 4°C until further analysis to preserve the RNA integrity. However, at the moment, there is not full agreement about the temperature and duration of the storage for the conservation of the samples before the analysis. Xiao et al.[16] indicated to maintain the samples at 4°C when the analysis is performed in the subsequent 24 h, otherwise for longer storage (>24 h) the samples are maintained at −70°C or, when not available, at −20°C.

**(3)** Inactivation of the virus: Some protocols of analysis include a step of inactivation of SARS-CoV-2 performed at 56°C per 30 min[44] to avoid any possible risk for the operator. However, most protocols now avoid the thermal inactivation step to preserve RNA integrity.[60,61]

Then, the whole chain for the quantification of the viral load in wastewater proceeds with molecular biology analysis, as follows.

**(4)** Viral enrichment is a very important passage because of extremely low viral titers in wastewater samples. Without concentration, detecting the virus in such samples, at the moment, is not achievable. Viral enrichment can be performed through various approaches that include centrifugation steps together with centrifugal ultrafiltration,[31,59] aluminum hydroxide adsorption-precipitation,[50] filtration through 0.22 μm filters combined with PEG precipitation,[66] or ultracentrifugation.[56] For example, La Rosa et al.[44] proposed the use of the standard WHO procedure for Poliovirus surveillance in wastewater (based on the use of polyethylene glycol and dextran) after some modifications in the concentration step.

**(5)** Viral RNA extraction is the step realized in order to isolate and purify RNA from virus and clean it from pollutants and inhibitors of PCR and is mostly carried out with commercially available RNA extraction kits with custom modifications.[31,44,48,50] In general, extraction consists of four phases: lysis (RNA is released from virus and proteins separated and denatured), binding (RNA is bound on a surface, i.e., silica membrane or magnetic silica), washing (with different special wash buffers RNA is removed of unwanted impurities such as salt, proteins, and other contaminants), and elution (pure RNA is free from the surface bond and ready to use in enzymatic PCR reaction).

**(6)** Nucleic acid amplification based on rRT-PCR targeting single or multiple regions of the SARS-CoV-2 genome, including the N, S, E, and RdRP genes encoding for the nucleocapsid, spike, envelope, and RNA-dependent RNA polymerase proteins, respectively.[7] The RdRP gene region also covers the ORF1ab gene, specifically targeted by a number of widely used primer sets. The Ct value measured in rRT-PCR is inversely proportional to the virus quantity. For example, a low Ct value means that a low number of amplification cycles are required to obtain a fluorescent signal over the threshold and thus that the SARS-CoV-2 viral load in the sample is higher. In the case of wastewater samples, as a consequence of the relevant dilution of

the virus, the Ct value to call a positive sample is often raised with respect to feces or other human specimens, even reaching values around 40.[55]

**(7)** Finally, the viral load in the wastewater sample can be expressed numerically as GU/mL.

## 6.5 Decay of SARS-CoV-2 in wastewater due to adverse environmental conditions

A certain reduction of the viral load may occur along the sewerage network, in the wastewater treatment, and in the environment in general, due to adverse conditions caused by pH changes, temperature variations, disinfectants, and various pollutants.[67] In particular, SARS-CoV-2 and the other CoVs belong to the enveloped viruses (characterized by a fragile lipid membrane called envelope) that are more susceptible to environmental factors[68] and more rapidly inactivated than enteric viruses, which are almost all nonenveloped.[69] For example, in wastewater, 90% inactivation of nonenveloped viruses can be observed in a relatively long period ranging from days to months, while it is shorter, from hours to days, in enveloped viruses, as demonstrated by Ye et al.[70] The lower resistance of the enveloped viruses is due to the lysis of the viral envelope—for example, caused by pollutants or detergents that break down the weak fatty layer around the SARS-CoV-2 virion—with the consequent damage of the lipid envelope that is required for the infection of the host cells.[71]

The stability and reduction of SARS-CoV-2 under some different environmental conditions was reported by Chin et al.[72] In particular, with regards to the effect of the sole temperature, SARS-CoV-2 remained highly stable at 4°C (with around a 0.7 log-unit reduction of infectious titer after 14 d), while it was sensitive to heat that inactivated the virus after 5 min at 70°C.[72] For a comparison, the previous SARS-CoV, when seeded into sewage, remained infectious for a period of 14 days at temperature of 4°C and only 2 days at 20°C.[65]

Comparing SARS-CoV-2 with other CoVs, the new coronavirus may have a greater resilience outside the body than expected.[73] In particular, Goh et al.[73] predicted by a model that SARS-CoV-2 could have one of the hardest outer shells among most CoVs. The virus may have the greatest resilience to hostile environmental conditions since the harder shell can protect the virion from damage. This aspect may also affect how long the virus remains infectious in the environment. However, this theoretical study is not exhaustive and further experimental research is needed in this direction.

The difference between SARS-CoV-2 and other CoVs is due to the fact that not all the CoVs have the same persistence or inactivation in water[68]: for example, even if belonging to the same genus, the time required for 90% inactivation of SARS coronavirus may be ninefold longer than another human coronavirus.[70]

It is worth noting that the investigations about the survival of CoVs in the environmental matrices should be carried out using the pathogen of interest, since viral persistence may differ even within different strains of the same viral species. Unfortunately, this is not always possible, and thus the research needs to use "surrogate" (model) viruses with similar properties. However, the choice of a surrogate virus is challenging because the exact relationships between phylogenetically different CoVs is still unclear.[74] The use of multiple surrogates instead of only one permits to cover the multiple aspects of a target virus.[75]

Although surrogates may not be exactly representative of the virus of interest, in absence of experimental data acquired for SARS-CoV-2, surrogates may offer some useful information about the potential behavior of SARS-CoV-2 in the contaminated water, as explained later in the chapter.

CoVs inactivation occurs more rapidly in sewage than in water due to various factors: high presence of organic matter, pollutants, or antagonistic bacteria.[76]

Casanova et al.[74] investigated the stability of two surrogate CoVs (transmissible gastroenteritis virus that is a diarrheal pathogen of swine, and mouse hepatitis virus that is a respiratory and enteric pathogen of laboratory mice) in pasteurized settled sewage at 4°C and 25°C. Experiments were carried out using viral stocks prepared as follows: (1) propagation by infecting confluent layers of host cell cultures in flasks, (2) harvesting of cell lysates, (3) clarification by centrifugation (3000× g, 30 min, 4°C), and (4) storage of supernatants as virus stock at −80°C.[74] In these experiments Casanova et al.[74] observed that the infectivity decreased at rate of 1.5−2.0 $\log_{10}$ per week at temperature of 25°C (99% reduction obtained at 7−9 d), while it decreased more slowly at rate of 0.2−0.3 $\log_{10}$ per week at 4°C.

When using raw sewage instead of pasteurized settled sewage, the rates of viral inactivation could be even faster because the pasteurization process could reduce the role of proteolytic enzymes that contribute to virus inactivation.

Pollution in water has a relevant effect on the virus inactivation. The study of Casanova et al.[74] indicated that the time required for the reduction of infectivity was about half in pasteurized settled sewage compared to clean

water. Another study on CoVs observed that 2 d at 23°C permitted to obtain ~2 log reduction in primary effluent, while 7–8 days were needed in tap water,[76] again indicating that the inactivation process may be accelerated in sewage.

The survival of a representative CoV (Human coronavirus 229E) was tested at 23°C in samples of primary effluent (presettled wastewater) and secondary effluent (after activated sludge and secondary settling, but prior to chlorination) taken from a WWTP.[76] The CoV was inactivated in wastewater with 3 log reduction between 2 and 4 d. In comparison with enteroviruses, Gundy et al.[76] indicated that this CoV was less stable in water, more rapidly inactivated, and less transmitted in aqueous environment.

## 6.6 Reduction of SARS-CoV-2 in wastewater treatment plants

WWTPs are characterized by a sequence of treatments (physical, biological, and disinfection), according to the typical flow-sheet shown in Fig. 6.1.

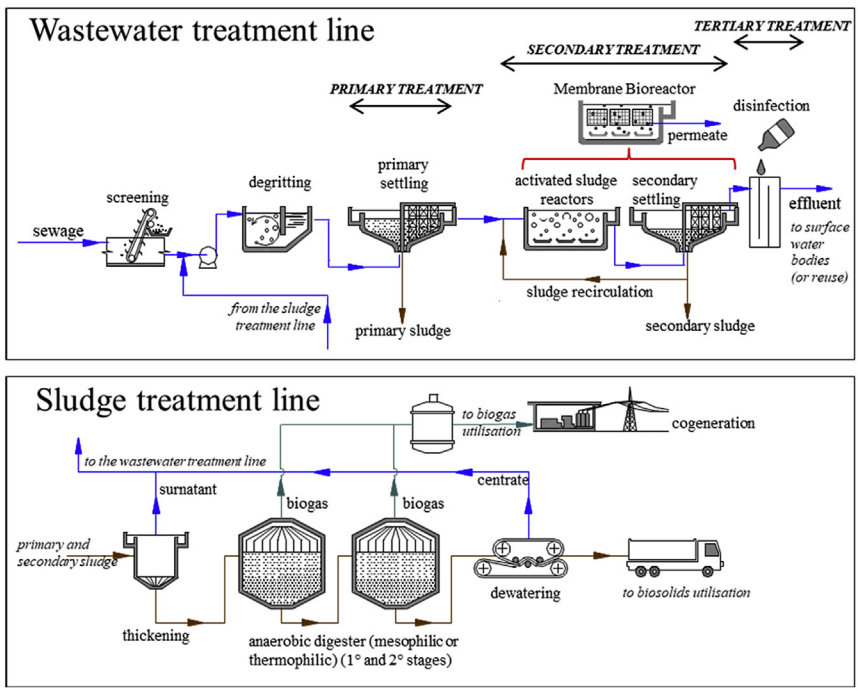


**Figure 6.1** Layout of a wastewater treatment plant divided in (1) wastewater treatment line that implements primary, secondary (activated sludge or membrane bioreactor), and tertiary treatments; (2) sludge treatment line.

WWTPs are designed to treat organic matter, solids, nutrients, and bacteria with high removal rates and efficiency, but are expected to be effective also against viruses when disinfection is implemented.

In primary treatment (settling) a reduction of up to 26% of two model enveloped viruses was observed (only 6% of reduction for nonenveloped viruses), according to Ye et al.[68] The viral envelope is hydrophobic and this favors the absorption to solids and thus the separation of the enveloped viruses in the sludge.[76]

Primary treatment and secondary treatment (activated sludge), shown in Fig. 6.1, may contribute only partially to the virus reduction, and removal in these stages is largely variable and viruses can remain viable from hours to days in absence of disinfection. Therefore, to improve the virus inactivation in WWTPs, disinfection must be implemented. In fact, SARS-CoV-2, being an enveloped virus with a fragile outer membrane, is susceptible to disinfection, even more than enteric viruses.[77]

Disinfection can be based on chlorination, ozonation, peracetic acid, or UV light.

With regards to chlorination, free chlorine inactivates SARS-CoV more effectively than chlorine dioxide.[78] The mechanism for the free chlorine inactivation is the reaction with proteins instead of genomes: the free chlorine molecules readily penetrate the lipid membrane, reacting with the proteins in the nucleocapsid.[70]

UV disinfection is based on exposure to UVC light emitted traditionally by UV mercury lamps. The fate of ssRNA viruses, like SARS-CoV-2, after exposure to UV is not completely clear, because the literature on RNA photochemistry is scarce compared to DNA photochemistry. In general, the susceptibility of enveloped viruses to UVC light is comparable to nonenveloped viruses because the inactivation mechanism targets primarily the genome and the lipid membrane cannot protect it from the UV light.[69]

When the secondary treatment is a membrane bioreactor (MBR) (Fig. 6.1), the biological process is coupled with the filtration of solids through a membrane system without the requirement of a secondary settler. Virus removal can be improved significantly[79] due to the small pore size of membranes (0.03−0.40 μm) and the biofouling that is an additional barrier for viruses.[80] The size of virions of SARS-CoV-2 is about 60−140 nm with projections of 9−12 nm, so MBRs installing membranes with a similar absolute pore size are effective in the removal. In particular, ultrafiltration has a cutoff rating of 0.005−0.01 microns and so it is more suitable in virus removal than microfiltration, and this latter requires a subsequent stage of

disinfection. No experimental data are available on the removal of SARS-CoV-2 in MBRs, but Lesimple et al.[81] and Tetteh et al.[82] indicate that MBR technology can be an effective technology for the removal of pathogens, including SARS-CoV-2. To have an idea of virus reduction, average removal of some enteric viruses in a full-scale MBR (absolute pore size of 0.1 μm) was 5.5 $\log_{10}$ for human adenovirus, 5.1 $\log_{10}$ per human enterovirus, and 3.9 log10 for norovirus.[83]

A summary of the studies that analyzed SARS-CoV-2 in the treated wastewater effluents or in the sludge separated from full-scale WWTPs is shown in Table 6.2.

In many investigations, the treated effluents were negative for SARS-CoV-2, especially in the plants equipped with tertiary treatments. Conversely, the implementation of a secondary treatment only does not ensure to reach viral loads below the detection limits. All the studies investigating the concentrations of SARS-CoV-2 in sludge, demonstrated the enrichment of the virus in primary, secondary, or thickened sludge.

## 6.7 Potential fecal-oral transmission associated with sewerage

The presence of SARS-CoV-2 in raw wastewater and treated effluents, measured in GU, is not always associated with viability and infectivity of the virus as indicated by many authors in the literature.[10,11,15,16,21,84,85] It is worth noting that the virus may cause infection only if it is able to retain viability.

Studies have found the presence of infectious virions of SARS-CoV-2 in feces[10,16,22] and in urine.[20] This postulates that SARS-CoV-2 may remain infectious also in wastewater. The infectious potential of untreated and treated wastewater was tested using a cell culture model.[54] Inoculation of differentiated Caco-2 cells for 10 d with wastewater indicated that treated wastewater was noninfectious even though viral RNA fragments were detected. It is important to underline that infectivity of SARS-CoV-2 was not yet observed in WWTPs up to now, either raw or treated samples, despite the presence of viral RNA in the samples.

As far as we know, the scientific literature reports one case of transmission caused by sewerage. Han and He[86] reported that the first known case of a sewage-associated transmission of SARS-CoV-2 was documented in a Chinese website (written in Chinese by Li P. and Bin H.). In particular, this COVID-19 outbreak occurred in a group of a few households, as a

**Table 6.2** Comparison among the studies which detected and quantified SARS-CoV-2 in treated effluents or in the sludge separated in the WWTPs.

| References | Location, date of sampling | Plants and type of sampling | Details on the study | SARS-CoV-2 load in effluent wastewater and sludge |
|---|---|---|---|---|
| Ampuero et al.[32] | Santiago, Chile. From March to June 2020. | • 2 WWTPs.<br>• 24-h composite samples. | • SARS-CoV-2 detected in treated wastewater samples. | Effluent from WWTP1:<br>25 May: 20 GU/mL<br>15 Jun: 167 GU/mL<br>Effluent from WWTP2:<br>25 May: 10 GU/mL |
| Arora et al.[33] | Jaipur, Rajasthan, India. From May 3 to June 14, 2020. | • 6 WWTPs.<br>• Samples collected from different units of the WWTPs. | • The treated wastewater was negative in all the WWTPs, even in those where raw wastewater was positive.<br>• The different wastewater treatments used (Moving Bed Biofilm Reactor and Sequencing Batch Reactor) permitted to reduce the viral load below the detection limit. | Negative effluents. |

*Continued*

**Table 6.2** Comparison among the studies which detected and quantified SARS-CoV-2 in treated effluents or in the sludge separated in the WWTPs.—cont'd

| References | Location, date of sampling | Plants and type of sampling | Details on the study | SARS-CoV-2 load in effluent wastewater and sludge |
|---|---|---|---|---|
| Balboa et al.[34] | Ourense, Spain. From April 6 to 21, 2020. | • 24-h composite samples taken twice a week. | • Negative samples in the secondary treatment effluent, confirming that the effluent is safe for reuse and discharge to water bodies.<br>• Viral RNA mainly retained in the sludge from the primary settler.<br>• Rarely detected in the biological sludge.<br>• Its concentration increased in the thickeners due to the long retention time (∼24 h) and the high solid content.<br>• Not detected in the digested sludge. | Negative effluents. Positive samples of sludge. |
| Haramoto et al.[39] | Yamanashi Prefecture, Japan. From March 17 to May 7, 2020. | • WWTP with conventional activated sludge process.<br>• Grab sampling before chlorination. | • SARS-CoV-2 RNA was detected in one (20%) secondary-treated wastewater sample. | Effluent from WWTP: $2.4 \times 10^3$ copies/L |
| Hong et al.[41] | Jeddah, Saudi Arabia. From April 15 to July 9, 2020. | • Grab samples collected in the supernatant of the activated sludge tank located in a hospital. | • 15.4% (8/52) of samples taken from the activated sludge tank (partially treated wastewater) were tested positive for N genes. | Effluent from secondary treatment:<br>N1: 81.1 GU/L<br>N2: 1115.8 GU/L<br>N3: 411.2 GU/L |

| | | | | |
|---|---|---|---|---|
| Kocamemi et al.[42] | Istanbul, Turkey. On May 7, 2020. | • 9 WWTPs.<br>• Grab sampling of primary sludge and waste activated sludge. | • SARS-CoV-2 was more concentrated in primary sludge and waste activated sludge than in the influent. | Range in sludge: $1.17 \times 10^4$ $-4.02 \times 10^4$ GU/L |
| Kumar et al.[43] | Gujarat, India. On May 8 and 27, 2020. | • Grab sampling of the final effluents after UASB and aeration pond. | • Final effluent samples were negative for 3 genes examined.<br>• The viral load was thus significantly reduced by the UASB treatment and aeration pond. | Negative effluents (CT values > 40) |
| Peccia et al.[58] | New Haven, Connecticut, USA. From March 19 to May 1, 2020 | • Samples taken daily at the outlet of a gravity thickener (solids content 2.6%−5%). | • All five measures traced the rise and fall of SARS-CoV-2 infections during the 10-week period studied. | Range in primary sludge: $1.7 \times 10^3$ $-4.6 \times 10^5$ GU/L |
| Randazzo et al.[50] | Region of Murcia, Spain. From March 12 to Apr 14, 2020. | • 6 WWTPs.<br>• 18 samples of secondary effluents.<br>• 12 samples of tertiary treated effluents. | • 11% (2/18) of secondary effluents were positive for at least one target.<br>• Tertiary effluent samples were all negative. | Secondary effluent 1 (N2 positive): 5.40 $\log_{10}$ GU/L<br>Secondary effluent 2 (3 targets positive): < quantification limit. |
| Rimoldi et al.[51] | Milano and Monza e Brianza, Italy. On April 14 and 22, 2020. | • WWTPs equipped with tertiary treatments.<br>• 2 grab samples of treated wastewater. | • Treated wastewater was negative. | Negative effluents |

*Continued*

**Table 6.2** Comparison among the studies which detected and quantified SARS-CoV-2 in treated effluents or in the sludge separated in the WWTPs.—cont'd

| References | Location, date of sampling | Plants and type of sampling | Details on the study | SARS-CoV-2 load in effluent wastewater and sludge |
|---|---|---|---|---|
| Sherchan et al.[52] | Southern Louisiana,USA. From January to April 2020. | • 2 WWTPs with conventional activated sludge and chlorine disinfection.<br>• Samples of secondary treated and final effluents after disinfection. | • Secondary-treated wastewater and final effluent samples tested negative.<br>• Thus the virus was removed in WWTPs to undetectable level. | Negative effluents |
| Westhaus et al.[54] | North Rhine-Westphalia, Germany. Apr 8, 2020. | • 9 WWTPs.<br>• Samples of treated wastewater. | • treated effluents were positive despite the different processes applied in the WWTPs | Range in treated effluents: 2.7−37 GU/mL |

consequence of the loss from a private combined sewer pipe that contaminated the surrounding environments causing the diffusion of the infection. The hypothesized route of transmission was confirmed by tracking the overflows from the broken pipe during another heavy rainfall event.[86]

The effects of the COVID-19 pandemic in the field of waste and wastewater services[87] are very complex routes and the knowledge about the potential fecal-oral transmission is only partial.[88] So, due to the presence of infectious SARS-CoV-2 virions in feces and urine and the potential presence in wastewater, the possibility of fecal-oral transmission cannot be excluded.[20]

From the state of the art, at the moment, in high-income countries it is unlikely that the environmental matrices could become a main transmission route for SARS-CoV-2, even if more research in this field is needed and appropriate caution is recommended.[86] Conversely, in low-income countries where pit toilets are the most common system for human excreta disposal, the inadequate sanitation may be a source of contamination of SARS-CoV-2 in soil and groundwater. Similarly, SARS-CoV-2 could be spread in the environment through "open defecation"; in 2017 nearly 950 million people in poor countries did not have basic sanitation and routinely practiced open defecation.[89]

### 6.7.1 Aerosolization of wastewater

The presence of viral RNA in sewage and the spread of air droplets cannot exclude the possibility of a viral presence in the aerosol. A well-known case occurred in Amoy Gardens in Hong Kong during the previous SARS outbreak, where the aerosols originated from the building pipes and containing the viruses reached a large apartment complex.[24] One study on the viability of SARS-CoV-2 in aerosol[90] indicated that the half-life (that is the time needed to halve the amount of the virus) was approximately 1.1 h.

WHO[77] has developed specific guidance for the workers in WWTPs during the SARS-CoV-2 outbreak. Briefly, and not exhaustively, workers should wear personal protective equipment (PPE; i.e., protective outerwear, gloves, boots, goggles or a face shield, and a face mask or FFP3 respirator mask), which continues to be effective in protecting against pathogens included SARS-CoV-2.[91]

### 6.7.2 Overflows in municipal combined sewer systems

The events of heavy rainfalls produce large stormwater runoff, which often exceeds the conveyance capacity of the pipes in the combined sewer systems, causing inevitable overflows of untreated sewage (called combined

sewer overflows, CSO). There is a large number of municipalities around the world still served by combined sewer systems, often characterized by aged structures and pipes.

CSO is a mixing of contaminated water that includes stormwater runoff and untreated sewage containing feces/urine, pathogenic microorganisms, and other pollutants. Due to the short residence time passed by feces/urine in sewers (approximately in the order of a few hours), the viruses excreted in feces could maintain viability and remain still infective.[68]

When CSO leave the sewerage network, they may spread quickly and widely into public areas or surface waters and may pose potential risks for the public health.[75]

### 6.7.3 Flooding events in urban areas

A preliminary retrospective analysis was performed by Han and He[86] in some locations characterized by a significant number of COVID-19 infected persons during flooding events in the period May—August 2020. Although any conclusive trends cannot be drawn, Authors discuss the potential risk of SARS-CoV-2 transmission in these flooded areas, where sewage overflows may be an additional factor for the virus spread.

In particular, during flooding events the population may be exposed to overflowed human excreta or human wastes derived from the overload in the combined sewer systems. This could contribute to the sewage-associated transmission of COVID-19 when heavy rainfalls are frequent.[86]

Therefore, in the municipalities served by combined sewer systems in areas subjected to flooding events, especially in summer, the network should be upgraded toward separated systems to reduce the risk of overflows of blackwaters.

### 6.7.4 Discharge of untreated or treated wastewater into surface water bodies

Although the presence of SARS-CoV-2 in wastewater has been confirmed in various researches, there are only few studies that have investigated on the viral dispersion in the receiving water bodies and the potential associated health risk.

In Rimoldi et al.,[51] three rivers (near Milan, Italy) were surveyed during the peak of pandemic in April 2020. Real-time RT-PCR analysis showed that samples taken from these receiving water bodies were positive for SARS-CoV-2. It is interesting to investigate more in depth the source of the viral contamination. In the study of Rimoldi et al.,[51] caffeine was used as a

clear indication of urban-only pollution due to untreated wastewater, because it is highly degradable in wastewater treatment and thus significantly reduced in the treated effluents. When the SARS-CoV-2 presence is associated with a significant detection of caffeine in the rivers, it means that wastewater treatment plants cannot be the origin of the virus presence. Instead the virus could relate to nontreated or inefficiently treated sewage discharged into surface waters as a consequence of illicit discharges, malfunction or leakages in the sewerage systems, noncollected domestic flows, or CSOs.

In some samples SARS-CoV-2 was detected but caffeine was not.[51] These cases suggest that a sporadic release of virus traces in the treated effluents from WWTPs cannot be completely excluded, especially when the WWTP is equipped with secondary treatment only.[51] An important fact is that, even in the cases when the viral RNA was detected, the infectivity and the risk of infection from surface water was not demonstrated.[51]

Similarly, the aim of the study of Haramoto et al.[39] was to detect SARS-CoV-2 RNA in rivers (Yamanashi Prefecture, Japan), in samples collected from March to May. In this study, none of the samples tested positive for SARS-CoV-2 RNA.

The situation in the study of Guerrero-Latorre et al.[92] was quite different. The study concerns urban rivers of Quito (Ecuador) where, as in many other cities worldwide, wastewater is directly discharged into surface water bodies. The samples were taken during a peak of COVID-19 cases and evaluated for water quality parameters, human adenovirus presence and SARS-CoV-2 presence. The water was concentrated, for viral analysis, using skimmed milk flocculation method and the results showed a high presence of SARS-CoV-2 in all the samples. However, the risks for health and ecology, especially in low-income countries should be further assessed.

## 6.8 Conclusions and future perspective

The recent global outbreak of SARS-CoV-2 has highlighted the limited knowledge on the fate of CoVs in sewerage and WWTPs. There is basis for thinking that SARS-CoV-2 is controlled in WWTPs and that viral loads in treated effluents are very low especially in the presence of a disinfection stage. Conversely, inappropriate discharges of sewerage can be a source of contamination of surface water and groundwater. Fecal-oral transmission of SARS-CoV-2 is unlikely even if caution is recommended in this field. This chapter summarizes the main aspects of the presence of SARS-CoV-2 in

wastewater, but alongside certain findings, a number of questions have emerged that need further research. Specific questions include

- viability and infectivity of SARS-CoV-2 in wastewater should be further investigated, since the identification of viral RNA is not evidence of infectious capacity;
- data on SARS-CoV-2 abundance in wastewater (and its variability) are still rare and this limits a shared conclusion on decay and removal in WWTPs;
- the fate of SARS-CoV-2 in biosolids (primary and secondary sludge from WWTPs) and related risks need to be investigated more in depth; and
- a challenge against future epidemic waves is based on the possibility of using the SARS-CoV-2 load in wastewater for epidemiological surveillance, as a tool capable of providing early warning of incipient outbreaks.

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