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Coronaviruses and SARS-CoV-2 in sewerage and their removal: Step by step in wastewater treatment plants

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ABSTRACT

The fate of Coronaviruses (CoVs) and in particular SARS-CoV-2 in wastewater treatment plants (WWTPs) has not been completely understood yet, but an adequate knowledge on the removal performances in WWTPs could help to prevent waterborne transmission of the virus that is still under debate. CoVs and SARS-CoV-2 are discharged from faeces into the sewer network and reach WWTPs within a few hours. This review presents the fate of SARS-CoV-2 and other CoVs in the primary, secondary and tertiary treatments of WWTPs as well as in sludge treatments. The viral loads decrease progressively along with the treatments from 20 to 3.0E+06 GU/L (Genomic Units/L) in the influent wastewater to concentrations below 2.50E+05 GU/L after secondary biological treatments and finally to negative concentrations (below detection limit) in disinfected effluents. Reduction of CoVs is due to (i) natural decay under unfavourable conditions (solids, microorganisms, temperature) for relatively long hydraulic retention times and (ii) processes of sedimentation, filtration, predation, adsorption, disinfection. In primary and secondary settling, due to the hydrophobic properties, a partial accumulation of CoVs may occur in the separated sludge. In secondary treatment (i.e. activated sludge) CoVs and SARS-CoV-2 loads can be reduced only by about one logarithm (~90%). To enhance this removal, tertiary treatment with ultrafiltration (Membrane Bioreactors) and chemical disinfection or UV light is needed. CoVs and SARS-CoV-2 in the sludge (1.2E+04–4.6E+08 GU/L) can be inactivated significantly in the thermophilic digestion (55 °C), while mesophilic temperatures (33–37 °C) are not efficient. Additional studies are required to investigate the infectivity of SARS-CoV-2 in WWTPs, especially in view of increasing interest in wastewater reclamation and reuse.

1. Introduction

Coronaviruses (CoVs) include among others the viruses responsible for the epidemic of severe acute respiratory syndrome (SARS) in 2002–2003, Middle East respiratory syndrome (MERS) in 2012, and, from the end of December 2019, the global pandemic of COVID-19, the infectious disease caused by SARS-CoV-2. Some CoVs, although considered respiratory viruses, have been found in significant concentration in the faeces of infected people and can be thus connected to wastewater and drinking water systems. Moreover, CoVs can survive in aqueous environments and may originate questions about the fecal-oral transmission route (Wigginton et al., 2015).

CoVs belong to the order Nidovirales and the family Coronaviridae. Of the four coronavirus genera (α, β, γ, δ), the CoVs infecting humans belong to the genera α-CoV and β-CoV. CoVs are enveloped viruses because the capsid is surrounded by a lipid envelope that consists of lipids and proteins and derives from the host cell membrane (WHO, 2020). Because of the fragility of the envelope, CoVs are less resistant to extreme pH values, pollutants, and disinfectants than nonenveloped viruses (Molnar and Gair, 2015; Tran et al., 2021). SARS-CoV-1, MERS-CoV, and SARS-CoV-2 are three kinds of CoVs belonging to the same genus β-CoV that share several similarities in genetic sequence (Rabaan et al., 2020). For this reason, the studies carried out on the previous outbreaks of SARS and MERS may provide a close reference for the recent pandemic of COVID-19. Therefore, the current knowledge about SARS-CoV-2 in sewerage, the risk of potential fecal-oral transmission, and its removal in wastewater treatment plants can be in part derived from the previous studies carried out on the other CoVs. In fact, at the moment, the knowledge available on SARS-CoV-2 in the wastewater context is limited (Kitajima et al., 2020). Because respiratory viruses can

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be transmitted with bioaerosols, for example from wastewater exposures (McKinney et al., 2006; van Doremalen et al., 2020), it is prudent to consider that CoVs could spread through wastewater. Therefore an in-depth knowledge of removal performances in wastewater treatment plants (WWTPs) could help to prevent waterborne transmission of the virus.

In the COVID-19 infected patients, but also in asymptomatic individuals, the SARS-CoV-2 virus can be excreted from the gastrointestinal tract in the stool as well as from other bodily secretions (saliva, sputum, urines) which are subsequently disposed of in wastewater (Sherchan et al., 2020; Kitajima et al., 2020). SARS-CoV-2 may be present in stool even after respiratory symptoms have ended (Wu et al., 2020b; Xing et al., 2020). In this way, the virus is discharged from faeces into the sewer network and can reach WWTPs within a few hours.

Nucleic acid fragments of SARS-CoV-2 have been frequently detected in raw wastewater during the recent outbreaks worldwide by using the RT-PCR and the quantitative RT-qPCR techniques (Ahmed et al., 2020b; Randazzo et al., 2020b; La Rosa et al., 2020a; Medema et al., 2020; Haramoto et al., 2020). RNA quantification is the most used approach and the recently proposed standard for wastewater-based surveillance by the EU recommendation (European Commission, 2021). However, this approach does not provide data on the infectivity of the virus, because the presence of fragments of viral RNA in wastewater does not necessarily imply that the virus is structurally intact and viable. Conversely, cell culture-based approaches would be the gold standard for assessing the infectivity of isolated viruses (Hamza et al., 2011). However, not all viruses are easy to propagate as they may replicate too slowly, the cell lines for their propagation may not be widely available, or in other cases, the methods are very difficult to be used routinely. Moreover, cell culture may be time-consuming and may have limited detection sensitivity. In the case of CoVs and SARS-oV-2, the presence of the envelope may results in a difficult isolation and detection of infectious virions in faeces and sewage (Sbaoui et al., 2021). Due to these drawbacks, PCR-based methods are commonly used in the analysis of CoVs and SARS-CoV-2 in wastewater, while the detection of infectious virions has rarely been investigated (Rimoldi et al., 2020; Bivins et al., 2020).

For this reason, data are not yet enough to prove the potential fecal-oral transmission route that is still under debate (Amirian, 2020; Kitajima et al., 2020). For example, the infectivity of SARS-CoV-2 resulted null in both inlet and outlet from some WWTPs, despite the presence of viral RNA in the samples (Rimoldi et al., 2020).

However, due to the limited information, a precautionary approach in risk assessment is advised and the potential presence of viable viral particles cannot be excluded (Rimoldi et al., 2020). Also, the role of wastewater in faecal-aerosol/droplet and faecal-fomite transmission has to be better understood (Olusola-Makinde and Reuben, 2020).

The limited knowledge of CoVs and their behavior in aqueous environments is due to the assumption that CoVs are structurally dissimilar to enteric viruses that are considered waterborne viruses (Carducci et al., 2020; Wigginton et al., 2015). Enteric viruses are viruses able to replicate in the gastrointestinal tract and fecal-oral transmitted; they are primarily transmitted by the waterborne route and recognized as a common cause of non-bacterial gastroenteritis worldwide. In general, enveloped viruses have not been associated with fecal-oral transmission and are considered more susceptible to inactivation in water (Wigginton et al., 2015). However, recently Bhatt et al. (2020), considering the presence of SARS-CoV-2 in wastewater and the potential waterborne transmission routes, elucidated that SARS-CoV-2 can be considered as both waterborne and non-waterborne virus.

This review presents the current state of knowledge about the fate of SARS-CoV-2 and other CoVs in WWTPs, from the raw wastewater and along the primary, secondary and tertiary treatments as well as in sludge treatments and aerosols. The experimental data about CoVs in WWTPs are currently largely limited and conclusions can differ among studies due to the different analytical methods, different sensitivity of the assays, and the use of surrogate viruses in some cases. Analogously, the quantification of SARS-CoV-2 in wastewater can differ among studies. Another major difficulty is to compare data measured in the different stages of WWTPs, because the operational conditions may vary largely and the viral loads decrease progressively along with the treatments with the concentrations that become very low (near or < LOD and LOQ).

Despite these limitations, this paper is an effort in the collection of the state of art on the fate of CoVs and SARS-CoV-2 in WWTPs and may help to enhance the design and implementation of suitable treatments for virus removal as well as to address future research directions. In fact, additional studies are required to investigate the presence and infectivity of SARS-CoV-2 in WWTPs, in order to understand better the efficiency of each treatment stage. More data on the occurrence of SARS-CoV-2 in the treated effluents could also aid in correlating the viral load with the increasing interest towards wastewater reclamation and reuse.

2. Raw wastewater entering the WWTPs

CoVs excreted in faeces by infected people reach the inlet of a WWTP in about 2–10 h from the excretion. For example, Rimoldi et al. (2020) estimated a period of about 6–8 h from stool emission to the arrival at the WWTP. Although many environmental factors (wastewater composition, temperature, pH, etc.) can affect the stability and infectivity of CoVs and other viruses along the sewer, the time spent in the sewer is not long enough to achieve their total inactivation, resulting in a certain presence of viable and infectious CoVs in raw wastewater (Amoah et al., 2020).

As mentioned above, the detection of viral RNA in wastewater performed using RT-PCR, which is the gold standard commonly accepted in clinical specimens (Amoah et al., 2020), does not always imply the presence of the infective viable virus. For this scope, other methods should be applied, such as isolation in cell cultures, but the application of these approaches on SARS-CoV-2 and other human CoVs is very rare and are conducted in a Biosafety Level 3 laboratory (CDC, 2020) or BSL4 (Bivins et al., 2020). However, the application of such cell culture-based methods may offer some advantages to test environmental matrices, because data on the presence of infectious viruses have important implications for water safety and can provide more reliable information for microbial risk assessment studies.

Most of the studies reporting the detection and quantification of SARS-CoV-2 in raw wastewater are focused on the application of wastewater-based epidemiology (WBE). WBE can help in the surveillance of the spreading of SARS-CoV-2 in a community, one or two weeks before the cases are detected through clinical testing (Randazzo et al., 2020b). Moreover, WBE is not biased by the number of clinical tests performed. Application of WBE is rapidly increasing worldwide and this is the reason for a rapid increase in the SARS-CoV-2 in wastewater data becoming available in the literature. Conversely, because it is not needed for WBE, data about the quantification of viral loads along the WWTPs and in the treated effluents are limited at the moment.

In order to focus on the removal of SARS-CoV-2 through the various WWTP stages, this review considered the available data of raw wastewater only when coupled with data in the subsequent stages, such as secondary and tertiary treatments. However, more data on SARS-CoV-2 and its viral RNA in raw wastewater can be found in Patel et al. (2020).

The concentrations of SARS-CoV-2 in raw wastewater may vary by several orders of magnitude from one study to another (Table 1). Firstly, this may be due to the differences in the prevalence of COVID-19 in the served community. Secondly, the different alternatives proposed for the concentration of the viral RNA, which are characterized by different recovery efficiencies, represent a critical step for the entire analysis and may affect significantly the amount of RNA recovered. The concentrations of SARS-CoV-2 in wastewater may be 4 orders of magnitude lower (Foladori et al., 2020) than the concentrations in infected faeces that range from 10^3 to 10^7 genomic units per mL of faeces (GU/mL),

Table 1
Available data on the detection of SARS-CoV-2 in raw wastewater and the subsequent treatment stages in the WWTP.

Reference	WWTPs	Concentration methods	Influent raw wastewater	Secondary treated wastewater	Effluent wastewater
Westhaus et al. (2021)	9 WWTPs in Germany	Centrifugal ultrafiltration unit	3–20 GU/mL	Activated sludge 2.7–37 GU/mL	
Sherchan et al. (2020)	2 WWTPs in the USA (Louisiana)	Two methods: 1. ultrafiltration 2. adsorption - elution using electronegative membrane	2/7 samples positive Titers: 3.1x10^3-7.5 × 10^3 GU/L	Activated sludge 0/4 samples positive Not detected with two methods of concentration LOD: 1.0 × 10^3 GU/L (ultrafiltration) 1.7 × 10^2 GU/L (adsorption-elution)	Chlorine disinfection All (4/4) effluent samples negative Not detected with two methods of concentration Volume used: 250 mL (method 1), 750 mL (method 2)
Haramoto et al. (2020)	1 WWTP in Japan	Two methods: 1. electronegative membrane-vortex (EMV) 2. adsorption-direct RNA extraction	0/5 samples positive LOD (200-mL volume used): 4.0x10^3-8.2 × 10^4 GU/L	Activated sludge 1/5 samples positive 2.4 × 10^3 copies/L (EMV method) Volume used: 5000 mL LOD: 1.4x10^2-2.5 × 10^3 GU/L	
Randazzo et al. (2020b)	6 WWTPs in Spain (Murcia)	Aluminum hydroxide adsorption - precipitation	35/42 samples positive 1.3x10^5-3.2 × 10^5 GU/L by using N1, N2, N3 assays LOQ: 2.8x10^4-8.1 × 10^4 GU/L for N1, N2, N3 assays	Activated sludge 2/18 samples positive <LOQ - 2.5 × 10^5 GU/L LOQ: 2.8x10^4-8.1 × 10^4 GU/L for N1, N2, N3 assays	Coagulation, flocculation, sand filtration, disinfection, UV, NaClO All (12/12) effluent samples negative Volume used: 200 mL
Randazzo et al. (2020a)	WWTPs in Spain (Valencia)	Aluminum-driven flocculation	All (12/12) samples positive 5.22–5.99 $\log_{10}$ GU/L	All (9/9) effluent samples negative Volume used: 200 mL	
Rimoldi et al. (2020)	3 WWTPs in Italy (near Milan)	No concentration to detect infectivity	All samples positive	Secondary treatment + peracetic acid or UV All effluent samples negative Volume used: 1 L and then 50 mL	
Arora et al. (2020)	3 WWTPs in India (Jaipur city) with SBR process	Two methods: 1. filtration and PEG adsorption 2. centrifugation at 7000 rpm	1/3 samples positive for at least two target genes Titers: not quantified	SBR process + Cl_2 All effluent samples negative	
Arora et al. (2020)	1 WWTP in India (Jaipur city) with MBBR process	Two methods: 1. filtration and PEG adsorption 2. centrifugation at 7000 rpm	Sample positive for at least two target genes	MBBR process + UV All effluent samples negative.	
Balboa et al. (2020)	1 WWTP in Spain	Centrifugation and PEG precipitation	5/5 samples positive <7.5–15 GU/mL	All (5/5) effluent samples negative.	
Ahmed et al. (2020a)	MBR on a cruise ship	Two methods: 1. ultrafiltration 2. adsorption – extraction with electronegative membrane	All samples positive	7/21 effluent samples negative for all assays 14/21 effluent samples positive for at least one assay Volume used: 100 mL (method 1), 200 mL (method 2)	
Kumar et al. (2020) Kumar et al. (2021)	UASB + aeration pond	filtration and PEG adsorption	All (2/2) samples positive Maximum concentration = 3.5 × 10^2 GU/L	All effluent samples negative LOQ: 1.7 × 10^2 GU/L	

depending on the day of sampling post-onset (Wölfel et al., 2020). The dilution is due to the large use of drinking water per capita, the presence of stormwater or infiltrations in the sewer network, and the limited percentage of positive cases among the population served by a WWTP. As a consequence of dilution, the viral titers measured in raw wastewater are very low and the detection of viral RNA may be affected by several interfering factors.

In raw wastewater, recent reports indicate that the concentration of SARS-CoV-2 may vary from 20 GU/L (Ahmed et al., 2020b; 100 mL sample and Ct 39) to 3 × 10^6 GU/L (Foladori et al., 2020), depending on the number of infected people in the community served by the WWTP. For a general comparison, the peak levels of adenoviruses and noroviruses in raw wastewater can reach 10^9/L and more (Gerba et al., 2017). The maximum concentrations of various human enteric viruses in raw wastewater have been reviewed in depth by Haramoto et al. (2018), who indicated values up to 10^9 copies/L for noroviruses, slightly less than 10^9 copies/L for adenoviruses, while values were higher than 10^{10} copies/L for pepper mild mottle virus. In this study, the maximum concentration was used as a key index because it indicates the worst-case scenario in the spread of the virus (Haramoto et al., 2018). The occurrence of rotaviruses was reported in the range of 10^6–10^8 GU/L (Mohan et al., 2021).

CoVs in wastewater enter WWTPs and are affected in two ways: (1) the natural decay of CoVs during the pronged time of residence in the plants under unfavourable conditions, as described in section 3; (2) the removal in the treatment stages where processes of sedimentation, filtration, predation, etc, are implemented, as presented in sections 4-5.

3. Natural decay in wastewater

The persistence and inactivation of CoVs and in particular SARS-CoV-2 in wastewater and related treatments are largely unknown and undocumented. Some knowledge can be derived from studies using surrogates, such as feline infectious peritonitis virus and human CoV 229E used by Gundy et al. (2009), mouse hepatitis virus (MHV strain A59) and *Pseudomonas* phage *Φ6* (surrogates for human enveloped viruses) used by Ye et al. (2016), transmissible gastroenteritis virus (TGEV) and MHV used by Casanova et al. (2009), bacteriophage *Φ6* as a surrogate of enveloped viruses used by Casanova and Weaver (2015) and murine hepatitis virus indicated as a good surrogate also for SARS-CoV-2 (Ahmed et al., 2020c).

However, it is worth noting that the stability in water of different types of viruses may be variable, and surrogates may be insufficient to describe exactly the behavior of a specific virus of interest such as in the case of SARS-CoV-2 (Aquino de Carvalho et al., 2017; Kitajima et al., 2020).

In general, SARS-CoV-2, similarly to other CoVs, finds in wastewater a difficult environment to survive and its fate is to undergo a spontaneous and progressive inactivation, also due to the strong influence by temperature and organic or microbial pollution (Carducci et al., 2020). Inactivation in wastewater is much faster for CoVs than for enteric viruses at ambient temperatures (Carraturo et al., 2020). However, the inactivation of CoVs may be highly variable depending on many factors, not all and always predictable. Referring specifically to SARS-CoV-2, the recent review of Carraturo et al. (2020) indicates a low capacity to survive in wastewater due to the organic matter or inhibiting matrix's autochthonous flora that may increase the decay of the viruses. With regards to environmental factors that can affect the persistence of CoVs and SARS-CoV-2 in wastewater, the main findings can be briefly summarised as follows.

CoVs vs. enteric viruses – Human enteric viruses (e.g. norovirus, rotavirus, astrovirus, adenovirus, pepper mild mottle virus; Haramoto et al., 2018) are mostly non-enveloped RNA viruses that use the enteric tract to infect human or animal cells. CoVs, and in particular SARS-CoV-2, are viruses that infect humans via the respiratory route (acute respiratory syndrome), but recently they are also described as enteric viruses because variably associated with gastrointestinal tract infections and enteric diseases (inter alia Siyuan and Liang, 2020; Sbaoui et al., 2021). Notably, SARS-CoV-2 is primary a respiratory virus, but the literature has already shown that the gastrointestinal tract is a target organ of SARS-CoV-2 that uses the ACE2 protein as a receptor (Wan et al., 2020; Sbaoui et al., 2021). For these reasons, the differences between CoVs and enteric viruses are found rather between enveloped and non-enveloped viruses. CoVs and SARS-CoV-2, being enveloped viruses, are less stable and more susceptible to inactivation in wastewater (e.g. by extreme pH, high temperatures) than most non-enveloped enteric viruses (WHO, 2020; La Rosa et al., 2020b), because the envelope is less resistant to environmental factors and disinfectants. For this reason, enveloped viruses are rarely associated with waterborne transmission.

Other microorganisms in the matrix - Many microrganims in wastewater are CoV antagonists of CoVs (Paul et al., 2021). The indigenous microorganisms naturally present in wastewater can contribute to increasing the degree of inactivation of surrogate CoVs in wastewater (Ye et al., 2016). In particular, mouse hepatitis virus was used in the study by Ye et al. (2016) to compare the influence of unpasteurized and pasteurized wastewater (heating at 70 °C, 3 h). The time required for 90% inactivation of MHV at 10 °C increased from 36 ± 5 h in raw wastewater to 149 ± 103 h in pasteurized wastewater (Ye et al., 2016). These results demonstrate that the virus inactivation rate is significantly slower in pasteurized wastewater due to the absence of antagonistic microorganisms that favour a longer viability of the virus. This antagonistic effect can be associated to protozoa or predatory bacteria such as *E. coli*, *Enterococcus* spp., *Bacillus* spp*.*, *Clostridium* spp. (Giacobbo et al., 2021).

Solids in the matrix - The solids in wastewater may act differently on the inactivation of CoVs: (i) surrogate CoVs survived longer in unfiltered wastewater with respect to filtered wastewater (Gundy et al., 2009), indicating that suspended solids and organic matter can offer protection from the oxidants present in wastewater; (ii) CoVs were more rapidly inactivated in pasteurized settled sewage in comparison with reagent grade water (Casanova et al., 2009), indicating the presence of inactivating agents in the solids. In this latter study, sewage was pasteurized and then inoculated with SARS-CoV-1 to reduce the effect of competing microorganisms and to evaluate only the influence of solids.

Inactivation time – Gundy et al. (2009) evaluated the inactivation time in settled wastewater at 23 °C of two CoVs: (1) feline infectious peritonitis virus, an enteric feline CoV, and (2) human coronavirus 229, a respiratory virus. In particular, in this study, the time required for 99.9% inactivation was 3.5 days (Gundy et al., 2009). In another study, Casanova et al. (2009) investigated the inactivation in settled wastewater at 25 °C of two CoVs: (1) transmissible gastroenteritis virus, a swine diarrheal pathogen, and (2) mouse hepatitis virus, a respiratory and enteric pathogen. The authors indicated that the time required for 99.9% inactivation of these CoVs was 10–14 days (Casanova et al., 2009). Casanova and Weaver (2015) estimated the inactivation kinetics of a surrogate of enveloped human viruses in sewage, indicating 5 logs of inactivation in 6 days at 22 °C. The authors underlined that longer holding times are advised at lower temperatures. Referring specifically to SARS-CoV-2 RNA, the time required for 90% reduction was 8 days in untreated wastewater (Ahmed et al., 2020c). The persistence of SARS-CoV-2 infectivity in wastewater was investigated by Bivins et al. (2020). The timing for the 90% reduction of viable SARS-CoV-2 in wastewater was 1.6–2.1 days at 20 °C. This result indicates that infectious SARS-CoV-2 is significantly less persistent than SARS-CoV-2 RNA and therefore detection of RNA alone is not strictly associated with the risk of infection.

Temperature - Considering the range of temperatures of wastewater over the year in temperate climatic zones, CoVs and surrogate CoVs persist longer at a temperature of 4 °C compared to 20 °C (Wang et al., 2005b; Casanova et al., 2009). At 4 °C, the time for 99.9% inactivation of two surrogate CoVs in pasteurized settled wastewater was 73–105 days, indicating long conservation of infectivity at low temperatures (Casanova et al., 2009). This indicates that the winter season may favour the persistence of CoVs, especially in the regions with cold climates and combined sewerages, where stormwater runoff or melting snow may cause a rapid drop in wastewater temperatures during the winters. In these cases, the longer survival of CoVs at low temperatures can potentially increase the risk of transmission from exposure to contaminated environmental sources such as sewage and bio-solids (Carraturo et al., 2020). The time required for 90% inactivation of a surrogate CoV (mouse hepatitis) in raw municipal wastewater at 10 °C and 25 °C was 36 ± 5 h and 13 ± 1 h, respectively (Ye et al., 2016). With regards to SARS-CoV-2, Chan et al. (2020) reported that the virus suspended in a solution (minimal essential medium containing 1% fetal bovine serum) retained viability for 7 days at 20–25 °C and up to 14 days at 4 °C. However, the virus retained its viability for only 1–2 days at hot temperatures of 33–37 °C (Chan et al., 2020).

pH – The typical range of pH in municipal wastewater (around 7–8) affects only slightly the inactivation of CoVs, as observed in surrogate CoVs by Casanova et al. (2009) at various temperatures. Lai et al. (2005) indicated that SARS-CoV-1 in stool survived for 1–5 days at alkaline pH of 8–9, while only for 3 h at acidic pH of 6. Conversely, SARS-CoV-2 appears more resistant than previous CoVs at extreme pH values. In particular, Chin et al. (2020) found SARS-CoV-2 extremely stable in a wide range of pH values from 3 to 10. This resistance to low pH may also explain the detection of SARS-CoV-2 in faeces that could derive from the swallowing of respiratory secretions and the potential resistance to gastric acidity in the stomach with the subsequent passage in the intestine. At the moment, the mechanism explaining the higher resistance of SARS-CoV-2 to extreme pH in aqueous environments is mostly unknown in the literature but should be explored further (Tran et al., 2021).

Kinetics and decay rate - Inactivation of a surrogate CoV (mouse hepatitis) at 10 °C and 25 °C in raw wastewater followed first-order kinetics (Ye et al., 2016). The data of Gundy et al. (2009) served as a basis for Hart and Halden (2020) to estimate some kinetic parameters for the attenuation of SARS-CoV-2 in wastewater at ambient temperature (20 °C) using a first-order kinetic. In particular, a half-life of 4.8–7.2 h, mean lifetime of 7–10 h, and decay rate of 0.096–0.143 h^{-1} were calculated (Hart and Halden, 2020). These values are comparable to the retention time in the sewerage, indicating that half of the viral load could be reduced during the travel in long municipal sewer networks. The natural CoVs decay rates could be important also in the implementation of WBE (i.e, a fraction of SARS-CoV-2 in the faeces may be lost in sewerage under summer temperatures and for long corrivation times). The decay rates of some human CoVs and their viral surrogates in wastewater were reviewed by Silverman and Boehm (2020). The decay rate increased with temperature in the range between 4 and 56 °C. The decay rate was higher in wastewater and lowest in laboratory water/buffer, suggesting that enveloped viruses may be affected by the constituents of wastewater such as enzymatic activity, predation, solvents, detergents, and organic matter (Silverman and Boehm, 2020). At 22–25 °C average values of decay rate were 2.9 ± 0.03 d^{-1} in sterilized wastewater (Silverman and Boehm, 2020). With regards to the use of surrogates, recently Ahmed et al. (2020c) did not find a statistically significant difference between the decay rate of SARS-CoV-2 RNA and the surrogate murine hepatitis virus, confirming the suitability of this surrogate. Ahmed et al. (2020c) determined the first-order decay rate constants of SARS-CoV-2 in untreated wastewater and values ranged from 0.084 d $^{-1}$ at 4 °C to 0.286 d^{-1} at 37 °C. Based on their experimental results on SARS-CoV-2, Ahmed et al. (2020c) concluded that moderate ambient temperatures (<37 °C) did not significantly affect SARS-CoV-2 RNA at HRT typical of wastewater collection systems (<24 h). Considering infectious SARS-CoV-2 in wastewater, the decay rate was 1.2–1.4 d $^{-1}$ and the half-life was 0.49–0.64 d (Bivins et al., 2020).

Despite the question of how long SARS-CoV-2 may survive in wastewater remains an open issue (Barcelo, 2020), some aspects appear clear. In synthesis, temperature is the most significant environmental variable for the reduction of SARS-CoV-2 in raw wastewater, followed by the influence of the heterogeneous matrix (Ahmed et al., 2020c). Moreover, during the COVID-19 outbreak, various antimicrobial compounds, e.g. used for hand washing or surface disinfection, were discharged into the sewer systems. Such compounds can inactivate the spike proteins of viruses. In particular, considering the large use of liquid chlorine or sodium hypochlorite, the excess reaches the domestic or hospital sewers where it can cause severe damage to the nucleic acids of the viruses as well as damaging effects on the capsid.

To enhance the removal of CoV, specific treatments (sedimentation, filtration, predation, adsorption, disinfection, etc.) play a crucial role, as discussed in sections 4-5. When CoVs are not inactivated/removed in these stages, they can reach: (i) the effluents and thus the receiving water bodies, (ii) the sludge treatments followed by sludge disposal. In particular, additional recommendations may be needed to ensure the safety of wastewater reuse in post-COVID-19 times (Oliver et al., 2020).

4. Fate of CoVs and SARS-CoV-2 through the stages of the wastewater treatment line

Apart from the pretreatments, aimed at separating coarse materials, grit, and oil/greases, the configuration of a WWTP (Fig. 1) is conventionally divided into a series of treatment stages: (1) primary treatment where raw wastewater is settled and the separated solids produce the primary sludge; (2) secondary treatment, based on biological processes, where biodegradable compounds are removed and inert particulate solids are separated; (3) tertiary treatment, aimed at improving the quality of the effluents with physical or chemical processes, included the final disinfection of the effluents.

Considering the entire wastewater treatment line from the influent to the final effluent, the overall removal of CoVs can be from one to several logs depending on the processes involved. For comparison, the overall removal of infectious enteric viruses in WWTPs may vary in the range of 1.9–5.0 log, with an average of 4.2 logs (Simmons and Xagoraraki, 2011).

Removal of CoVs in WWTPs cannot be derived from typical physico-chemical parameters of the influent/effluent such as COD (Chemical

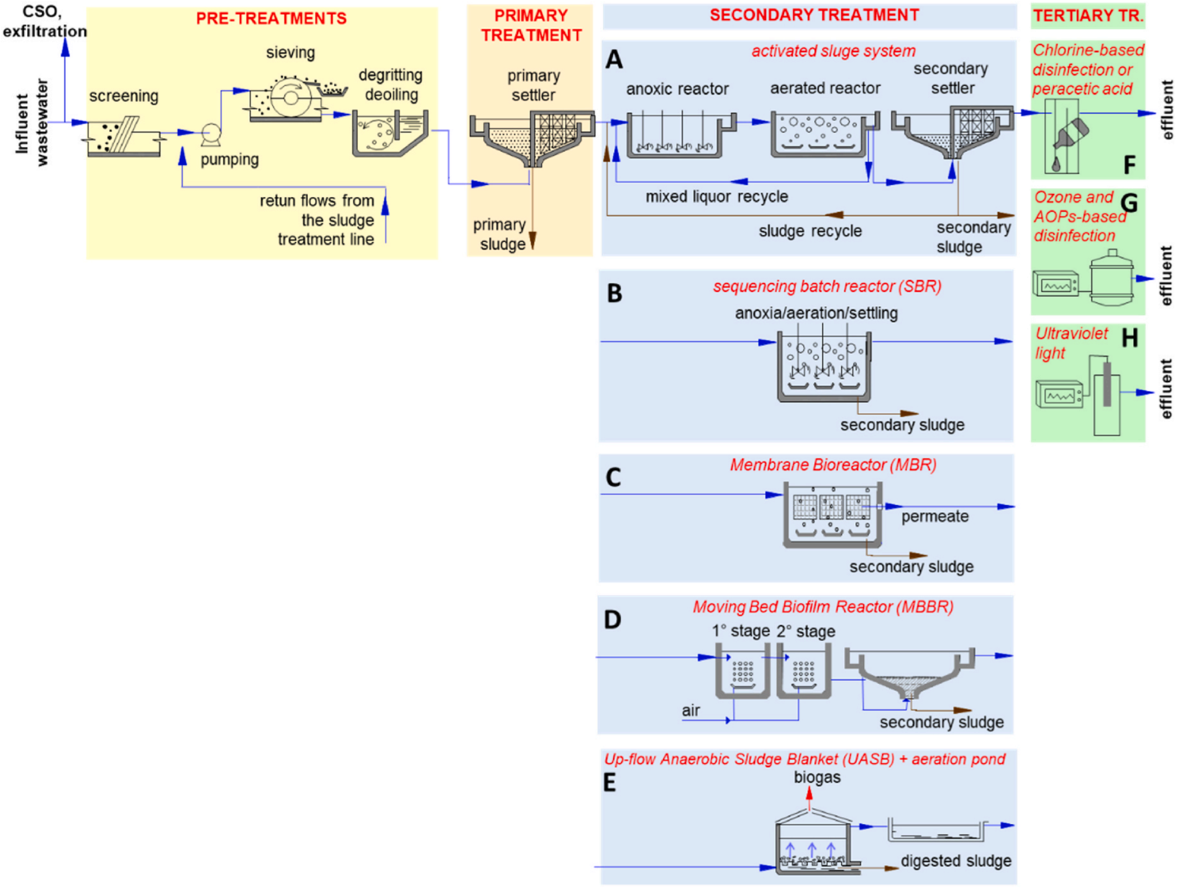

Fig. 1. Flow-sheet of various configurations of WWTPs. Secondary treatments: (A) Activated sludge with nutrient removal; (B) Sequencing Batch Reactors; (C) Membrane Bioreactors; (D) Moving Bed Biofilm Reactor; (E) UASB. Tertiary treatments: (F) Chlorine-based disinfection; (G) Ozone and AOPs-based disinfection; (H) Ultraviolet light.

Oxygen Demand) or BOD_5 (Biochemical Oxygen Demand) because there is no correlation between these routine parameters and the viral load. However, COD and BOD_5 in raw wastewater are used for estimating the population size of the sewershed served by the WWTP, aimed at calculating the virus loads per capita per day required for surveillance of SARS-CoV-2 and WBE applications (European Commission, 2021).

4.1. Removal in the pre-treatments: screening, sieving, degritting

Pre-treatments consist of preliminary processes such as mechanical screening (separation of particles with size >5 mm), sieving (>0.25 mm), degritting, deoiling/degreasing, and pumping. These treatments cannot affect significantly the levels of viruses in the wastewater. For comparison, preliminary treatment using fine screens can remove about 0.2–0.4-log of pathogenic bacteria and fecal indicators (Zhou et al., 2015). CoVs can be aerosolized in these stages or diffused with droplets, especially during the pumping and movement of wastewater or during the extraction of the separated materials (wastes, grits, greases, etc.).

The routine activity of workers is the inspections and preventive maintenance of these stages, and in some cases the manual cleaning of coarse screening. Workers and laboratory staff have the task of collecting wastewater samples for physico-chemical analyses and the pre-treatments are the point for the sampling of the influent raw wastewater. During the work activities, they can be potentially exposed to a variety of infectious agents (Zaneti et al., 2021). In particular, in the study of Zaneti et al. (2021), quantitative microbial risk assessment (QMRA) was performed at the entrance of two WWTPs, with the aim of assessing the risk for workers during COVID-19 outbreaks. Pre-treatments were composed by manual (coarse) and automatic (fine) screening, followed by degritting; all these treatment units were not covered or equipped with collective protective equipment as splashes barriers. SARS-CoV-2 was considered in the risk assessment model, in comparison with *E. coli* which is a common fecal indicator (Zaneti et al., 2021). The major health risk for wastewater treatment workers was recognized during the manual cleaning of coarse screening (Zaneti et al., 2021). In an extreme scenario, the estimated risks were up to 30 times higher than the risk assessed for *E. Coli* under the same exposure route (Zaneti et al., 2021).

Therefore, it is mandatory for workers that perform these operations to use appropriate personal protective equipment (PPE). Especially in developing countries, the occupational exposure for workers may be higher since the protocols of PPE use is not as stringent as in developed countries (Zaneti et al., 2021).

Pumping stations are usually placed close to pre-treatments, where raw wastewater is lifted from the underground pit to higher elevations to permit further transportation by gravity. The pumped wastewater flows through pressurized pipes where typical pressures are in the order of a few bars. To our knowledge, this pressure does not have a significant impact on virus inactivation, while only high-pressure processing is required to provide efficient means for virus inactivation (Silva et al., 1992; Roos, 2020).

4.2. Removal in the primary treatment

The first major process in some WWTPs, but not in all, is the primary sedimentation aimed at removing the settleable solids with the consequent production of primary sludge which is thickened by gravity. Primary sludge has a dry solids content (total solids) of 1–2%, higher than 0.01–0.05% in raw wastewater (Peccia et al., 2020). Viruses are small particles with a density similar to water that cannot settle spontaneously and efficiently in the primary sedimentation. The capacity of separation of the viral particles may moderately increase when they adsorb on larger suspended solids able to settle. The flocculation process forms particles with larger volume and higher density which have an enhanced settling velocity (Bhatt et al., 2020). In particular, CoVs have a hydrophobic envelope, which renders CoVs less soluble in water and increases the tendency to adsorb on solids (Gundy et al., 2009).

The partitioning between liquid and solids of two human enveloped virus surrogates in wastewater was investigated by Ye et al. (2016). These Authors demonstrated that 26% of the virus adsorbed to the solids in wastewater at equilibrium (reached after a couple of hours). The adsorption kinetic in samples indicated that enveloped viruses were more adsorbed on solids than non-enveloped viruses (Ye et al., 2016). These findings confirm that a fraction of the CoVs load in raw wastewater can be removed and accumulated in primary sludge.

Despite the partial removal in primary treatment, this is not the main mechanism for the removal of CoVs from wastewater. In absence of data about CoVs, an approximative estimation can be derived from the removal of enteric viruses in primary sedimentation which is 0.1–1.0 log (Simmons and Xagoraraki, 2011; Simmons et al., 2011).

The concentration of SARS-CoV-2 in primary sludge was investigated by Peccia et al. (2020) during the Covid-19 outbreak in a metropolitan area. In the sludge matrix, the qRT-PCR cycle threshold (CT) value was 38.75 and was used as a detection threshold for the applied method. Viral RNA of SARS-CoV-2 was detected in all the samples and concentrations ranged from 1.7×10^3 to 4.6×10^5 GU/mL. In particular, the presence of SARS-CoV-2 in primary sludge was 2–3 times higher than the values in raw wastewater derived from the literature (Peccia et al., 2020).

The presence of SARS-CoV-2 in two samples of primary sludge collected from two WWTPs in Istanbul was investigated by Kocamemi et al. (2020). All samples were tested positive with a CT of 34.7–35.9. Concentrations of SARS-CoV-2 in primary sludge were 1.25×10^4 and 2.33×10^4 GU/L. These values were similar in primary and secondary sludge and both were higher than the copy numbers observed in the influent wastewater, confirming an effect of partial accumulation of SARS-CoV-2 in the sludge.

Westhaus et al. (2021) investigated the partitioning of SARS-CoV-2 in influent wastewater, comparing the aqueous and the solid phase of the samples, separated by centrifugation. The Authors found that the SARS-CoV-2 RNA copy number in the solid phase was one log unit higher than in the aqueous phase: in particular, 25 copies/mL were measured in the solid phase in comparison with 1.8 copies/mL in the aqueous phase of the influent wastewater (Westhaus et al., 2021). This is another confirmation of the fact that a part of SARS-CoV-2 can accumulate in the solids and thus in the primary sludge.

Peccia et al. (2020) suggested that primary sludge can be used to accurately track outbreaks in a community as an alternative matrix for the monitoring of raw wastewater. In fact, CoVs accumulate in the primary sludge after an acceptable delay from the excretion: the retention time in the sewerage and approximately 1–3 h residence time in the primary settler.

4.3. Removal in the secondary treatment

The secondary treatments often implement suspended-growth biological processes such as activated sludge that utilizes dense microbial cultures maintained in suspension in aerated or anoxic tanks and followed by settling units for the separation of sludge from the secondary-treated effluent.

Typical HRTs of the activated sludge processes range from 5 to 15 h but may reach 24 h or more in low-loaded processes used to reduce sludge production or to treat slowly biodegradable wastewater. The HRT in the biological reactors, calculated as the ratio between the reactor volume (V) and the flow rate (Q), is an important parameter in inactivating CoVs because longer HRTs favour a higher spontaneous decay rate of the viruses (Amoah et al., 2020).

Theoretically, CoVs removal in activated sludge processes may take place both during the permanence in the aerated/anoxic tanks where they are affected by competition and predation by microorganisms in activated sludge and during sedimentation in the secondary settling. However, full-scale monitoring revealed that the secondary treatment based on the conventional activated sludge may reach only a limited reduction of CoVs and SARS-CoV-2, estimated in approximately one logarithm (~90%). An overview of the case studies available in the literature about the quantification of SARS-CoV-2 in the secondary-treated wastewater is summarised in Table 1.

4.3.1. Activated sludge

The activated sludge process can contribute effectively with a >3 $\log_{10}$ removal efficiency of enteric viruses. However, human viruses (adenovirus, polyomavirus, and torque teno virus) persisted in the secondary treated effluent with concentrations of 10^2–10^3 GU/L (Mohan et al., 2021). Therefore, tertiary treatment is required before reuse of the treated effluent.

Nine municipal WWTPs, all based on activated sludge configurations and designed for 120,000–2,400,000 Population Equivalent, were investigated for the presence of SARS-CoV-2 in influent and secondary-treated effluents (Westhaus et al., 2021). All wastewater samples showed detectable virus RNA and the concentrations varied in a range of 3–20 GU/mL in the inflow and 2.7–37 GU/mL in the secondary-treated wastewater (Westhaus et al., 2021). These results indicated that titers in influent and treated effluents were comparable and thus a poor removal of SARS-CoV-2 was observed in these conventional activated sludge WWTPs.

In some other studies, the concentration of SARS-CoV-2 in the secondary-treated wastewater was not detectable. This does not always mean that the virus was absent, but in some cases, the concentration of the viral RNA may be below the assay limit of detection. Therefore the results should always be associated with the LOD of the specific method applied.

In the study of Sherchan et al. (2020), the secondary-treated wastewater taken from two WWTPs in the USA tested negative for SARS-CoV-2 RNA, indicating that the virus was removed in the activated sludge stage to an undetectable level. In this study, the LOD was 1.0×10^3 copies/L using ultrafiltration as a method of concentration and 1.7×10^2 copies/L using a method based on adsorption-elution (Sherchan et al., 2020).

Haramoto et al. (2020) investigated the secondary-treated wastewater before chlorination in a WWTP in Japan. Despite none of the 5 influent samples tested positive for SARS-CoV-2, 1 out of 5 secondary effluents resulted positive with a concentration of 2.4×10^3 GU/L. The reason for this discrepancy in the results of influent and secondary-treated wastewater was attributed to the difference in the limit of detection (LOD) in the two types of wastewater: (1) in the analysis of untreated wastewater a 200-mL volume was filtered for concentration and LOD was 4.0×10^3–8.2×10^4 copies/L; (2) in the secondary-treated wastewater a 5000-mL volume was filtered which leads to lower LOD of 1.4×10^2–2.5×10^3 copies/L (Haramoto et al., 2020). Moreover, the relatively low concentration of SARS-CoV-2 RNA observed in wastewater by Haramoto et al. (2020) could be associated with the low prevalence of COVID-19 cases in the area served by the studied WWTP.

In Randazzo et al. (2020b), the SARS-CoV-2 genetic material was quantified both in the primary wastewater that is the flow entering the activated sludge stages and in secondary-treated wastewater. Comparing the respective concentrations, 1.3×10^5–3.2×10^5 GU/L were measured in the inlet, while only a sample was above LOQ in the outlet with a concentration of 2.5×10^5 GU/L (theoretical LOQ was 2.8–8.1 $\times 10^4$ GU/L). These data indicate that the difference of SARS-CoV-2 loads from inlet to outlet is limited in activated sludge.

The scarce or slight removal of CoVs in activated sludge is in agreement with the reduction of other types of viruses such as Norovirus and Sapovirus, which was 1.2–1.4 log units (Taboada-Santos et al., 2020). In activated sludge processes, the main removal mechanism of CoVs could be attributed to the adsorption on suspended solids (biological flocs) followed by settling in the secondary clarifier (Mohapatra et al., 2020). This is the consequence of the high hydrophobicity that characterises the lipoprotein layer of CoVs that make them more prone to adsorption on solids.

To enhance the limited removal obtained in activated sludge, the aid of ultrafiltration, chemical disinfection, or UV light is needed (see sections 4.3.4 and 4.4), otherwise, the virus may remain for days in discharged effluents and spread in receiving water bodies. In particular, a residual amount of CoVs and SARS-CoV-2 could remain viable after the secondary treatment (see Section 3) because the inactivation times in wastewater are longer (in the order of days, depending on temperature) than the typical HRTs in the secondary stages in WWTPs (in the order of hours). Secondary-treated wastewaters after disinfection with peracetic acid and UV lamps were analysed for the detection of SARS-CoV-2 RNA in Italy (Rimoldi et al., 2020). All samples of untreated wastewater tested positive for SARS-CoV-2, while all the effluents resulted negative (Rimoldi et al., 2020).

4.3.2. Sequencing Batch Reactors

When the activated sludge process is implemented in a batch reactor instead of a continuous flow reactor, the configuration is a sequencing batch reactor (SBR, Fig. 1). Arora et al. (2020) investigated SARS-CoV-2 in two SBR plants with tertiary treatment (disinfection with Cl_2) and one SBR without tertiary treatment. In these WWTPs, the average concentrations of BOD_5 and COD were 200–300 mgBOD_5/L and 400–700 mgCOD/L in the influent wastewater while they became 5–9 mgBOD_5/L and 24–50 mgCOD/L in the treated effluents, confirming the high removal efficiency of organic matter in the plants (Arora et al., 2020).

With regards to the investigation on SARS-CoV-2, samples were collected in the summer months at ambient temperatures up to 45 °C (Arora et al., 2020). This study reports the first evidence of SARS-CoV-2 in raw and treated wastewater under the hottest months in the summer. The temperature can significantly affect CoVs survival and inactivation (see section 3) in the sewerage. Moreover, the removal of SARS-CoV-2 in the SBR process could be enhanced by the unfavourable temperatures, because the viral survival is shorter under high temperatures.

The study of Arora et al. (2020) showed the presence of at least two target genes in untreated wastewater samples from 1 out of 3 SBR plants, thus confirming the presence of the SARS-CoV-2 genome in 1 plant. After secondary treatment, effluents were negative for any detectable presence of viral genome. Negative samples discharged from SBR processes equipped with Cl_2 disinfection indicated the quality of the treated effluents that are intended to be used for irrigation in the Indian context (Arora et al., 2020).

4.3.3. Moving bed biofilm reactors

The influent and effluent from a secondary treatment based on a moving bed biofilm reactor (MBBR) were monitored in Arora et al. (2020) in a WWTP treating part of the municipal wastewater of Jaipur city. The MBBR was followed by a tertiary treatment with final UV disinfection. The average values of BOD_5 and COD in the influent wastewater of 363 mgBOD_5/L and 1055 mgCOD/L were reduced to 43 mgBOD_5/L and 98 mgCOD/L in the treated effluents (Arora et al., 2020).

In the study of Arora et al. (2020) the raw wastewater samples entering the MBBR plant tested positive for SARS-CoV-2. Instead, the effluents from the MBBR + UV showed negative results for the presence of viral RNA. This configuration based on MBBR coupled with UV was thus effective and able to decrease the viral particles below the detection limit, reducing also the risk to public health with the reuse of treated water for irrigation (Arora et al., 2020). The evaluation of SARS-CoV-2 removal in WWTPs effluents is particularly important in India, analogously to other developing countries, because the effluents may be used in nearby gardens and agricultural areas for irrigation reuse (Arora et al., 2020).

4.3.4. Membrane Bioreactors

Membrane Bioreactors (MBRs) couple a biological reactor with suspended biomass (similar to conventional activated sludge but often at higher solids concentrations) with a high-efficient membrane technology. The MBR process replaces the secondary biological treatment, the secondary sedimentation, and the tertiary treatment such as microfiltration or sand filtration. Therefore it can be considered a secondary and, in part, tertiary treatment.

In MBRs the separation of viral particles is based on the principle of size exclusion and thus the size of the viruses has an important role in the retention by the membrane. When viruses attach to mixed liquor flocs, they are unlikely to pass through the membrane pores. Common membranes used in full-scale WWTPs fall in the field of microfiltration (MF, nominal pore size of 0.1–10 μm) and ultrafiltration (UF, nominal pore size <0.1 μm).

The virions of CoVs have an almost spherical size with a diameter ranging from 80 nm to 220 nm, while the diameters of SARS-CoV-1 and SARS-CoV-2 virions are similar and in the range of 60–140 nm (Foladori et al., 2020). Considering these sizes, the recommended commercial membranes for the removal of CoVs - and in particular of SARS-CoV-2 - should be in the field of UF, while MF could not ensure a high removal.

The main mechanisms that affect virus removal in MBR are: (i) adsorption on mixed liquor solids; (ii) retention by the membrane cake layer; (iii) natural inactivation (Chaudhry et al., 2015). Comparing the contributions of these mechanisms to the global virus removal, the most important factor was backwashing, followed by inactivation, cake layer, and adsorption on solids (Chaudhry et al., 2015).

When compared to conventional activated sludge coupled with secondary sedimentation, MBRs permit a higher removal of viruses (Simmons and Xagoraraki, 2011). Viral removal of 2–3 $\log_{10}$ has been reported for different types of viruses in MBRs (Miura et al., 2018). Other studies reported removal of 4–5 $\log_{10}$ for pathogenic viruses in full-scale MBRs with 0.04 μm membrane (Chaudhry et al., 2015; Simmons et al., 2011). However, different viruses have different behaviour in the adsorption on the mixed liquor solids, causing virus type-specific removal during the MBR treatment process as shown by Miura et al. (2018). In the specific case of CoVs, the tendency of these viruses to attach to solids (see section 4.2) suggests that their retainement by the membranes could be favoured.

Influent and effluent wastewater from an MBR plant was sampled in a cruise ship on April 23, 2020, after passenger disembarkation and with only the crew on board (Ahmed et al., 2020a). All untreated wastewater collected from the cruise ship tested positive. In the effluent from the MBR, 7/21 samples were negative for all assays and 14/21 were positive for at least one assay (unfortunately, the cut off of the membranes was not indicated).

4.3.5. *Up-flow anaerobic sludge blanket + aeration pond*

Influent wastewater was treated in an up-flow anaerobic sludge blanket (UASB) and aeration pond-based secondary treatment (Kumar et al., 2020). This plant was designed to obtain effluents with concentrations <20 mgBOD$_5$/L, <30 mgTSS/L and <100 mgCOD/L. All influent samples tested positive for SARS-CoV-2, with the estimated maximum concentration of 3.5×10^2 GU/L. The plant was efficient to remove SARS-CoV-2 and all effluent samples tested negative with CT values > 40 (Kumar et al., 2020).

The reduction of SARS-CoV-2 RNA after an UASB treatment and a treatment in an aeration tank and polishing pond was explained also in Kumar et al. (2021). SARS-CoV-2 RNA concentrations in raw wastewater and UASB inlet samples were detected above the LOQ, while the concentration in the effluent from the UASB and the final effluent was not quantifiable (concentration $<$ LOQ of 1.7×10^2 copies/L; Kumar et al., 2021). Considering the LOQ as a maximum concentration in the effluents, the reduction of SARS-CoV-2 during the UASB treatment was higher than 1.3 $\log_{10}$ (Kumar et al., 2021).

4.4. *Disinfection*

WWTPs play thus a fundamental role in protecting public health, because the effluents may be used for irrigation, recreational purposes, or discharged in rivers where water is derived for the production of drinking water. Because WWTPs have this important role to prevent the transmission of water-borne human enteric pathogens, fecal indicators and *E. coli* are monitored according to local regulations. However, almost no or very weak correlation can be found between faecal indicators or *E. coli* and viruses in the treated effluents (Osuolale and Okoh, 2017), indicating the need for the assessment of wastewater quality in terms of viral contamination.

To control the viral and pathogen transmission in the environment, the secondary-treated wastewater undergoes a final disinfection treatment before being discharged to the receiving water bodies. There is evidence that CoVs are less resistant to disinfection than enteric viruses - such as adenoviruses, norovirus, rotavirus, and hepatitis A - for which a wide literature exists in WWTPs (Simmons and Xagoraraki, 2011; Ye et al., 2016; Gundy et al., 2009). Disinfection of wastewater is commonly applied using liquid chlorine, sodium hypochlorite, chlorine dioxide, and UV light (Wang et al., 2020a), all expected to effectively denature human CoVs in wastewater, due to their fragile envelope (Gundy et al., 2009). Because of the genetic similarities, these disinfection technologies are effective also against SARS-CoV-2 (García-Ávila et al., 2020).

In Randazzo et al. (2020b), the persistence of SARS-CoV-2 was tested in some WWTPs after disinfection with NaClO and a combination of NaClO and UV (but without specifications about dosages, contact times). Although 2/18 samples of secondary-treated wastewater tested positive, none (0/12) of the tertiary-treated and disinfected samples tested positive (Randazzo et al., 2020b), as summarised in Table 1, confirming the efficacy of the disinfection implemented in the WWPTs against SARS-CoV-2.

The selection of a disinfectant depends on various factors such as investment and cost of operation, safety, flow rate, availability and level of operation management (Mandal et al., 2020). Other disinfection strategies such as solar irradiation (Chauhan, 2020) or heat (Kampf et al., 2020) have been proposed but further research is needed for their efficiency in water and economic sustainability.

4.4.1. *Chlorine-based disinfectants*

CoVs, being enveloped viruses, are generally more sensitive to chlorine than non-enveloped viruses and thus faster inactivated (Wang et al., 2005a). This fact is well known, considering that chlorine-based disinfectants (e.g. bleach) are commonly used for cleaning surfaces and to prevent the spread of SARS-CoV-2. The mechanism of chlorine dioxide against CoVs is based on the denaturation of some proteins, such as tryptophan and tyrosine and cysteine residues and when chlorine dioxide reacts with them the virus inactivation results very rapid (Ogata, 2007; Kály-Kullai et al., 2020).

Wang et al. (2005a, 2005b) investigated the effect of sodium hypochlorite (NaClO) and chlorine dioxide (ClO_2) at various concentrations (5–40 mg/L) and contact times on the inactivation of SARS-CoV-1 in wastewater and other matrices. Free chlorine was found to inactivate SARS-CoV better than chlorine dioxide (Wang et al., 2005a). Free residue chlorine >0.5 mg/L for chlorine or 2.19 mg/L for chlorine dioxide permits to obtain the complete inactivation of SARS-CoVs in wastewater (Wang et al., 2005a).

It is interesting to compare the inactivation of CoVs with *Escherichia coli*. Under the same experimental conditions, *Escherichia coli* presented always a lower inactivation rate with both NaClO and ClO_2 (Wang et al., 2005a, 2005b), indicating that dosages applied for disinfection of *E. coli* surpass largely those required for CoVs.

These observations are confirmed at full-scale: effluents tested were always negative for SARS-CoV-2 RNA after chlorination in the studies of Sherchan et al. (2020), Randazzo et al. (2020b), and Arora et al. (2020).

To limit the spread of SARS-CoV 2 through wastewater, some countries have mandated to strengthen disinfection processes in WWTPs, through increased use of chlorine (Zambrano-Monserrate et al., 2020).

With regards to the use of excessive chlorination, a drawback is associated with the production of disinfection by-products (DBP) that pose some ecological risks to the receiving water bodies and human health (Zhang et al., 2020). In the environment in general, an excess of chlorine effluent from WWTPs can be a risk for the ecosystem and chlorine can react with organic matter producing halogenated organic compounds, which are toxic for aquatic organisms.

4.4.2. *Peracetic acid*

Peracetic acid, or peroxyacetic acid (PAA), is a strong oxidant that produces reactive oxygen species able to disrupt cell membranes in bacterial pathogens, but variably efficient against viruses (Kumar et al., 2020). Despite the mechanism of PAA is not yet clear, the action of PAA is to modify viral proteins of the envelope and the capsid, which suggests a lower resistance of enveloped viruses than non-enveloped viruses. As far as we know, in the studies about the presence of SARS-CoV-2 in WWTPs, only Rimoldi et al. (2020) considered two plants where disinfection with peracetic acid was implemented. In this study, all disinfected effluents tested negative for SARS-CoV-2.

4.4.3. *Ozone*

Ozone is composed of three atoms of oxygen and must be produced on-site due to its short half-life at room temperature. Ozone is a powerful oxidant that is injected into water as gas and inactivates viruses leading to spontaneous oxygen gas formation. Enveloped viruses such CoVs are more sensitive to ozone than non-enveloped viruses because ozone interacts with the envelope composed of a lipid bilayer (Dev Kumar et al., 2020). With regards to SARS-CoV-2, further research is needed about the disinfection with ozone to determine the efficacy of commercial devices and the minimum ozone CT values (residual concentrations and contact time) to meet the required $\log_{10}$ virus reduction. The current knowledge about the efficacy of ozone for the inactivation of other viruses suggests that ozone is likely to be highly effective at inactivating SARS-CoV-2 in water (Morrison et al., 2021). This statement derives from many studies which demonstrated that ozone at low CT was effective at rapidly inactivating various types of viruses in wastewater or potable water with reductions of 4-$\log_{10}$ (Morrison et al., 2021).

4.4.4. *Ultraviolet light*

Ultraviolet irradiation at a wavelength of 100–280 nm (UV–C) targets the viral genomes, causing pyrimidine dimers and breakage in nucleic acids, affecting thus the viral replication (Dev Kumar et al., 2020; Shirbandi et al., 2020). However, the mechanism of inactivation of viruses in wastewater with UV has not been completely understood in the literature (Ye et al., 2018).

Ye et al. (2018) indicated that enveloped and nonenveloped viruses were characterised by comparable inactivation kinetics by UV_{254}, because inactivation with UV_{254} is based on a reaction with the genome. UV irradiation was proven to be effective against CoVs, considering that CoVs have one of the largest single-stranded RNA genomes (26–32 kb) among RNA viruses and the inactivation rate increases with the length of the RNA transcript.

Irradiation of UV for 1–2 min on SARS-CoVs in culture medium destroyed viral infectivity (Ansaldi et al., 2004). Compared to chlorine-based disinfectants, the inactivation rate of enveloped viruses with UV is much lower than with free chlorine (Ye et al., 2018). However, UV irradiation has the advantage of a lower amount of by-products than chlorine.

5. Fate through the stages of the sludge treatment line

Primary and secondary sludge separated from the wastewater treatment line are sent to the so-called sludge line, aimed at reducing water content (through thickening and dewatering) and degrading organic matter (using aerobic stabilisation or anaerobic digestion). In some WWTPs, stabilisation of sludge is completed with drying or chemical treatment by the addition of lime. According to US-EPA, biosolids in Class A are pathogen-free and can be used for gardening, while Class B biosolids may contain some pathogens included viable viruses, both enveloped and non-enveloped (i.e. CoVs).

A fraction of CoVs in raw wastewater may adsorb on solids, and thus they can accumulate in the sludge (Kitamura et al., 2021; Mohan et al., 2021). The presence of SARS-CoV-2 was investigated in 2 samples of primary sludge and 7 samples of secondary sludge produced in biological stages with sludge retention time of 12–26 days (Kocamemi et al., 2020). All samples tested positive and CT values of sludge samples were 33.5–35.9. Titers of SARS-CoV-2 were in the range of 1.17×10^4 - 4.02×10^4 GU/L, with similar values among primary and secondary sludge.

Available data on the detection of SARS-CoV-2 in primary and secondary sludge is collected in Table 2.

Due to the long time passed in the sludge line - that can range from some days to weeks - a certain inactivation of CoVs is expected before the final disposal of sludge. Moreover, at the high temperatures required for thermophilic digestion (55 °C) or thermal treatments (>100 °C), complete inactivation of SARS-CoV-2 can be obtained (Bardi and Oliaee, 2021) as presented in detail in section 5.2.

During the removal and transportation of primary and secondary sludge from a stage to another, viral particles may deposit on fomites or surfaces and enter in contact with operators that should wear proper PPE (Yang et al., 2020).

5.1. *Thickening and dewatering*

Thickening and dewatering (belt presses, centrifuges, filter presses) are physical or mechanical units used to reduce the moisture content of sludge.

Sludge thickener was proposed as a suitable spot for the sampling of sludge for the detection of SARS-CoV-2 aimed at WBE application (Balboa et al., 2020). Considering that enveloped viruses have an affinity towards biosolids, it was considered that the concentration of SARS-CoV-2 genetic material in the sludge can be higher than in wastewater. The concentration of SARS-CoV-2 may increase in the thickeners also as a consequence of the relatively long retention time of about 24 h (longer than in primary settlers which is about 1–2 h) and the high solid content in the thickened sludge (Balboa et al., 2020). However, such a long retention time in the thickener can affect the WBE due to: (i) the reduced amount of virus that can be found in the thickened sludge due to the low stability of RNA over time; (ii) the delay in the generation of thickened sludge with respect to the influent flow rate; (iii) the lack of information on the daily loads that are calculated considering 24-h samples and the corresponding 24-h flow rate.

Belt presses and filter presses are open devices that may cause direct exposure of operators to the viral particles during the management of the sludge. Centrifuges instead are closed and thus minimize the production of aerosol and droplets. The dewatering unit could be another point of exposure route for workers in WWTPs (Amoah et al., 2020). QMRA was performed by Westrell et al. (2004) considering various types of viruses and bacteria (rotavirus, adenovirus, hemorrhagic *E. coli*, *Salmonella* spp.). In this study, the highest individual health risk from a single exposure was caused by the aerosols produced from the belt press during the sludge dewatering (Westrell et al., 2004). The risk of viral transmission must be managed using appropriate PPE to reduce exposition to infectious viral particles (see section 6).

5.2. *Digestion*

When thickened sludge undergoes the treatment in aerobic stabilisation or anaerobic digestion, the sludge retention time is prolonged by a period of 1–2 weeks. This relatively long retention time contributes to the progressive natural decay of CoVs and SARS-CoV-2. However, a part of these viruses may survive and maintain infectivity, especially at low

Table 2
Available data on the detection of SARS-CoV-2 in various types of sludge and along the sludge treatment line in WWTPs.

Reference	WWTPs	Concentration methods	Primary sludge	Secondary sludge	Thickened sludge	Digested sludge
Kocamemi et al. (2020)	7 WWTPs in Turkey	PEG adsorption	All (2/2) samples positive 1.25×10^4 - 2.33×10^4 GU/L	All (7/7) samples positive 1.17×10^4 - 4.02×10^4 GU/L		
Balboa et al. (2020)	1 WWTP in Spain	Centrifugation and PEG precipitation	4/5 samples positive 10^4 - 4×10^4 GU/L	1/10 samples positive 7.5×10^3 - 10^4 GU/L	9/10 samples positive $<7.5 \times 10^3$ - 2×10^4 GU/L	Thermal hydrolysis and anaerobic digestion All (10/10) samples negative
Peccia et al. (2020)	1 WWTP in the USA		All samples positive 1.7×10^6 - 4.6×10^8 GU/L			

temperatures and thus, for a precautionary approach, proper PPE must be used by the personnel involved in the digested sludge management.

Anaerobic digestion of sludge can be applied at mesophilic temperatures in the range of 35–37 °C, or thermophilic temperatures in the range of 50–55 °C (Ahring et al., 2002). Studies on the effect of temperature on SARS-CoV-2 specifically contained in the sludge are not available. However, information can be derived from studies in which SARS-CoV-2 inactivation was investigated in other matrices or in the context of laboratory analyses of wastewater where pasteurization is necessary for reducing the risk for technicians.

High temperatures contribute to disrupting the envelope and consequently enveloped viruses such as CoVs can be inactivated (Chan et al., 2011). With regards to the thermophilic field, infectivity of SARS CoV-1 was lost at a temperature of 56 °C for 15 min (Chan et al., 2011). Some protocols for the detection of SARS-CoV-2 in wastewater use thermal inactivation, which is performed by heating the samples at 56 °C for 30 min (La Rosa et al., 2020a) or at 60 °C for 90 min (Arora et al., 2020; Wu et al., 2020a). These approaches confirm that the effect of heating contributes to a significant inactivation of CoVs and SARS-CoV-2, with more efficiency in the thermophilic field. It is worth noting that despite the virus can be effectively inactivated at those temperatures, the viral RNA is preserved. Therefore, sludge after thermophilic digestion could be tested positive for SARS-CoV-2 using real-time RT-PCR methods, even in presence of an effective heat inactivation.

Mesophilic temperatures are not so efficient in the inactivation of SARS-CoV-2 that remained stable at 37 °C for at least 24 h (Wang et al., 2020b). The sludge applied and treated in the mesophilic anaerobic digestion of 5 WWTPs was investigated by Bibby and Peccia (2013). The influent sludge was a mixture of primary and secondary sludge, while digested sludge was collected before dewatering. CoVs were present in 80% of samples (all samples of influent sludge and 60% of samples of digested sludge) and were the second most prevalent type of RNA virus. This study highlighted that respiratory viruses may be prevalent in sludge, in addition to those which are transmitted by ingestion, and this suggests the potential transmission due to aerosol exposure in the management of biosolids.

5.3. *Sludge disposal*

The investigation of Bibby et al. (2011) indicated that CoVs were among the most abundant human viruses in the biosolid samples tested. In particular, Bibby et al. (2011) tested Class B biosolid generated from primary and secondary sludge treated in mesophilic anaerobic digestion (HRT of 15 d) followed by dewatering to 17% solid content.

Biosolids generated in the WWTPs are generally recycled or disposed of by composting, landspreading, incineration, or landfilling. The reuse for agricultural land application permits to exploit useful nutrients for crops. About half of the enteric viruses present in raw wastewater may accumulate in the sludge (Simmons and Xagoraraki, 2011). Beyond enteric viruses, the dewatered sludge may potentially contain CoVs and SARS-CoV-2, depending on the types of sludge treatments implemented in the plant. However, even if SARS-CoV-2 can be present in these matrices, the fecal-oral transmission route of SARS-CoV-2 in the biosolids from WWTPs has not yet been demonstrated.

The recycling and disposal of the residual biosolids from WWTPs could expose operators in close contact with the biosolids to the viruses if remained infective after the sludge treatment and thus proper PPE is always advised (Yang et al., 2020).

6. Aerosolization in WWTPs

Bioaerosols are emitted at various points of a WWTP, but mainly through mechanical mixing or aeration in secondary treatment, especially when surface turbines are used (Balboa et al., 2020) and in many procedures of sludge treatment such as dewatering (Yang et al., 2020).

The scarcity of data about the quantification of SARS-CoV-2 in aerosols from WWTPs is the major limitation to understand the most relevant points and afford a risk analysis. As far as we know, the first study about the risk of COVID-19 infection derived from exposure to aerosols of WWTPs was published by Gholipour et al. (2021). In this study, the viral RNA of SARS-CoV-2 was detected in 40% (6/15) of air samples collected in the WWTP during a period of high prevalence of COVID-19 in the region. The highest concentration of SARS-CoV-2 RNA was detected in the pumping station (Gholipour et al., 2021).

van Doremalen et al. (2020) indicated that SARS-CoV-2 remained viable in aerosols for 3 h, and the half-live (that is the time needed to halve the amount of the virus) was approximately 1.1 h. This result was comparable to that observed for SARS-CoV-1, confirming an analogous behavior among these two types of CoVs (van Doremalen et al., 2020). However, in the laboratory experiments of Fears et al. (2020), SARS-CoV-2 was able to produce viral bioaerosols that may remain infectious over longer periods via airborne transport.

Aerosol formation in WWTPs is an aspect to be considered for the protection of the employees involved in wastewater management operations. Personal and collective protective equipment (PPE and CPE) are indicated to protect WWTPs workers during routine manual cleaning, sampling or analyses of wastewater, and inspection or supervision of plants (Zaneti et al., 2021). Regarding CPEs, treatment tanks should be covered where possible, or equipped with splash barriers to avoid splashing and sprays of sewage in the mouth or on the face (Zaneti et al., 2021). Appropriate PPE is protective outerwear, face shield, face masks, safety googles, gloves, liquid-repellent work clothing and boots as recommended by the World Health Organization (WHO, 2020) and Water Environment Federation (WEF, 2020).

7. Loss of sewage: CSO, exfiltration, and poor sanitation

Raw wastewater may reach receiving water bodies or public areas causing serious pollution in the cases of: (i) combined sewer overflows (CSO) during stormwater events when the flow rate surpasses the capacity of the network; (ii) faults and leakage from sewage pipes or connections; (iv) WWTPs not working adequately; (iii) lack of adequate sanitation infrastructure (lack of WWTPs, open sewers). In these cases, faecal material and associated CoVs may reach natural water bodies (Al Huraimel et al., 2020). Exposure to raw wastewater may pose a higher risk than exposure to treated effluents because wastewater is fresh and thus viral loads and infectivity may still be high (Amoah et al., 2020).

Viral loads of SARS-CoV-2 were detected in river streams contaminated with raw municipal wastewater (Guerrero-Latorre et al., 2020; Rimoldi et al., 2020). In the study of Rimoldi et al. (2020) the presence of SARS-CoV-2 RNA was attributed to the discharge of non-collected domestic wastes or urban runoff from domestic wastewater. Despite the detection of SARS-CoV-2 RNA, no viable viral particles were found in this study based on cell cultures (Rimoldi et al., 2020). Despite the transmission of SARS-CoV-2 via sewage or wastewater systems has not been proven yet, the preliminary nature of data reported in the literature suggests the need for further research in this field.

8. Conclusions

WWTPs play an essential public health service and recently, during the ongoing COVID-19 pandemic, their role in the reduction of SARS-CoV-2 from raw wastewater has been highlighted. Insufficient sanitation or inadequate wastewater management may pose potential risks of fecal-oral or fecal-air transmission. The present work reviews the fate of SARS-CoV-2 in WWTPs composed of pre-treatments, primary, secondary, and tertiary treatments as well as sludge treatment and disposal. This analysis focuses on the various physical, chemical, biological processes implemented in WWTPs that contribute to the removal/inactivation of SARS-CoV-2 entered with the influent raw wastewater. This study provides the groundwork for further research on SARS-CoV-2 inactivation in WWTPs; in fact, further efforts are needed to understand in depth the fate of SARS-CoV-2 and the quantitative data about reduction. This knowledge is a prerequisite to predict the quality of the treated effluents and surplus sludge and their impact on reuse and public health.

Secondary and tertiary treatments are efficient in removing SARS-CoV-2 reducing the risk associated with wastewater. Discrepancies may exist among published articles because the analytical methods are not the same and ongoing. Based on recent literature, there is no evidence of the survival of SARS-CoV-2 in disinfected effluents from WWTPs when chlorine-based disinfectants (NaClO, ClO_2) or UV lamps are used. Therefore WWTPs may play a medium-term role to reduce the risk of virus shedding during the ongoing pandemic and in case of future new waves.

Author contributions

Paola Foladori: Conceptualization, Data curation, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. Francesca Cutrupi: Conceptualization, Data curation, Methodology, Resources, Writing – original draft, Writing – review & editing. Maria Cadonna: Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing. Serena Manara: Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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