Science of the Total Environment 881 (2023) 163453

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

SARS-CoV-2 in the environment: Contamination routes, detection methods, persistence and removal in wastewater treatment plants

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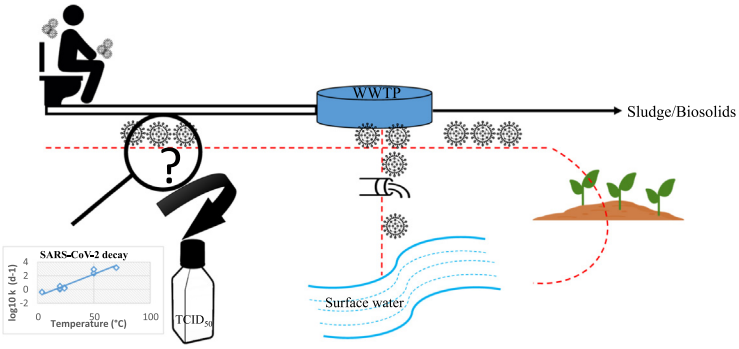
HIGHLIGHTS

- Lack of evidence for the presence of infectious SARS-CoV-2 in environmental samples
- Fecal-oral transmission of SARS-CoV-2 associated with wastewater is highly unlikely.
- Decay of SARS-CoV-2 is higher in wastewater and lowest in water matrices.
- SARS-CoV-2 RNA is detected in sludge but removed in thermophilic sludge treatments.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damia Barcelo

Keywords:
SARS-CoV-2
Detection methods
Wastewater
Wastewater treatment plants
Decay
Fate

ABSTRACT

The present study reviewed the occurrence of SARS-CoV-2 RNA and the evaluation of virus infectivity in feces and environmental matrices. The detection of SARS-CoV-2 RNA in feces and wastewater samples, reported in several studies, has generated interest and concern regarding the possible fecal–oral route of SARS-CoV-2 transmission. To date, the presence of viable SARS-CoV-2 in feces of COVID-19 infected people is not clearly confirmed although its isolation from feces of six different patients. Further, there is no documented evidence on the infectivity of SARS-CoV-2 in wastewater, sludge and environmental water samples, although the viral genome has been detected in these matrices. Decay data revealed that SARS-CoV-2 RNA persisted longer than infectious particle in all aquatic environment, indicating that genome quantification of SARS-CoV-2 does not imply the presence of infective viral particles. In addition, this review also outlined the fate of SARS-CoV-2 RNA during the different steps in the wastewater treatment plant and focusing on the virus elimination along the sludge treatment line. Studies showed complete removal of SARS-CoV-2 during the tertiary treatment. Moreover, thermophilic sludge treatments present high efficiency in SARS-CoV-2 inactivation. Further studies are required to provide more evidence with respect to the inactivation behavior of infectious SARS-CoV-2 in different environmental matrices and to examine factors affecting SARS-CoV-2 persistence.

Contents

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http://dx.doi.org/10.1016/j.scitotenv.2023.163453
Received 30 December 2022; Received in revised form 7 April 2023; Accepted 7 April 2023
Available online 12 April 2023

1. Introduction

SARS-CoV-2, a virus belonging to the subgenus Sarbecovirus of the genus Betacoronavirus in the *Coronaviridae* family, is the causative agent of the pandemic coronavirus disease 2019 (COVID-19) which is the most global health crisis since the era of the influenza pandemic of 1918 (Cascella et al., 2022). It has a positive sense single- stranded RNA genome enclosed within a protein capsid coated with a bilayer lipid envelope. The genome of ~30,000 nucleotides contains four structural proteins, that include envelope (E), nucleocapsid (N), membrane (M), and spike (S), and 25 nonstructural proteins (Feng et al., 2020). According to the World Health Organization data, as of 28 December 2022, over 649 million confirmed cases and 6.6 million deaths have been reported worldwide (WHO, 2022).

Although SARS-CoV-2 is predominantly a respiratory virus, it can cause gastrointestinal symptoms such as nausea, abdominal pain, vomiting and diarrhea, with the latter being the most frequent (Moura et al., 2022; Lin et al., 2020). It has been demonstrated that people infected with SARS-CoV-2 shed the virus in feces in addition to saliva, nasopharyngeal secretions, sputum (Ahmed et al., 2020c; Cevik et al., 2021) and to lesser extent in urine (Jeong et al., 2020), which are collected in sewerage. Accordingly, numerous studies have reported the presence of SARS-CoV-2 genome in human feces (Wölfel et al., 2020; Wang, et al. 2020a; Xiao et al., 2020; Yong et al., 2020), where approximately 50 % of COVID-19 patients shed fecal RNA in the week after infection and 4 % of patients shed fecal viral RNA up to 10 months after diagnosis (Natarajan et al., 2022; Zhou et al., 2022; Zhang et al., 2021). Further, SARS-CoV-2 genome has been detected throughout different processes of WWTPs, where the viral RNA was found not only in WWTP effluent, primary sludge and secondary sludge, but also in some treated sludge (biosolids) (Foladori et al., 2022). Besides, the occurrence of SARS-CoV-2 genome has also been reported in surface water, sediments and aquatic biota (Rimoldi et al., 2020; Guerrero-Latorre et al., 2020; Kolarević et al., 2021; Tandukar et al., 2022; Polo et al., 2021; Mancusi et al., 2022; Yang et al., 2022).

Following the detection of SARS-CoV-2 RNA in water bodies, the risk of virus transmission to human via the water route was discussed and the key question is whether the detected SARS-CoV-2 is infectious or not?

In fact, the positive detection of SARS-CoV-2 RNA does not provide evidence on the infectivity of the virus in these environmental samples and the possible oral-fecal transmission, because the presence of fragments of viral genome in environmental sample does not necessarily imply that the virus is structurally intact and viable (Bivins et al., 2020; Foladori et al., 2022; de Oliveira et al., 2021; Ahmed et al., 2020a). Cell culture assay remains the only method used to assess the infectivity of such samples as it provide the more reliable information to evaluate the risk of transmission of SARS-CoV-2 in the environment.

To date, viable cases of SARS-CoV-2 have been reported in the feces of only six different patients (Xiao et al., 2020; Zhang et al., 2020; Dergham et al., 2021; Wang et al. 2020b). Moreover, no cases of infection through contact with fecally contaminated samples have been reported and the few published studies revealed the absence of infectious SARS-CoV-2 in environmental samples.

The small number of studies addressing the infectious potential of SARS-CoV-2 in environmental samples may be due to the limited access to biosafety level 3 laboratories to work with SARS-CoV-2 in cell culture assays (CDC, 2021) and to the presence of toxic compounds and microorganisms, which would hinder cell culture assay (Monteiro et al., 2022).

Persistence and decay data are necessary to evaluate the infectivity risk of the detected virus in wastewater, sludge, biosolids, and other environmental matrices. Actually, there is still little knowledge about the infectivity of this virus in these matrices. However, inactivation and decay studies demonstrated that SARS-CoV-2 RNA persisted longer than infectious viruses when seeded in wastewater, surface water and seawater.

The objective of this review was to (i) provide an overview on the environmental contamination with SARS-CoV-2; (ii) discuss on the applicability and limitations of the relevant methods used for its detection in the environmental samples; (iii) collect the available decay data of infectious SARS-CoV-2 and its genome in different aquatic environments and discuss the parameters influencing its persistence; and (iv) present the efficiency of different treatments in WWTPs on the removal of SARS-CoV-2.

2. SARS-CoV-2 in the environment and potential contamination modes

SARS-CoV-2 is an airborne virus whose transmission routes involve human-to-human that occurs mainly by aerosol droplets released from the infected person's mouth and nose (Patel et al., 2021). High viral loads have been found in the respiratory tract of infected individuals whose can shed the virus and its genetic material via their sputum, nasopharyngeal secretions and saliva (Patel et al., 2021; Cevik et al., 2021).

It has been demonstrated that SARS-CoV-2 RNA can be shed from people infected with COVID-19 (Wang et al., 2020b; Wölfel et al., 2020; Xiao et al., 2020; Yong et al., 2020; Kim et al., 2020). Fecal shedding of SARS-CoV-2 RNA was also observed in asymptomatic individuals and it was reported that the duration of fecal RNA shedding persisted from 1 to 50 days following the complete resolution of symptoms (Foladori et al., 2020; Park et al., 2021; van Doorn et al., 2020; Gupta et al., 2020). Since SARS-CoV-2 RNA can be shed in the feces of individuals with symptomatic or asymptomatic infection, it can be discharged into the sewerage to the central WWTP (Fig. 1) and hence could be transmitted to the environment by several routes (Wölfel et al., 2020; Foladori et al., 2020).

Treated wastewater, such as discharged secondary effluents, may release SARS-CoV-2 RNA into the aquatic ecosystems (Fig. 1) and, in particular surface water systems (e.g. rivers, lakes, seawater, ponds and estuaries) and then to sediments, bivalve shellfish and aquatic biota (Bosch et al., 2006; Polo et al., 2021; Le Guernic et al., 2022).

Moreover, sewer overflows caused by heavy rainfalls, leakage from sewage network systems like sewers and septic tanks can act as the source of viral contamination to the surface water (Fig. 1) (Bernard et al., 2022). Additionally, poor treatment at WWTPs, or lack of complete infrastructure in some countries are factors that can result in SARS-CoV-2 environmental contamination (Bogler et al., 2020).

In WWTPs, a partial accumulation of SARS-CoV-2 may take place in the separated solids due to its hydrophobic properties. Sewage sludge (solids) generated in the WWTPs is usually treated before disposal or recycling. Once treated, biosolids can be recycled or disposed of using three main routes: recycling to agriculture via landspreading, incineration or landfilling (Li et al., 2021). In this regards, surface water may also be contaminated through stormwater runoff from agricultural soil (Bernard et al., 2022). Further, it has also been proposed that groundwater may become contaminated with SARS-CoV-2 from a sewage sludge landfill (Anand et al., 2022), through agricultural soils, or from fecally contaminated surface water (Fig. 1).

Despite the detection of SARS-CoV-2 RNA in various environmental matrices in different studies (as shown by bold arrow in Fig. 1), infectivity of SARS-CoV-2 was not detected (Rimoldi et al., 2020; Westhaus et al., 2021) or not investigated in these matrices. However, based on the this evidence and the efficacy of most WWTPs in virus reduction, Ahmed et al. (2021); Albert et al. (2021); Sobsey (2022); Cerrada-Romero et al. (2022) asserted that fecal-oral transmission of SARS-CoV-2 associated with wastewater is likely to be low compared to well-documented person-to-person transmission via respiratory droplets/aerosols.

3. Detection methods of SARS-CoV-2 in wastewater

Most environmental monitoring of SARS-CoV-2 to date uses quantitative PCR-based methods to detect viral RNA. Given the low concentrations of SARS-CoV-2 RNA in wastewater (concentrations ranged between 20 GC/L and 3×10^6 GC/L), several concentration/enrichment protocols, such as ultracentrifugation, ultrafiltration, electronegative membrane filtration and precipitation with polyethylene glycol (PEG), have been developed and applied before viral RNA extraction (Ahmed et al., 2020b; Kumblathan et al., 2021; Ahmed et al., 2022). SARS-CoV-2 RNA detection and quantification is then performed using reverse transcription quantitative real-time PCR (RT-qPCR) based on a calibration curve, or reverse transcription digital PCR (RT-dPCR) without the necessity for a calibration curve (Kumblathan et al., 2021; Ahmed et al., 2022). Several gene targets specific to the SARS-CoV-2 have been used in molecular assays including a combination of structural (i.e., envelope (E), nucleocapsid (N) and spike (S)) and/or non-structural (i.e., ORF1ab, RdRp) genes in simplex or multiplex formats (Corman et al., 2020). However, data in the literature underline the absence of information regarding the SARS-CoV-2 recovery efficiency of different concentration methods and the lack of method standardization. This, is turn, highlights the challenge that need to be addressed to obtain accurate quantification, especially when low viral RNA quantities are present in the environmental samples. Another limiting factor for SARS-CoV-2 RNA detection is that wastewater contains a wide range of PCR-inhibitors such as proteins, fats, carbohydrates, polyphenols, metal ions, and RNAses (Ahmed et al., 2022) that can affect the proper PCR amplification and can also give false negative results (Foladori et al., 2021).

Nonetheless, quantitative PCR-based methods do not provide information on the presence of the infective viable virus in wastewater because viral genome detection does not necessarily indicate the presence of infective viable virus (Foladori et al., 2022). A new method referred as viability RT-qPCR or capsid integrity was employed to assess SARS-CoV-2 infectivity in environmental samples (Desdouits et al., 2021; Polo et al., 2021; Monteiro et al., 2022; Cuevas-Ferrando et al., 2021). This technique combines the use of photoactivatable dye pretreatment, such as ethidium monoazide (EMA), propidium monoazide (PMA) or platinum chloride ($PtCl_4$), with qPCR. These molecules penetrate only damaged or destroyed capsids where they

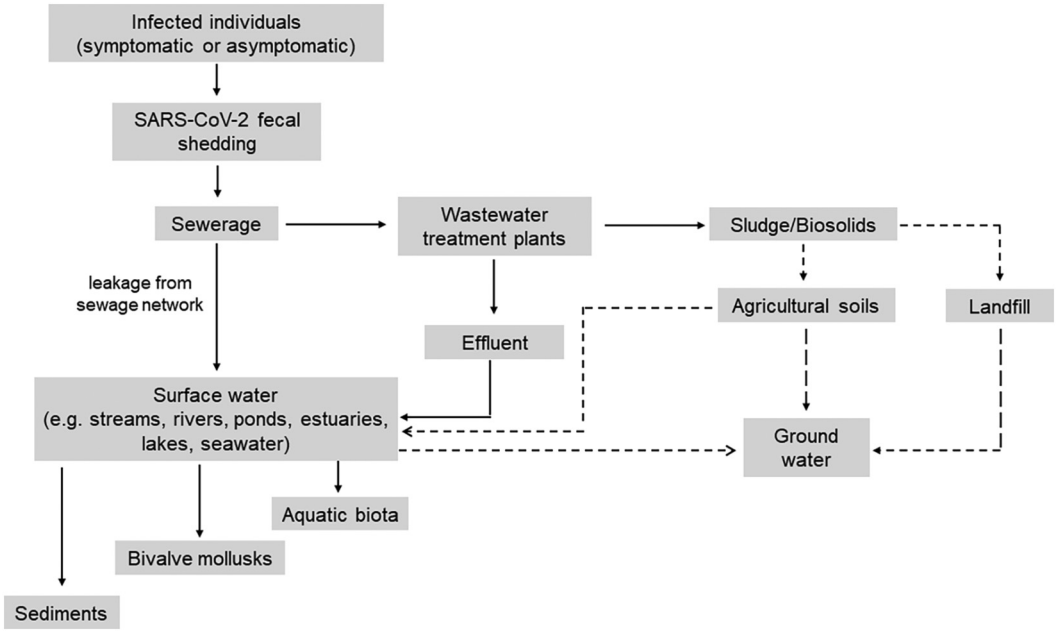

Fig. 1. Potential modes of environmental contamination by SARS-CoV-2. Dashed arrow indicates suspected contamination routes where no data has been collected.

intercalate covalently into viral genome RNA, interfering with PCR amplification (Elizaquível et al., 2014). However, the efficacy of such strategy is limited to many factors such as dye concentration, type of the light source, and incubation conditions (Leifels et al., 2021). Actually, robustness of these methods should be evaluated considering the diversity of wastewater characteristics and composition that could widely vary according to location and weather.

Cell culture has long been considered the gold standard approach for isolating infectious virus particles. Nevertheless, several factors make it difficult to use this method to determine the possible presence of SARS-CoV-2 particles in wastewater:

i) the requirement for a biosafety level 3 (BSL-3) laboratory for SARS-CoV-2 manipulation (CDC, 2021).
ii) the considerable costs and time needed to establish cell culture tests.
iii) the diversity of toxic compounds and micro-organisms resulting from wastewater concentration that are difficult to eliminate before sample inoculation, which would hinder cell culture assay (Monteiro et al., 2022).
iv) the variation in cell line behavior in response to infection by the SARS-CoV-2 virus. In this regard, Decimo et al. (2022) examined the behavior of Vero E6 / kidney cell line originating from monkeys using 4 sub clones from 4 different laboratories. In light microscopy, Vero E6 cells were grouped under 2 morphological phenotypes, the fibroblastic phenotype and the epithelial one. Both phenotypes varied in response to infection by the SARS-CoV-2. For instance, cells of fibroblast phenotype were detached between 48 and 72 h after infection and continuously produced virus at high titers ($> 10^6$ PFU/mL) without the cell layer being damaged and this type of cells could be used for cell line production. In contrast, the cells of the epithelial phenotype were partially or totally destroyed within 48 and 72 h and this type of cells could be used for $TCID_{50}$ or phage lysis assays. Transcriptomic analyzes carried out 24 h after infection confirmed these results (Decimo et al., 2022).
v) It is well known that Vero E6 kidney cell line is widely used in coronavirus research for virus stock propagation and antiviral assays. Although it does express the ACE2 receptor for SARS-CoV-2 attachment, it lacks the TMPRSS2 protease required for entry into human cells. Instead, viral entry into Vero E6 is likely cathepsin-mediated and may not accurately mimic the infection event in human cells (Mautner et al., 2022). Besides, Caco-2 cells, an intestinal epithelium cell line originating from humans, and Calu-3 cells, a pulmonary epithelium cell line also originating from humans seem to be preferential modeling cell lines. Different viral isolates replicate similarly in Caco-2 cells, but show very different replicative capacities in Calu-3 cells (de Souza et al., 2021).

4. Occurrence and infectivity evaluation of SARS-CoV-2 in feces and environmental matrices

4.1. Feces

SARS-CoV-2 RNA shedding by infected patients has been detected in feces at concentrations between 10^6 and 10^{10} genome copies per L of feces (GC/L). The detected levels varied according to the day of sampling post infection initiation (Wölfel et al., 2020; Foladori et al., 2020), where the highest levels were recorded during the first week of symptoms. Even though the detection of SARS-CoV-2 genetic signal in feces samples, the presence of infectious particles in these samples is not confirmed. In fact, studies have examined the presence of infectious SARS-CoV-2 in feces from infected individuals with contradictory results. Until date, viable SARS-CoV-2 has been reported in the feces of only six different patients (Table 1), but with no data on quantities of infectious SARS-CoV-2. Using the Vero E6 cell line, Xiao et al. (2020) were able to find infectious SARS-CoV-2 in 2 fece samples from one infected patient, while Wölfel et al. (2020) were unable to detect infectious SARS-CoV-2 in two separate laboratories by using the same cell line, despite the positive RT-qPCR tests with high RNA load. In another study, Jeong et al. (2020) failed to demonstrate the presence of viable SARS-CoV-2 in fecal samples using Vero cells, but viable SARS-CoV-2 was isolated from the nasal washes of ferret inoculated with one COVID-19 patient's stool. Additionally, Zhang et al. (2020) were able to observe viral particles with typical morphology of a coronavirus using electron microscopy after inoculating stool suspension into Vero cells (Table 1).

It is well known that other human coronaviruses, such as SARS-CoV-1 and Middle East respiratory syndrome coronavirus (MERS-CoV), are excreted in the stools of infected patients and remain viable under conditions that could facilitate fecal–oral transmission (Cuicchi et al., 2021), whereas, there is no current evidence showing that SARS-CoV-2 could also be transmitted via this route. Potential SARS-CoV-2 infection in the gastrointestinal tract has been discussed in regard to the intestine expression of ACE2 receptors required for SARS-CoV-2 infection and to the prolonged viral shedding (Giacobbo et al., 2021; Cerrada-Romero et al., 2022). Recently, Cerrada-Romero et al. (2022) evaluated the viability of the SARS-CoV-2 viral particles excreted in 79 stools sample collected from 62 adult COVID-19 patients. They showed that SARS-CoV-2 RNA was detected in stools samples from 27 (43.5 %) out of the 62 patients. However, SARS-CoV-2 replication, assessed by the generation of cytopathic effects followed by viral load quantification by RT-PCR assay, was not revealed in any of stool samples, suggesting that SARS-CoV-2 replicative capacity is null or very limited in stool samples, and thus, they suggested that the fecal–oral transmission of SARS-CoV-2 as an alternative infection route is highly unlikely. Furthermore, Zang et al. (2020) reported that SARS-CoV-2 entered the intestinal lumen was inactivated by simulated human colonic fluid, and hence the virus is likely to be inactivated before it is expelled.

4.2. Wastewater

The viral RNA concentrations measured in raw wastewater were at least 4 $\log_{10}$ unit lower than those detected in feces and varied between 20 GC/L and 3×10^6 GC/L (Ahmed et al., 2020a; Foladori et al., 2020). It was reported that this viral dilution is due to many factors such as the large domestic daily water consumption per person, the presence of rainwater and parasitic inflow in the sewerage, industrial discharges, and the limited percentage of COVID-19 positive persons in the community served by the WWTP (Foladori et al., 2022).

Due to the strict biosafety requirements for SARS-CoV-2 and the fast evolving COVID-19 pandemic, research has been concerned primarily on the detection of SARS-CoV-2 RNA in wastewater and only a few investigations have examined the potential presence of infectious SARS-CoV-2 in raw and treated wastewaters with negative results (Table 1). Rimoldi et al. (2020) used the RNA positive and negative raw and treated wastewater samples to inoculate Vero E6 cells. Infectivity was assessed daily by screening cells for cytopathic effects under reverse-phase light microscope. No infectious SARS-CoV-2 particles was detected in the analyzed samples.

Using the CaCo-2 cell line, the absence of infective viable SARS-CoV-2 was also obtained by Westhaus et al. (2021) in influent raw wastewater and in both effluent and tertiary treated effluent wastewater samples despite the presence of SARS-CoV-2 genome in all the analyzed samples.

Recently, Robinson et al. (2022) used 10 raw wastewater samples with SARS-CoV-2 RNA levels ranging from 16.9×10^4 to 3.255×10^6 GC/L to inoculate Vero E6 cells within one week from collection. The authors did not find infectious SARS-CoV-2 in the analyzed samples. Moreover, according to Monteiro et al. (2022), the detected SARS-CoV-2 RNA in secondary treated wastewater at concentrations up to 10^4 GC/L was found to be non-infectious in cell culture using Vero E6 for 5 days.

These results align with recent evidence suggesting that wastewater does not appear to be a be a route of SARS-CoV-2 transmission (Albert et al., 2021; Cerrada-Romero et al., 2022; Ahmed et al., 2021; Sobsey, 2022).

4.3. River water

There are only few studies that have examined the occurrence of SARS-CoV-2 in the receiving water systems. Rimoldi et al. (2020) surveyed three rivers (near Milano, Italy) during the epidemic peak outbreak in April 2020 (Table 2). They found SARS-CoV-2 RNA in all investigated river samples. However, the viral load was not reported by the authors. Because SARS-CoV-2 detection was correlated with caffeine detection in the river samples,

Table 1
Infectious SARS-CoV-2 evaluation in stool and wastewater samples.

Reference	Country	Type of samples	Cell culture assay	Results
(Xiao et al., 2020)	China	Stool	3 Positive stool samples for SARS-CoV-2 RNA obtained from 2 patients were tested for infectivity using the Vero E6 cell line	2 out of 3 were positive for infectious viral particles
(Wölfel et al., 2020)	Germany	Stool	Experiment conducted using the same cell line used by Xiao et al. (2020), in two separate laboratories	No infectious SARS-CoV-2 particles was detected despite the high viral RNA load detected by RT-qPCR
(Zhang et al., 2020)	China	Stool	Vero cells were used for viral isolation from stool samples of unreported number of COVID-19 patients The presence of SARS-CoV-2 was confirmed by electron microscopic observation	A virus particle with typical morphology of coronavirus was observed in one sample
(Jeong et al., 2020)	Republic of Korea	Stool	3 Positive qPCR samples were subjected to SARS-CoV-2 isolation in Vero cells. One fecal specimen was selected to experimentally infect ferret and then viable virus titres in nasal washes were checked on 2, 4, 6 and 8 days post infection	No cultures were positive, however viable SARS-CoV-2 was isolated from the nasal washes of the stool treated ferret
(Kim et al., 2020)	Republic of Korea	Stool	129 stool samples were tested for infectivity test using CaCo-2 cell line	No cultures were positive
(Wang et al., 2020a)	China	Stool	Four SARS-CoV-2 positive fecal specimens with high copy numbers were cultured to detect live virus. No details on cultivation method was reported	Viable SARS-CoV-2 was observed in the stool sample from 2 patients
(Albert et al., 2021)	Spain	Stool and sewage	Fecal sewage samples with highest RNA concentrations were used to inoculate Vero E6 cells	No cytopathic effect on Vero E6 cells was observed in any of the analyzed samples
(Dergham et al., 2021)	France	Stool	Vero cells were used for viral isolation from 106 stool samples of 46 COVID-19 patients	Viable SARS-CoV-2 was detected in 2 stool samples from 1 patient undergone a kidney transplant 21 years ago
(Cerrada-Romero et al., 2022)	Spain	Stool	Vero cells were used for viral isolation from 79 stools sample collected from 62 adult COVID-19 patients	No cultures were positive
(Rimoldi et al., 2020)	Italy	Raw and tertiary treated wastewater	SARS-CoV-2 infectivity in positive and negative samples for SARS-CoV-2 RNA was evaluated using Vero E6 cells	No infectious SARS-CoV-2 particles was detected in all samples
(Westhaus et al., 2021)	Germany	Influent raw wastewater, effluent and effluent after tertiary treatment	Infectivity of purified and concentrated influent and effluent samples (in both liquid and solid phases) were evaluated using differentiated Caco-2 cells	No infectious SARS-CoV-2 particle was detected in all samples
(Robinson et al., 2022)	USA	Raw wastewater	10 positive raw wastewater samples for SARS-CoV-2 RNA were tested for infectivity using Vero E6 cells	No cytopathic effects were obtained
(Monteiro et al., 2022)	Portugal	Secondary treated wastewater (effluents)	Positive secondary-treated wastewater samples for SARS-CoV-2 RNA were tested for infectivity using Vero E6 cells	No infectious SARS-CoV-2 viral particles was obtained despite the high viral RNA load detected by RT-qPCR

Table 2
Detection of SARS-CoV-2 RNA in river water and marine environment.

Reference	Country	Type of samples	Location	Target regions used for RT-qPCR detection	Concentration range (GC/L)
(Rimoldi et al., 2020)	Italy	River water	Lambro River, Vettabbia Canal, and Lambro Meridionale River in the two provinces of Milan and Monza and Brianza	Nucleocapsid (N), ORF1ab and E	Not reported
(Guerrero-Latorre et al., 2020)	Ecuador	River water	Quito's river	Nucleocapsid N1 and N2	2.91×10^5 to 3.19×10^6 GC/L (N1) 2.07×10^5 to 2.22×10^6 GC/L (N2)
(Kolarević et al., 2021)	Serbia	River water	Danube River	Nucleocapsid (N1 and N2) and Envelope (E)	5.96×10^3 to 1.30×10^4 GC/L
(Tandukar et al., 2022)	Nepal	River water	Bagmati River	CDC-N1, CDC-N2, NIID_2019-nCOV_N, and N_Sarbeco	4–5.1 $\log_{10}$ GC/L
(Yang et al., 2022)	China	River water	Beijing, China	E_Sarbeco, CDC_N1	9.7×10^1 and 9.52×10^2 GC/L
(Polo et al., 2021)	Spain	Bivalve mollusks: *Ruditapes philippinarum* and *Ruditapes decussatus* Marine sediments	Estuaries and river catchment in Galicia (NW of Spain)	IP4, E and N1	Below limit of quantification to 4.48 $\log_{10}$ GC/g of digestive tissue Below limit of quantification to 3.60 $\log_{10}$ GC/g of sediment
(Mancusi et al., 2022)	Italy	Bivalve mollusks (*Mytilus galloprovincialis)*	Coastal sites from Gulf of Naples (Campania region, Italy).	Orf1b nsp14, RdRp and E	7.8×10^1 to 2.6×10^3 GC/g (Orf1b nsp14) 7.2×10^1 to 4.9×10^3 GC/g (RdRp) 1.3×10^2 to 5.0×10^2 GC/g (E)
(Yamazaki et al., 2022)	Japan	Cultivated oysters (*Crassostrea gigas*)	Kyoto Hiroshima, Okayama, Hyogo and Yamaguchi prefectures, Japan	Nucleocapsid N2	SARS-CoV-2 was not detected in the analyzed oysters samples
(Ransome et al., 2023)	UK & Serbia	Cultivated oysters (*Crassostrea gigas*), river water and sediments	The River Thames, UK Sava and Danube rivers, Serbia	Nucleocapsid N1 and E genes	None of the collected samples were positive for the N1 or E gene, and no infectious SARS-CoV-2 was recovered from any of these samples

they related viral presence to untreated or ineffectively treated wastewater discharged into surface waters. Infectivity of positive RNA samples was evaluated by screening cells for cytopathic effects under reverse-phase light microscope. No infectious SARS-CoV-2 was detected in all positive samples.

Guerrero-Latorre et al. (2020) examined the presence of SARS-CoV-2 RNA in rivers from urban streams in Quito, Ecuador, where wastewater is discharged directly into receiving waters (Table 2). SARS-CoV-2 RNA detected in the analyzed samples ranged from 2.91×10^5 to 3.19×10^6 GC/L using N1 assay and from 2.07×10^5 to 2.22×10^6 GC/L using N2 assay. The higher SARS-CoV-2 RNA concentration was recorded during COVID-19 peak. A study conducted in Serbia to detect the SARS-CoV-2 RNA in Danube River showed that RNA viral load in the analyzed sampling sites ranged from 5.96×10^3 up to 1.30×10^4 GC/L (Table 2). SARS-CoV-2 genome detection was associated with the discharge of untreated wastewaters (Kolarević et al., 2021) and no infectious virus was recovered in any environmental samples. Tandukar et al. (2022) detected SARS-CoV-2 RNA in 9/13 river water samples collected from the Bagmati River in Nepal (Table 2). The mean concentration of the viral RNA ranged from 4 to 5.1 $\log_{10}$ GC/L according to the RT-qPCR. In another study, 9 river samples were taken from 3 locations from upstream to downstream of a river in Beijing, China (Yang et al., 2022). Samples were collected 17 days before to 19 days after the end of the second wave of the COVID-19 epidemic. Results showed that SARS-CoV-2 RNA was detected in 9 river samples and concentrations ranged between 9.7×10^1 and 9.52×10^2 GC/L (Table 2). In Argentina, La Caldera, Mojotoro, and Arenales Rivers were monitored for the presence of SARS-CoV-2 (Maidana-Kulesza et al., 2022). SARS-CoV-2 RNA was found in about half of samples in low concentrations in La Caldera and Mojotoro Rivers, while it was high in Arenales River (concentrations between 10^6 and 10^7 GC/L).

Recently, river water samples spiked with infectious SARS-CoV-2 showed that infectious SARS-CoV-2 inoculum is stable in water and sediment for <3 days, while SARS-CoV-2 RNA is detectable for at least seven days (Ransome et al., 2023).

4.4. Marine environment

Polo et al. (2021) detected SARS-CoV-2 genome in 9/12 bivalve mollusks and 3/12 estuarine sediments (Table 2). For bivalve mollusks samples, the quantification values ranged from below limit of quantification to 4.48 $\log_{10}$ GC/g of digestive tissue. Concerning the marine sediment samples, the detected SARS-CoV-2 load ranged from below limit of quantification to 3.60 $\log_{10}$ GC/g of sediment. However, using viability RT-qPCR assay, they showed that the detected SARS-CoV-2 RNA did not correspond to intact capsids and, therefore, to infectious viral particles.

Recently, Mancusi et al. (2022) reported the presence of SARS-CoV-2, using RT-ddPCR, in 27/179 (15.1 %) of bivalve mollusks (*Mytilus galloprovincialis*) harvested from Southern Italy area (Table 2). Viral concentration range was 7.8×10^1 to 2.6×10^3 GC/g using Orf1b nsp14 region, 7.2×10^1 to 4.9×10^3 GC/g using RdRp gene and 1.3×10^2 to 5.0×10^2 GC/g using E gene.

Yamazaki et al. (2022) examined cultivated oysters sold in Japan for the presence of SARS-CoV-2 between October 2021 and April 2022 to clarify the extent of viral contamination and evaluate the risk of food-borne transmission of SARS-CoV-2. Despite a marked increase in infections caused by the Omicron variant from January to April 2022 in Japan, SARS-CoV-2 was not detected in any of the 145 raw oyster samples surveyed from Kyoto Hiroshima, Okayama, Hyogo and Yamaguchi prefectures (Yamazaki et al., 2022).

5. Persistence of infectious SARS-CoV-2 and its genome in wastewater

Human enveloped viruses, like coronaviruses, are considered to have a rapid decay rate in the water environment (Kampf et al., 2020; Ye et al., 2016). After the SARS epidemic of 2003–2004, an experimental study showed that SARS-CoV-1 stability under an infectious form was only 2 days at 20 °C, but 14 days at 4 °C (Wang et al., 2005) in urban and hospital wastewater. Survival of SARS-CoV-2 and other viruses in wastewater could be influenced by several factors. These factors include viral structure, the composition of the wastewater, pH, temperature (Amoah et al., 2020) or even the microbial composition (Wurtzer et al., 2021). Because of the limited reports about the presence and behavior of infective SARS-CoV-2 in wastewater and the absence of suggested evidence that water and wastewater play a role in SARS-CoV-2 transmission, inactivation and persistence data may allow us to evaluate the infectivity risk of the virus in water and wastewater.

In this section, we present the persistence data of infectious SARS-CoV-2 and SARS-CoV-2 RNA in wastewater and discuss the different factors, mainly temperature, influencing its persistence. In addition, the persistence of SARS-CoV-2 in wastewater is compared to other water matrices.

5.1. Persistence of infectious SARS-CoV-2 in wastewater

Bivins et al. (2020) studied the persistence of infectious SARS-CoV-2 in wastewater spiked with high (10^5 $TCID_{50}$ /mL) and low (10^3 $TCID_{50}$/mL) titers of infective SARS-CoV-2 at 20 °C. They reported that the virus decay at both titers was not significantly different and observed that it takes 1.6–2.1 days at high and low titers respectively for 90 % inactivation (Table 3). Further, the authors reported that infective SARS-CoV-2 could be detected for the entire 7 days at 20 °C during the higher titer experiment (10^5 $TCID_{50}$ /mL) and for 3 days during the lower titer experiment (10^3 $TCID_{50}$ /mL).

5.2. Persistence of SARS-CoV-2 RNA in wastewater

SARS-CoV-2 RNA was found to be significantly more persistent than infectious particles where T_{90} for SARS-CoV-2 RNA at 20 °C was 3.3 and 26.2 days in wastewater spiked at high (10^5 $TCID_{50}$ /mL) and low (10^3 $TCID_{50}$/mL) titers, respectively (Bivins et al., 2020) compared to 1.6 and 2.1 days for infectious SARS-CoV-2, respectively (Table 3).

Hokajärvi et al. (2021) also observed high SARS-CoV-2 genome persistence (Table 3). They found that T_{90} of SARS-CoV-2 RNA in wastewater at 4 °C was in the range 36–52 days compared to 5.5 days for infectious SARS-CoV-2 at 4 °C (de Oliveira et al., 2021).

Recently, Yang et al. (2022) studied the persistence of endogenous SARS-CoV-2 in wastewater sample where its initial load was $\sim 5 \times 10^3$ GC/L. They showed that the T_{90} values of SARS-CoV-2 RNA were 17.17 and 7.68 days, respectively, at 4 °C and 26 °C smaller than that obtained by Ahmed et al. (2020b) 27.8 and 12.6, respectively at 4 °C and 25 °C (Table 3). This was interpreted by the authors as being related to the fact that their decay experiment was performed with endogenous SARS-CoV-2 and not with spiked one as achieved by Ahmed et al., 2020b and others studies (Hokajärvi et al., 2021; Bivins et al., 2020), where incomplete viral structure may present in the wastewater making viral RNA more prone to degradation. In this context, Wurtzer et al. (2021) indicated that SARS-CoV-2 viral genome could persist under several forms in wastewaters: RNA protected within an infectious particles, RNA protected in a non-infectious particles and free total or partial RNA. Moreover, they showed that <10 % of the total viral RNA was under a protected form in raw wastewater samples collected in Greater Paris area, suggesting the presence of minor part of intact particles in the analyzed samples (Wurtzer et al., 2021).

5.3. Effect of temperature on the persistence

Infectivity of SARS-CoV-2 in wastewater was shown to be affected by temperature. The persistence was significantly decreased with increasing temperature (Table 3), where T_{90} values for infective SARS-CoV-2, seeded at 10^5 $TCID_{50}$ /mL, reduced to 15 and 2 min at 50 °C and 70 °C respectively compared to 1.6 days at 20 °C (Bivins et al., 2020). Similar finding was reported by Varbanov et al. (2021) where they showed that T_{90} for infective SARS-CoV-2 in wastewater, spiked with 10^5–10^6 $TCID_{50}$ /mL titer was 18 h and 4 min at 20 °C and 50 °C, respectively (Table 3).

It was reported that the decrease in virus survival with increasing temperature could be associated to the denaturation of proteins and nucleic acids

Table 3
Persistence of SARS-CoV-2 particles and RNA in different water matrices.

Reference	Matrix	Test conditions	Method of measurement	Temperature	T_{90}
(Bivins et al., 2020)	Wastewater influent	Samples were inoculated with low titer (10^3 $TCID_{50}$/mL) of SARS-CoV-2. The experiment lasted for 7 days	$TCID_{50}$/mL	20 °C	2.1 days
			RNA quantification		26.2 days
Bivins et al., 2020)	Wastewater influent	Samples were inoculated with high titer (10^5 $TCID_{50}$/mL) of SARS-CoV-2. The experiment lasted for 7 days	$TCID_{50}$/mL	20 °C	1.6 days
			RNA quantification		3.3 days
Bivins et al., 2020)	Wastewater influent	Samples were inoculated with high titer (10^5 $TCID_{50}$/mL) of SARS-CoV-2. The experiment lasted for 7 days	$TCID_{50}$/mL	50 °C 70 °C	15 min 2.2 min
Bivins et al., 2020)	Tap water	Samples were inoculated with high titer (10^5 $TCID_{50}$/mL) of SARS-CoV-2	$TCID_{50}$/mL	20 °C	2 days
			RNA quantification		33.2 days
(Ahmed et al., 2020a)	Wastewater influent	Samples were spiked with gamma irradiated SARS-CoV-2: 7.03 ± 0.19 log_{10} GC in 15 mL. The experiment lasted for 33 days	RNA quantification	4 °C 15 °C 25 °C 37 °C	27.8 days 20.4 days 12.6 days 8.04 days
(Ahmed et al., 2020a)	Wastewater influent	Samples were autoclaved and spiked with gamma irradiated SARS-CoV-2: 7.03 ± 0.19 log_{10} GC in 15 mL. The experiment lasted for 33 days	RNA quantification	4 °C 15 °C 25 °C 37 °C	43.2 days 29.9 days 13.5 days 5.7 days
(Ahmed et al., 2020a)	Tap water	Samples were spiked with gamma irradiated SARS-CoV-2: 7.03 ± 0.19 log_{10} GC in 15 mL. The experiment lasted for 33 days	RNA quantification	4 °C 15 °C 25 °C 37 °C	58.6 days 51.2 days 15.2 days 9.4 days
(Varbanov et al., 2021)	Wastewater influent	Samples were spiked with SARS-CoV-2 (10^5–10^6 $TCID_{50}$/mL). The experiment lasted for 7 days	$TCID_{50}$/mL	20 °C 50 °C	18 h 4 min
(Fukuta et al., 2021)	Mineral water	Samples were spiked with SARS-CoV-2 (10^5 PFU/mL). The experiment lasted for 11 weeks	PFU/mL	4 °C	175.43 days
(Fukuta et al., 2021)	Tap water	Samples were spiked with SARS-CoV-2 (10^5 PFU/mL). The experiment lasted for 11 weeks	PFU/mL	4 °C	50.25 days
(Fukuta et al., 2021)	Distilled water	Samples were spiked with SARS-CoV-2 (10^5 PFU/mL). The experiment lasted for 11 weeks	PFU/mL	4 °C	85.47 days
(Sala-Comorera et al., 2021)	Sterilized filtered River water	Samples were spiked with SARS-CoV-2 (3.16×10^4 $TCID_{50}$/mL). The experiment lasted for 20 days	$TCID_{50}$/mL	4 °C 20 °C	3.77 days 2.27 days
(Sala-Comorera et al., 2021)	Sterilized filtered sea water	Samples were spiked with SARS-CoV-2 (3.16×10^4 $TCID_{50}$/mL). The experiment lasted for 20 days	$TCID_{50}$/mL	4 °C 20 °C	2.15 days 1.13 days
(de Oliveira et al., 2021)	Autoclaved River water	Samples were spiked with SARS-CoV-2 (2×10^4 PFU/mL). The experiment lasted for 15 days	PFU/mL	4 °C 24 °C	7.7 days 1.9 days
(de Oliveira et al., 2021)	Autoclaved filtered River water	Samples were spiked with SARS-CoV-2 (2×10^4 PFU/mL). The experiment lasted for 15 days	PFU/mL	24 °C	3.3 days
(de Oliveira et al., 2021)	Autoclaved wastewater	Samples were spiked with SARS-CoV-2 (2×10^4 PFU/mL). The experiment lasted for 15 days	PFU/mL	4 °C 24 °C	5.5 days 1.2 days
(de Oliveira et al., 2021)	Autoclaved filtered wastewater	Samples were spiked with SARS-CoV-2 (2×10^4 PFU/mL). The experiment lasted for 15 days	PFU/mL	24 °C	1.5 days
(Hokajärvi et al., 2021)	Wastewater influent	Samples were spiked with SARS-CoV-2 (titer was not specified). The experiment lasted for 25 days	RNA quantification	4 °C	52 days for *E*-Sarbeco 36 days for N2
(Roldan-Hernandez et al., 2022)	Wastewater primary settled solids from 2 wastewater treatment plant (A and B)	Study performed with endogenous SARS-CoV-2 at concentrations determined using N1 and N2: 4.48 ± 3.93 log_{10} GC/g (A) and 4.60 ± 3.69 log_{10} GC/g (B) for N1	RNA quantification	4 °C 22 °C 37 °C	95 (A), 64.6 (B) 85.9 (A), 36.5 (B) 36.6 (A), 25.3 (B) for N1 gene
		4.45 ± 3.89 log_{10} GC/g (A) and 4.55 ± 3.56 log_{10} GC/g (B) for N2 The experiment lasted for 10 days	RNA quantification	4 °C 22 °C 37 °C	214.7 (A), 75.4 (B) 107.3(A), 26(B) 49.4 (A), 23.5 (B) for N2 gene
(Yang et al., 2022)	Raw wastewater	Study performed with endogenous SARS-CoV-2 (5×10^3 GC/L). The experiment lasted for 4 days	RNA quantification	4 °C 26 °C	17.17 days 8.4 days

(Gundy et al., 2009; Ye et al., 2016; Ahmed et al., 2020b; Hokajärvi et al., 2021).

On the other hand, studies have shown that low temperature favors viral persistence. de Oliveira et al. (2021) studied the persistence of infectious SARS-CoV-2 in autoclaved wastewater and found that it takes 5.5 days at 4 °C for 90 % inactivation while T_{90} at 24 °C was 1.2 days (Table 3). These findings suggest that SARS-CoV-2 may persist longer in wastewater in temperate or colder regions than in tropical regions.

As for infectious SARS-CoV-2, the persistence of viral RNA is also negatively affected by increasing the temperature. Observations by Ahmed et al. (2020b) showed that times for 1-log_{10} reduction (90 % reduction) of SARS-CoV-2 RNA in untreated wastewater under 4, 15, 25 and 37 °C were 27.8, 20.4, 12.6, 8.04 days, respectively (Table 3). As the increase of temperature could have an impact on the SARS-CoV-2 RNA persistence, this parameter should be recorded when the genome is used to follow virus circulation in the population using a wastewater surveillance between summer and winter. Hence, the interest of integrating the collection temperature in the metadata associated with wastewater.

5.4. Effect of wastewater characteristics/composition on the persistence

Besides temperature, another factor affecting the persistence of SARS-CoV-2 is the wastewater characteristics/composition. In the study of Wurtzer et al. (2021), the persistence of infectious SARS-CoV-2 was studied by spiking five negative wastewater samples. After 24 h-incubation, they observed that SARS-CoV-2 infectivity was reduced by about 1-log_{10} in all samples at 4 °C while a reduction of >3 log_{10} and <1-log_{10} was observed in 3 out of 5 wastewater samples and 2 over 5 samples respectively at 20 °C. This variation in

SARS-CoV-2 infectivity at 20 °C may be explained by the difference in chemical and/or microbial composition of wastewater samples. In another study, de Oliveira et al. (2021) reported that decay rate of infectious SARS-CoV-2 was lower in autoclaved filtered wastewater (T_{90} = 1.5 days) than in autoclaved wastewater (T_{90} = 1.2 days) at 24 °C (Table 3).

With respect to SARS-CoV-2 RNA, Roldan-Hernandez et al. (2022) reported a high persistence of SARS-CoV-2 RNA in primary settled solids compared to persistence in raw wastewater. Persistence study was conducted with endogenous SARS-CoV-2 RNA in two WWTPs (POTW A, 14.07 % of solids and POTW B, 16.57 % of solids) at 4 °C, 22 °C and 37 °C. T_{90} values ranged from 24 to 214 days, according to temperature conditions, target gene and the degree of sorption to solids where faster RNA decay rates were obtained at POTW B compared to POTW A (Table 3).

As for infectious SARS-CoV-2, genome persistence in wastewater was also shown to be influenced by microbial activities in raw wastewater. In autoclaved wastewater, T_{90} values of SARS-CoV-2 RNA were 5.71 to 43.2 days with decreasing temperature from 37 to 4 °C, and were higher than the T_{90} for untreated wastewater (8.04 to 27.8 days) at all temperatures except 37 °C (Ahmed et al., 2020b).

5.5. Effect of pH on the persistence

There is limited information on the impact of pH on the survival of infectious SARS-CoV-2 in wastewater matrix. However, Varbanov et al. (2021) investigated the effect of a pH range of 9–12 on the inactivation of infectious SARS-CoV-2 in wastewater for 10 min at room temperature. They observed a slight decrease (<1-$\log_{10}$ unit) in infective SARS-CoV-2 at pH 9 or 10, while an approximately 5.5 $\log_{10}$ units reduction was observed at pH 11. They also reported that the inactivation of infectious SARS-CoV-2 was not affected when the exposure time was increased at pH 9 or 10 from 10 to 60 min.

This considerable reduction of infective SARS-CoV-2 at pH >11 may provide useful information about the stability of the virus during lime treatment of sludge. In fact, lime applied to sludge leads to an enormous increase in pH, where it usually elevates the pH to higher than 11 and even 12 (Parmar et al., 2001) and may have a rigorous effect on pathogen reduction and the associated risk of exposure.

Infectious SARS-CoV-2 was shown to be stable over a wide range of pH (3 − 10) in suspension at room temperature (Chin et al., 2020) in contrast to SARS-CoV-1 where its survival was shown to be affected by the pH of feces (Lai et al., 2005). The survival time of SARS-CoV-1 ranged from three hours in slightly acidic feces of a newborn to four days in diarrheal feces of an adult with a pH of up to 9 (Lai et al., 2005). Xie et al. (2022) showed that the complex structures of hACE2 and the S proteins of SARS-CoV/SARS-CoV-2 are stable at pH values ranging from 7.5 to 9. Moreover, the presence of a polybasic cleavage site in SARS-CoV-2 spike may be the factor in the increased survival of SARS-CoV-2 over wide range of pH compared to SARS-CoV-1 (Winstone et al., 2021).

5.6. SARS-CoV-2 persistence in other water matrices

SARS-CoV-2 can persist longer in different aquatic matrices (Bivins et al., 2020; Ahmed et al., 2020b) than in wastewater. Bivins et al. (2020) showed that T_{90} of infectious virus particles in tap water at room temperature was 2 days compared to 1.6 days in sewage (Table 3). In another study, starting with a spiked concentration of 10^5 PFU/mL, the half-life value of infectious SARS-CoV-2 were 52.79, 25.77 and 15.123 days in mineral water, distilled water and tap water, at 4 °C respectively (Fukuta et al., 2021), the values which represented a calculated T_{90} values of 175.4, 50.25 and 85.4 days, respectively (Table 3). Further, de Oliveira et al. (2021) demonstrated that SARS-CoV-2 persisted more in river water than in wastewater with T_{90} values were 7.7–1.9 days for river water and 5.5–1.2 days for wastewater at 4–24 °C, respectively. Sala-Comorera et al. (2021) showed there was rapid inactivation of infectious SARS-CoV-2 in sterilized filtered seawater (T_{90} = 2.15 and 1.14 at 4 °C and 20 °C, respectively) compared to sterilized filtered river water (T_{90} = 3.77 and 2.28 at 4 °C and 20 °C, respectively) (Table 3).

With respect to RNA, Bivins et al. (2020) showed that the SARS-CoV-2 RNA could persist in tap water at room temperature for up to 33.2 days, while it persisted for 3.3 days in wastewater. Ahmed et al. (2020b) observed 90 % RNA reduction of SARS-CoV-2 at 4–37 °C after 9.4–58.6 days in tap water compared to 8.04–27.8 days in raw wastewater (Table 3).

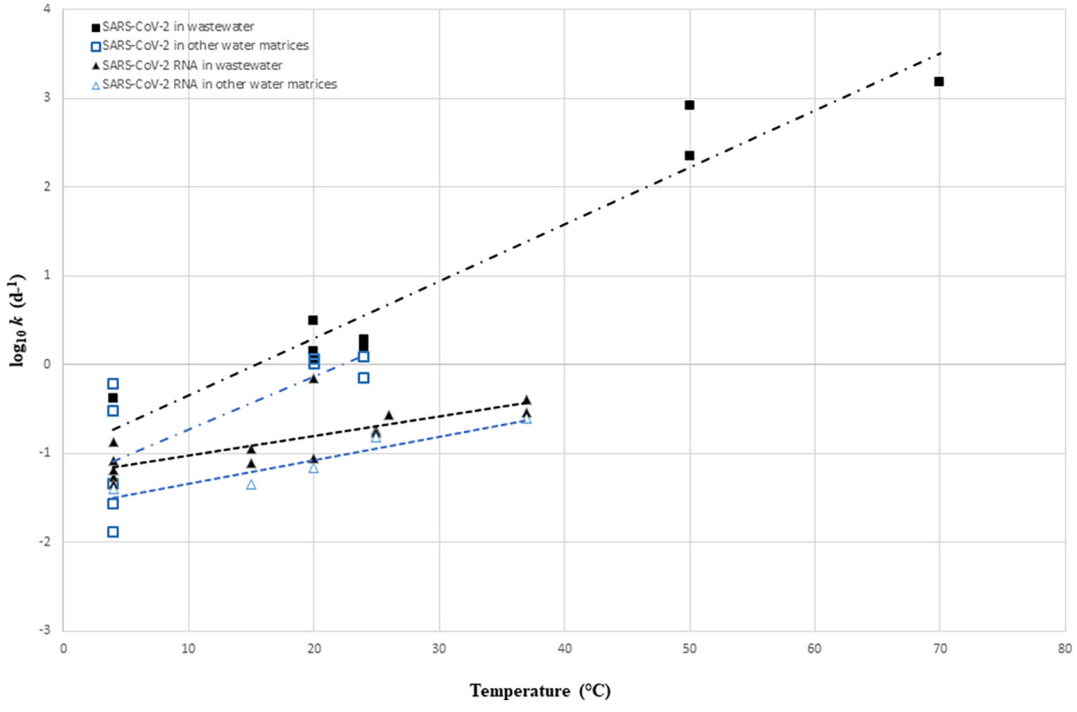

Fig. 2. $\log_{10} k$ of infections SARS-CoV-2 and SARS-CoV-2 RNA in wastewater and other water matrices at different temperatures. Dashed lines represent the linear regression fits for each target. Individual k values are obtained from previous studies and presented in Table S1.

5.7. Temperature sensitivity of SARS-CoV-2 decay in aquatic environment

First-order decay rate constants (k) of both infectious SARS-CoV-2 and SARS-CoV-2 RNA in wastewater and other water matrices (tap water, mineral water and river water) were collected from previous works (Table 3) and presented in units of inverse days (d^{-1}). In case a study only reported T_{90} values, they were converted to first-order decay rate constants according to Chick's law:

$$T_{90} = -\mathrm{Ln}\,(0.1)/k$$

Data compiled from this analysis are presented in Table S1.

To evaluate the change in the decay rate constant with temperature, k at different temperatures of infectious SARS-CoV-2 and SARS-CoV-2 RNA in wastewater and other water matrices were $\log_{10}$ transformed ($\log_{10} k$) and a linear regression was performed (Fig. 2).Values of the linear regression for each target are summarized in Table 4.

Fig. 2 showed that k of infectious SARS-CoV-2 and SARS-CoV-2 RNA increase with temperature regardless of the experimental water matrix. As shown in Fig. 2, the first-order decay rate constant for infectious SARS-CoV-2 showed slightly higher sensitivity to temperature in wastewater (slope = 0.064) than in water matrices (slope = 0.059) (Table 4). Moreover, infectious SARS-CoV-2 is more persistent in water matrices than in wastewater at a similar range of temperature (lower y-intercept in water matrices) (Fig. 2, Table 4). In comparison to other studies, similar slopes were obtained from the linear regression performed for infectious coronaviruses in wastewater (slope = 0.065) by Silverman and Boehm (2020) and for infectious SARS-CoV-2 in wastewater (slope = 0.07) performed by Bivins et al. (2020).

As for infectious SARS-CoV-2, comparable trend was observed for SARS-CoV-2 RNA in both wastewater and other water matrices in relation to temperature (Fig. 2, Table 4). Notably, within the range of environmentally relevant temperatures (i.e., 4–37 °C) infectious SARS-CoV-2 decayed at a faster rate than SARS-CoV-2 RNA in wastewater and other water matrices. For instance, in wastewater, when temperature increase 1 °C, $\log_{10} k$ increase by 0.064 (i.e k increase by 1.15) for infectious SARS-CoV-2 and increase by 0.022 (i.e k increase by 1.05) for SARS-CoV-2 RNA. It is important to note that SARS-CoV-2 RNA is less affected by temperature increase regardless the type of matrix than the infectious particle.

Additionally, SARS-CoV-2 RNA showed higher persistence than infectious SARS-CoV-2 in other water matrices than in wastewater at a similar range of temperature (lower y-intercept in other water matrices).

In conclusion, k is higher in wastewater and lowest in water matrices for both infectious SARS-CoV-2 and SARS-CoV-2 RNA. Several studies have indicated the low capacity of SARS-CoV-2 to persist in wastewater because of the presence of organic matter, pollutants and microbes that may increase the inactivation rates of the viruses (Foladori et al., 2022; Pinon and Vialette, 2018).

6. Elimination of SARS-CoV-2 in wastewater treatment plants

Traditional wastewater treatment techniques are intended to efficiently remove organic matter, suspended solids and bacteria (Adelodun et al., 2020). However, it has been shown to present an efficiency in removing viruses when disinfection is applied (Foladori et al., 2021). Wastewater treatment line comprises a series of steps: pre-treatments, primary treatment, secondary treatment, and tertiary treatment. It was reported from the literature that the efficiency to eliminate SARS-CoV-2, from the influent to the effluent wastewater, depends on the processes involved in the WWTP.

Table 4
Values of slopes, y-intercept and regression coefficients (R^2) of linear regression of the $\log_{10}$-transformed first-order decay rate constants (in units of inverse days) of infectious SARS-CoV-2 and SARS-CoV-2 RNA as a function of temperature in wastewater and other water matrices.

Virus	Wastewater			Other water matrices		
	Slope	y-intercept	R^2	Slope	y-intercept	R^2
Infectious SARS-CoV-2	0.064	−0.989	0.93	0.059	−1.3307	0.55
SARS-CoV-2 RNA	0.022	−1.239	0.56	0.026	−1.6054	0.89

6.1. Pre-treatments stage

Pre-treatments involve the separation of coarse materials by mechanical screening (removal of particles over 5 mm in size), sieving (removal of particles over 0.25 mm in size), grit removal, and oil or grease removal (Zhou et al., 2015; Foladori et al., 2022). It was reported that these treatments might not have significant effects in reducing the viral load in wastewater (Zhou et al., 2015).

6.2. Primary treatment

Primary treatment aims to remove the settleable solids by sedimentation and producing primary sludge, which has 1–2 % total solids content, higher than 0.01–0.05 % in raw wastewater (Peccia et al., 2020). The effluent from primary sedimentation units is referred to as primary effluent. The retention time in the primary sedimentation tank or clarifier is between 2 and 3 h (Foladori et al., 2022). Because virus particles are small and having similar density to water, they cannot settle spontaneously and efficiently during this treatment. However, SARS-CoV-2, as in the case of others enveloped viruses, has a hydrophobic envelope, which decreases virus solubility in water and increases its ability to adsorb on solids (Gundy et al., 2009). Thus, this makes the virus to concentrate in sludge when it adsorbs on settled suspended solids. Moreover, the capacity of viral separation may increase during the flocculation process, where viral particles aggregate in the suspension and form larger particles with higher density (Bhatt et al., 2020). About 1-$\log_{10}$ removal of SARS-CoV-2 RNA was obtained after primary treatment by Abu Ali et al. (2021), indicating that most of viral RNA was adsorbed to settled solids while a 0.48 ± 1.17 $\log_{10}$ removal of SARS-CoV-2 RNA was obtained by Serra-Compte et al. (2021).

6.3. Secondary treatment

Secondary treatment involves biological treatments aiming at the removal of biodegradable compounds and the separation of inert particulate solids. The commonly adopted method in most WWTPs is the activated sludge process. It mainly consists of two steps. The first one involves aeration or anoxic tank, in which wastewater is treated with the aid of active microorganisms (i.e., activated sludge). During the second step, the secondary treated effluent and the activated sludge are separated in the sedimentation tank, or secondary clarifier (Amoah et al., 2020). Due to the hydrophobicity of the viral envelope, SARS-CoV-2 removal may be due to the adsorption on suspended solids (biological flocs) and subsequent sedimentation in the secondary clarifier (Mohapatra et al., 2021) where an additional 1-log removal was obtained after secondary settling by Abu Ali et al. (2021). Moreover, the retention time of the activated sludge process may contribute to viral removal through a spontaneous decaying process (Amoah et al., 2020). Randazzo et al. (2020) published a preliminary study on SARS-CoV-2 presence in wastewater after secondary treatment by activated sludge process and showed that 11 % of samples were positive to SARS-CoV-2 RNA. Balboa et al. (2021) confirmed these results when studying the presence of SARS-CoV-2 in several point of a real WWTP in Spain. Recently, no infectious SARS-CoV-2 viral particles was detected by Monteiro et al. (2022) in secondary treated wastewater samples despite their high viral RNA load.

When using a membrane bioreactor (MBR) system as secondary treatment, the biological process is combined with membrane filtration without the need for a secondary settler (Foladori et al., 2022). The degradation of biomass is performed inside the bioreactor tank, while the solid–liquid separation is achieved in a membrane module (Al-Asheh et al., 2021). Microfiltration and ultrafiltration, with size ranges of 0.1–0.2 μm and 0.005–10 μm, respectively, are the most common types of filtration utilized in MBR procedures (Foladori et al., 2022). A higher viral removal efficiency was achieved in MBR compared to conventional activated sludge process

Table 5
Data on the detection of SARS-CoV-2 RNA in various types of samples along the wastewater and sludge treatment lines in WWTPs.

Reference	Country	Sample type and/or treatment conditions	No of samples	number of positive samples or % of detection by RT-qPCR	Concentration range (GC/L)
Peccia et al., 2020	USA	Primary sludge	44	44	1.7×10^3–4.6×10^3
Kocamemi et al., 2020	Turkey	Primary sludge	2	2	12.5×10^3 –23.3×10^3
		Secondary sludge	7	7	11.7×10^3–40.2×10^3
Westhaus et al., 2021	Germany	Influent raw wastewater	9	9	3×10^3–2×10^4
		Effluent	2	2	2.7×10^3–37×10^3 for all
		Effluent after tertiary treatment	1	1	untreated and treated effluent
		Effluent after ozonation and filtration	1	1	samples
Balboa et al., 2021	Spain	Influent raw wastewater	5	5	2.15×10^3–9.8×10^3
		Treated effluent	5	0	ND[a]
		Primary sludge	5	4	1.3×10^3–24.5×10^3
		Biological sludge	10	1	1.9×10^3
		Thickened sludge	10	9	1.9×10^3–18.8×10^3
		Digested sludge (Thermal hydrolysis and anaerobic digestion)	10	0	ND
Serra-Compte et al., 2021	France and Spain	Primary treated effluent	5	40 %	Not reported
		Secondary treated effluent	30	23.3 %	
		Effluent after activated sludge plus nutrient removal	11	18.2 %	
		Tertiary treated effluent	2	0 %	
		Membrane bioreactor treatment	11	0 %	
		Primary sludge	6	83.3 %	
		Activated sludge	14	57.1 %	
		Thickened sludge	13	69.2 %	
		Anaerobic digested sludge	7	71.4 %	
		Anaerobic digested sludge plus thermal hydrolysis	5	0 %	
D'Aoust et al., 2021	Canada	Post grit sludge	24	79.2 % (N1) and 82.3 % (N2)	1.7×10^3–78×10^3 for all
		Primary clarified sludge	24	92.7 % (N1) and 90.6 %(N2)	positive samples
Pourakbar et al., 2022	Iran/East Azerbaijan	Raw wastewater	4	4	3.8×10^3–28×10^3
		Final chlorination effluents	8	0	ND
		Primary sludge	4	2	3.2×10^3–13×10^3
		Activated sludge	4	3	7.1×10^3–31×10^3
		Anaerobically digested sludge	8	0	ND
Carraturo et al., 2022	Italy	Mature digestate collected during 13 months from the storage tank of a full-scale anaerobic digestion plant	11	0	ND
Yang et al., 2022	China	Influent samples from 4 municipal WWTPs in Beijing	15	6	8.5×10^1–8.8×10^3
		Hospitals influent	6	3	1.60×10^2–9.61×10^2
		Secondary treated effluents	5	3	3.7×10^2–2.34×10^3
		Tertiary treated effluent water	12	8	4.64×10^2–8.62×10^2
		Sewer sediment	2	2	4×10^3

[a] ND: not detected.

(Simmons and Xagoraraki, 2011). For instance, a 1.96 $\log_{10}$ removal of SARS-CoV-2 was obtained after MBR treatment compared to 1.03 $\log_{10}$ removal after activated sludge treatment (Serra-Compte et al., 2021). These results indicate that sludge obtained after MBR treatment may contain higher SARS-Cov-2 load than the sludge obtained after activated sludge process.

6.4. Tertiary treatment

Tertiary treatment involves the disinfection of the effluents with physical or chemical processes such as chlorination, UV irradiation, or ozonation. These treatments showed high efficiency in complete SARS-CoV-2 inactivation (Patel et al., 2021). Moreover, SARS-CoV-2 RNA was not detected in the tertiary effluent in most of the conducted studies. In the preliminary study of Randazzo et al. (2020), all samples were negative to SARS-CoV-2 RNA after tertiary treatment (using disinfection with NaClO and in some cases coupled with UV). This finding was also confirmed by the study of Balboa et al. (2021). Additionally, cell culture assays showed the absence of infectious SARS-CoV-2 particle in tertiary treated effluent wastewater samples (Rimoldi et al., 2020; Westhaus et al., 2021).

7. Partitioning and fate of SARS-CoV-2 RNA along the water and sludge treatment lines

Several studies have evaluated the partitioning and fate of SARS-CoV-2 during wastewater treatment processes (Table 5). Kocamemi et al. (2020) investigated the presence of SARS-CoV-2 in two primary sludge samples and seven secondary sludge samples collected from two WWTPs in Istanbul. The copy number values of SARS-CoV-2 in sludge samples ranged between 11.7 and 40.2 GC/mL and were higher than that observed in influent wastewater, indicating a partial accumulation of SARS-CoV-2 in the sludge. This finding was also confirmed by Westhaus et al. (2021) who studied SARS-CoV-2 partitioning in influent wastewater by comparing the aqueous and solid phases of the samples after centrifugation. They observed that SARS-CoV-2 RNA copy number was higher in the solid fraction (25 GC/mL) by approximately one order of magnitude than in the aqueous fraction (1.8 GC/mL).

In a collaborative study performed by six laboratories across the USA, Kim et al. (2022) compared SARS-CoV-2 RNA in primary settled solids obtained from primary clarifiers and raw wastewater influent samples collected from five publicly owned treatment works (POTWs). They showed that SARS-CoV-2 RNA concentrations, on a mass equivalent basis, were approximately 3 $\log_{10}$ unit higher in primary settled solids than in influent samples (Table 5).

In another study, D'Aoust et al. (2021), quantified SARS-CoV-2 RNA in 24 wastewater influent solids or post grit solids (PGS), and 24 primary clarified sludge (PCS) samples in two water resource recovery facilities in Canada, using CDC N1 and N2 assays. SARS-CoV-2 RNA was detected in high frequency in PCS (92.7 and 90.6 % for N1 and N2, respectively) as compared with PGS samples (79.2 and 82.3 % for N1 and N2, respectively) (Table 5).

Furthermore, Balboa et al. (2021) studied the prevalence of SARS-CoV-2 in various samples in a WWTP located in north-western Spain (Table 5). The analyzed samples were 5 influent raw wastewater, 5 treated effluent,

5 primary sludge, 10 biological sludge, 10 thickened sludge and 10 anaerobically digested sludge. SARS-CoV-2 RNA was detected in 100 % of influent samples at a generally low concentration, at most up to 9.8×10^3 GC/L but reached $>2 \times 10^4$ GC/L in some sludge samples. No SARS-CoV-2 RNA was detected in the effluent samples as they were adsorb to solids. Hence, SARS-CoV-2 RNA was found in the majority of the primary (5/4) and thickened sludge (9/10) samples. Interestingly, no RNA signal was detected in the digested sludge samples, which is likely due to the high temperature applied during anaerobic digestion (Balboa et al., 2021). The authors hypothesized that the primary settler and the sludge thickeners could act as "concentrators" of SARS-CoV-2 RNA. Similarly, Serra-Compte et al. (2021) detected SARS-CoV-2 RNA with high frequency in primary, activated and thickened sludge samples (Table 5). It was also detected in anaerobically digested sludge samples but was eliminated in sludge samples when thermal hydrolysis was applied during anaerobic digestion.

Pourakbar et al. (2022) also studied the fate of SARS-CoV-2 RNA in two different WWTPs in Iran using sequencing batch reactor (SBR) and conventional activated sludge (CAS) (Table 5). They showed that SARS-CoV-2 RNA was detected in all raw wastewater samples and was absent in the final chlorination effluents. They observed that the viral RNA in the WWTPs has higher affinity to biosolids rather than liquid phase, with higher concentrations in the secondary sludge (0.71×10^4– 3.1×10^4 GC/L) than the primary sludge (0.32×10^4– 1.3×10^4 GC/L) of the conventional activated sludge process. This fact could be due to the higher solids retention time in the secondary treatment units, which was about 16–20 days. However, SARS-CoV-2 RNA was shown to be completely destroyed during anaerobic digestion with solids retention time value of about 30 days (Pourakbar et al., 2022).

Recently, Carraturo et al. (2022) studied the effectiveness of a full-scale thermophilic (55 °C) anaerobic digestion process by monitoring the hygienic characteristics of mature digestate samples collected during 13 months. They showed the absence of SARS-CoV-2 RNA in all samples despite it has been detected in the inlet flux of organic solids (Table 5).

According to the Italian National Institute of Health (COVID-19, 2020) and U.S. National Research Council (Council, 2002), the presence of viruses in sludge is not directly indicative of a potential hazard of the matrix as an effective transmission capacity of the pathogen is not proven.

8. Concluding remarks

The occurrence of SARS-CoV-2 RNA and the evaluation of virus infectivity were reviewed in different compartments presented in Fig. 1. Viral RNA of SARS-CoV-2 have been detected in wastewater, sludge, effluent, river water, sediments and bivalve mollusks. However, data are lacking for certain compartments, for example in groundwater, that allows not to have a global overview of the spreading of SARS-CoV-2 in the environment.

Based on the information reviewed in this study, there is no clear evidence for the presence of infectious SARS-CoV-2 in feces although its isolation from stool samples of six different patients. Additionally, infectivity of virus was not revealed in wastewater and sludge samples when SARS CoV-2 RNA were detected. However, cell culture method, which actually considered the gold standard technique to confirm the presence of infectious particle, remain complex to use especially when concentrated wastewater is tested. Furthermore, samples containing high level of microorganisms and the diversity of chemical compound could provide interference, which complicate the interpretation. The use of new methods combining different strategy such as PMA, $PtCl_4$, EMA-RT-PCR should allow, in the future, obtaining more data on this aspect.

Data on the partitioning and removal of SARS-CoV-2 RNA during the wastewater and sludge treatments revealed that

(a) tertiary treatments of wastewater present high efficiency in complete removal of SARS-CoV-2
(b) SARS-CoV-2 RNA copy number was higher in the solid fraction than in the aqueous fraction and genome concentrations increase during the sludge thickening process
(c) Sludge digestion treatments, mainly in thermophilic condition show an efficiency in SARS-CoV-2 inactivation.

Regarding SARS-CoV-2 persistence, SARS-CoV-2 RNA was found to be more persistent than infectious particles in aquatic environment and SARS-CoV-2 decay occurs at a higher rate in wastewater than in others water matrices at a similar range of temperature. Besides temperature, SARS-CoV-2 persistence in wastewater was shown to be also influenced by pH and wastewater characteristics and composition. The high persistence of SARS-CoV-2 RNA makes its detection and quantification a useful indicator to monitor the disease among the population. More studies are needed to understand the different mechanisms of SARS-CoV-2 particles and RNA inactivation in wastewater. Finally, it is also important to study in depth the fate of SARS-CoV-2 and the mechanism of its decay through the stage of wastewater and sludge treatment lines.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.163453.

CRediT authorship contribution statement

Ali Atoui: Writing - Original draft preparation, Conceptualization, Writing - Reviewing and Editing.
Christophe Cordevant: Writing - Reviewing and Editing.
Thierry Chesnot: Writing - Reviewing and Editing.
Benoit Gassilloud: Conceptualization- Writing - Reviewing and Editing.

Data availability

Data will be made available on request.

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.

Acknowledgments

The authors are grateful to Gilles Salvat, the General Director for Research and Reference at Anses, for his critical reviewing and support. We also thank Damien Mouly and Frédéric Jourdain from Santé publique France for reading the manuscript and for providing criticism and helpful suggestions. This work was supported by One Health European Joint Programme under grant agreement No 773830: COVRIN One Health research integration on SARS-CoV-2 emergence, risk assessment and preparedness.

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