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Viruses in wastewater: occurrence, abundance and detection methods

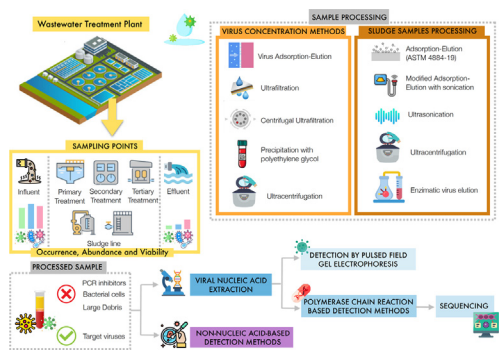
Mary Vermi Aizza Corpuz [a], Antonio Buonerba [b,c], Giovanni Vigliotta [d], Tiziano Zarra [b,c], Florencio Ballesteros Jr [a,e], Pietro Campiglia [f], Vincenzo Belgiorno [b,c], Gregory Korshin [g], Vincenzo Naddeo [b,c,*]

[a] Environmental Engineering Program, National Graduate School of Engineering, University of the Philippines, 1101 Diliman, Quezon City, Philippines
[b] Sanitary Environmental Engineering Division (SEED), Department of Civil Engineering, University of Salerno, 84084, Fisciano (SA), Italy
[c] Inter-University Centre for Prediction and Prevention of Major Hazards (C.U.G.RI.), Via Giovanni Paolo II, 84084, Fisciano (SA), Italy
[d] Laboratory of Microbiology, University of Salerno, 84084 Fisciano, Italy
[e] Department of Chemical Engineering, College of Engineering, University of the Philippines, 1101 Diliman, Quezon City, Philippines
[f] Department of Pharmacy, University of Salerno, 84084 Fisciano, Italy
[g] Department of Civil and Environmental Engineering, University of Washington, Box 352700, Seattle, WA 98105-2700, United States

HIGHLIGHTS

- An overview on virus occurrence, detection and viability in wastewater and sludge
- Molecular methods are extensively used to detect viruses in wastewater.
- Use of sequencing to monitor virus abundance and diversity in wastewater and sludge
- Detection and quantification of viruses in wastewater to monitor disease outbreaks
- Research need: studies on detection methods of emerging viruses such as SARS-CoV-2

GRAPHICAL ABSTRACT



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ABSTRACT

This paper presents an updated and comprehensive review on the different methods used for detection and quantification of viruses in wastewater treatment systems. The analysis of viability of viruses in wastewater and sludge is another thrust of this review.

Recent studies have mostly focused on determining the abundance and diversity of viruses in wastewater influents, in samples from primary, secondary, and tertiary treatment stages, and in final effluents. A few studies have also examined the occurrence and diversity of viruses in raw and digested sludge samples. Recent efforts to improve efficiency of virus detection and quantification methods in the complex wastewater and sludge matrices are highlighted in this review.

Abbreviations: ASTM, American Society for Testing Materials; CER, Cation exchange resin; cDNA, Complementary DNA; cPCR, Conventional Polymerase Chain Reaction; COVID-19, Coronavirus disease 2019; CPE, Cytopathic effects; DNA, Deoxyribonucleic Acid; EV, Enterovirus; EVE, Enzymatic virus elution; ELISA, Enzyme Linked Immunosorbent Assay; EFM, Epifluorescence Microscopy; FCM, Flow Cytometry; GUI, Graphical user interface; HAV, Hepatitis A virus; HFUFS, Hollow fibre ultrafiltration system; HAdV, Human adenovirus; HAdV2, Human adenovirus 2; HAdV 40, Human adenovirus 40; HEV, Hepatitis E virus; HSV1, Herpes Simplex Virus Type 1; HHV, Human herpesvirus; IFA, Immunofluorescence Assay; ICC-PCR, Integrated Cell Culture-Polymerase Chain Reaction; ICC-RT-qPCR, Integrated Cell Culture-real time RT-PCR; JCPyV, JC Polyomavirus; HPV, Human papillomavirus; HPyV, Human polyomavirus; HTtV, Human Torque teno virus; μPAD, Microfluidic paper analytic device; MMTV, Mouse mammary tumour virus; MHV, Murine Hepatitis Virus; NGS, Next Generation Sequencing; NoV, Norovirus; NoV GI, Norovirus GI; NoV GII, Norovirus GII; PMMV, Pepper Mild Mottle Virus; PTA, Phosphotungstic acid; PEG, Polyethylene Glycol; PCR, Polymerase Chain Reaction; PV, Poliovirus; PV1, Poliovirus 1; φ6, Pseudomonas phage; PFGE, Pulsed-Field Gel Electrophoresis; qPCR, Real-Time Polymerase Chain Reaction; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; RT-qPCR, Real Time RT-PCR; RNA, Ribonucleic Acid; RoV, Rotavirus; RoV-A, Rotavirus A; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; TEM, Transmission Electron Microscopy; VIRADEL, Virus Adsorption-Elution.

* Corresponding author at: Sanitary Environmental Engineering Division (SEED), Department of Civil Engineering, University of Salerno, 84084 Fisciano, Italy.
E-mail addresses: mamorales1@upd.edu.ph (M.V.A. Corpuz), abuonerba@unisa.it (A. Buonerba), gvigliotta@unisa.it (G. Vigliotta), tzarra@unisa.it (T. Zarra), fcballesteros@up.edu.ph (F. Ballesteros), pcampiglia@unisa.it (P. Campiglia), vbelgiorno@unisa.it (V. Belgiorno), korshin@uw.edu (G. Korshin), vnaddeo@unisa.it (V. Naddeo).

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A summary and a detailed comparison of the pre-treatment methods that have been utilized for wastewater and sludge samples are also presented. The role of metagenomics or sequencing analysis in monitoring wastewater systems to predict disease outbreaks, to conduct public health surveillance, to assess the efficiency of existing treatment systems in virus removal, and to re-evaluate current regulations regarding pathogenic viruses in wastewater is discussed in this paper. Challenges and future perspectives in the detection of viruses, including emerging and newly emerged viruses such as the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), in wastewater systems are discussed in this review.

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1. Introduction

Viruses are ubiquitous and persistent in raw wastewater and treated wastewater as well as in the receiving water bodies (Fumian et al., 2010). One of the main sources of viruses, including viral pathogens in wastewater is the human fecal matter, particularly that from infected persons (Gerba et al., 2017; Hellmér et al., 2014a; Symonds et al., 2009). Sewage systems receive enteric viruses excreted by infected individuals. An infected person sheds 10^5 to 10^{12} viral particles per gram of fecal matter (Gerba, 2000). In addition to human pathogenic viruses, waterborne viruses that originate from food production, animal husbandry, seasonal surface runoff and other sources are present in wastewater (Corsi et al., 2014). The bodies that receive treated wastewater are oftentimes used for recreational activities and agriculture, and as a source of raw water for drinking water production (Corsi et al., 2014; Dias et al., 2019). Effluents from wastewater treatment plants (WWTPs) are widely used for irrigation and for aquifer recharge (Gerba et al., 2017). The presence of potentially pathogenic viruses in wastewater is of concern since it can pose risks to human health (Carducci et al., 2009; Naddeo and Liu, 2020). Sludges from wastewater treatment plants are also utilized for agricultural applications as soil conditioner or fertilizer (Andreadakis et al., 2002; Bibby and Peccia, 2013; Grobelak et al., 2019). Aside from the concerns related to reuse of wastewater and sludge, exposure of WWTP workers to viral pathogens is a potential risk. Aeration facilities in WWTPs generate and diffuse bioaerosols containing chemicals and microorganisms including viruses that are typically non-waterborne (Pasalari et al., 2019; Wang et al., 2020b). Aerosols are also emitted during the handling of sludge and land application of biosolids (Bibby and Peccia, 2013).

2. Occurrence of viruses in wastewater and sludge

The abundance and diversity of pathogenic viruses in wastewater has been shown to reflect the pattern of infection in human population (Fumian et al., 2010; Montazeri et al., 2015). Adenovirus (HAdV), rotavirus (RoV), hepatitis A virus (HAV), and other enteric viruses, such as noroviruses (NoV), coxsackievirus, echovirus, reovirus and astrovirus are some of the principal human pathogens viruses transmissible via water media. Enteric viruses cause waterborne diseases such as diarrhea in children and in adults (Pietruchinski et al., 2006) and are associated with other disease outbreaks (Thongprachum et al., 2018). Enteric viruses may lead to symptoms notably nausea, vomiting, and fever (Bishop and Kirkwood, 2008). In addition to diarrhea and other gastrointestinal diseases, some enteric viruses have been related to respiratory diseases such as bronchiolitis (Kocwa-Haluch, 2001).

The aims of quantification of viruses in wastewater include: *i*) the determination of the level of risk associated to waterborne diffusion of viruses, *ii*) the evaluation of the efficiency of disinfection as measure of control of virus, *iii*) the surveillance on the extent of diffusion of viruses in a population (Barcelo, 2020). These objectives can be met by determining both the concentration of virus and its infectivity. Virus requires a host species for replication therefore, in absence of the latter, the concentration of a virus in an environment can be constant or progressively reduced by inactivation. Table 1 and Fig. 1 report literature data concerning the range of concentrations and viability of representative viruses that can populate wastewater both continually (e.g., enteric viruses) and sporadically (e.g., coronaviruses during an epidemic). It can be observed that WWTP processes are not completely effective in the reduction of viral genomes' concentrations of most viruses in wastewater (Table 1). For a complete information on the presence of viruses in water, as well as in other environments, their viability and corresponding potential infectivity should also be investigated. This information is an essential input for determining pathways of virus transmission, quantifying the extent of disease risk, and identifying interventions through the use Quantitative Microbial Risk Assessment (QMRA) and Infectious Disease Transmission Modeling (IDTM) (Brouwer et al., 2018).

Enteric viruses are non-enveloped viruses with an enhanced resistance. Some enteric viruses such RoV have been shown to be resistant to UV disinfection. The resistance is attributed to its structure, specifically to the presence of a double-stranded RNA and three-layered capsid protein (Li et al., 2009). On the contrary, enveloped viruses are more susceptible to inactivation in wastewater. The presence of solvents, detergents and disinfectant in wastewater rapidly compromise the lipidic viral envelope or the surface protein. Severe Acute Respiratory Syndrome (SARS) coronavirus (SARS-CoV-1), an enveloped virus, was found to be persistent in raw sewage at 20 °C for 2 days and 14 days at 4 °C (Wang et al., 2005b, 2005c). A limited number of studies have shown that the viability of SARS coronaviruses has not been observed in effluent from WWTPs (see Table 1). SARS-CoV-1 has also been reported by Wang et al. (2005c) to be inactivated by free chlorine with concentration greater than 0.5 mg/L and by chlorine dioxide with concentration greater than 2.19 mg/L. Mechanisms of removal of viruses from WWTP include adsorption of viruses on larger aggregated particles that are separated from wastewater by sedimentation (Verbyla and Mihelcic, 2015), retention by membrane and biofilm layers, predation and enzymatic breakdown in membrane bioreactors (Chaudhry et al., 2015) , and inactivation by disinfection processes such as UV, chlorination, and ozonation (Xagoraraki et al., 2014). As discussed, the resistance of the viruses to inactivation mechanisms is influenced by the viral structure. Double-stranded viruses, including reovirus, HAdV and RoV, have been shown to be generally more resistant to UV radiation than single-stranded ones (Calgua et al., 2014; Harris et al., 1987; Li et al., 2009). This behavior of the double-stranded viruses is attributed to the capability to repair their genomes during replication in the host cells (Beck et al., 2014; Calgua et al., 2014). In addition to the influence of the viral structure to inactivation, some WWTP processes also reduce the rate of inactivation of viruses. Although adsorption of viruses to solids contributes to their removal from wastewater, this also provides a degree of protection of the viruses from inactivation (Hejkal et al., 1981).

This paper aims to present and compare the currently developed methods of detection and quantification of viruses in wastewater. It also aims to present a review of the different pre-treatment steps needed to increase the efficiency of detection and quantification methods of viruses in wastewater and associated products. In this paper, particular attention is paid to the detection and quantification of the recently emerged SARS-CoV-2. This virus belongs to the *Coronaviridae* family, characterized by a protein capsid, containing the viral RNA, protected by a bilipidic envelope with embedded spike proteins. This virus can be transmitted by inhalation of infected respiratory particles (Chan et al., 2020), though other potential routes of

Table 1
Occurrence and viability of viruses in wastewater and sludge.

Virus	Concentration					Viability		
(size; typology)	WWTP	Influent (genome copies/L)	Effluent (genome copies/L)	Sludge (genome copies/kg of dry solids)	Reference	Sample matrix	Number samples with infectious virus/total number of samples	Reference
HEV (27–34 nm; non-enveloped, linear, non-segmented, positive-sense ssRNA virus)	Municipal	7.80×10^3 to 5.80×10^8	4.00×10^3 to 1.00×10^4	**	Izopet et al., 2019* Di Profio et al., 2019; Masclaux et al., 2013; Iaconelli et al., 2020; Beyer et al., 2020	Effluent	30/30	Baez et al., 2017
RoV (70 nm; non-enveloped, linear, segmented, positive-sense dsRNA virus)	Municipal	1.00×10^1 to 6.31×10^4	1.01×10^3 to 4.47×10^6	8.00×10^3 to 8.00×10^5[b] (primary sludge)	Drancourt, 2017* He et al., 2011 Osuolale and Okoh, 2017; Qiu et al., 2018; Prado et al., 2014	Effluent pre-UV Effluent post-UV Activated sludge	3/51 4/50 3/12	Qiu et al., 2018 Qiu et al., 2018 Schlindwein et al., 2010
RoV-A (70 nm; non-enveloped, linear, segmented, positive-sense, dsRNA virus)	Hospital	2.08×10^6 to 8.7×10^8	4.50×10^6 to 1.10×10^7 9.12×10^6 to 3.72×10^7[a]	**	Drancourt, 2017* Prado et al., 2011; Prado et al., 2019	**		–
HAdV (70–90 nm; non-enveloped, linear, non-segmented, dsDNA virus)	Municipal	5.00×10^4 to 3.30×10^{10}	7.76×10^3 to 7.60×10^9 5.50×10^4 to 7.59×10^5[a]	1.80×10^4 to 1.20×10^9[b] (Primary sludge) 9.40×10^9 to 3.90×10^{11} (Thickened sludge) 9.10×10^7 to 6.90×10^9 (Anaerobically digested sludge)	Payne, 2017* Calgua et al., 2011; Qiu et al., 2018; Schlindwein et al., 2010; La Rosa et al., 2020; Prado et al., 2019; Sidhu et al., 2018; Prado et al., 2014; Wong et al., 2010	Effluent Secondary effluent Effluent pre-UV Effluent post-UV Activated sludge Thickened sludge Anaerobically digested sludge	15/16 6/12 4/12 16/51 8/12 10/50 12/12 2/2 1/8	Thompson et al., 2003 Li et al., 2010 Li et al., 2010 Qiu et al., 2018 Schlindwein et al., 2010 Qiu et al., 2018 Schlindwein et al., 2010 Wong et al., 2010 Wong et al., 2010
	Hospital	1.70×10^5 to 2.30×10^7	6.60×10^4 to 2.90×10^6	**	Prado et al., 2011	**	**	–
NoV GI (27–35 nm; non-enveloped, linear, non-segmented, positive-sense ssRNA virus)	Municipal	1.90×10^9 to 9.30×10^9	1.55×10^3 to 2.00×10^9	5.00×10^7 (Anaerobically digested sludge)	Cunliffe et al., 2014* Qiu et al., 2018; La Rosa et al., 2020; Wong et al., 2010	**	**	–
Nov GII (27–35 nm; non-enveloped, linear, non-segmented, positive-sense ssRNA virus)	Municipal	1.48×10^5 to 2.60×10^9	1.41×10^4 to 9.90×10^8	1.60×10^4 to 4.90×10^5[b] (Primary sludge) 1.50×10^8 (Anaerobically digested sludge)	Cunliffe et al., 2014* Masclaux et al., 2013; Qiu et al., 2018; La Rosa et al., 2020; Prado et al., 2014; Wong et al., 2010	**	**	–
	Hospital	0.00 to 1.80×10^6	3.20×10^4 to 9.60×10^7	**	Prado et al., 2011	**	**	–
HAV (27–32 nm; non-enveloped, linear, non-segmented, positive-sense ssRNA virus)	Municipal	1.20×10^5 to 8.90×10^5	1.70×10^5 to 3.80×10^5	Activated sludge: 3.10×10^5 to 5.20×10^5[b]	Rasche et al., 2019* Schlindwein et al., 2010; Villar et al., 2007; Prado et al., 2014	Effluent Activated Sludge	8/12 2/12	Schlindwein et al., 2010 Schlindwein et al., 2010

(continued on next page)

Table 1 (*continued*)

Virus	Concentration					Viability		
(size; typology)	WWTP	Influent (genome copies/L)	Effluent (genome copies/L)	Sludge (genome copies/kg of dry solids)	Reference	Sample matrix	Number samples with infectious virus/total number of samples	Reference
EV (27–30 nm; non-enveloped, linear, non-segmented, positive-sense ssRNA virus)	Municipal	3.80×10^8 to 2.40×10^9	5.40×10^7 to 1.60×10^9	5.47×10^7 to 1.15×10^8 (Primary sludge) 5.66×10^6 to 1.44×10^7 (Activated Sludge) 1.44×10^7 to 2.40×10^8 (Thickened Sludge) 2.60×10^7 to 7.60×10^7 (Anaerobically digested sludge)	Rajtar & Majek, 2008* Schvoerer et al., 2001; Monpoeho et al., 2000; Wong et al., 2010	Effluent Effluent Pre-UV Effluent Post-UV Thickened Sludge Anaerobically Digested Sludge	9/25 17/51 7/50 2/2 1/8	La Rosa et al., 2020 Qiu et al., 2018 Qiu et al., 2018 Wong et al., 2010 Wong et al., 2010
JCPyV (40 nm; non-enveloped, circular, non-segmented dsDNA virus)	Municipal	8.33×10^4 to 3.20×10^8	1.00×10^6 to 1.00×10^8 2.82×10^5 to 7.76×10^{6a}	Zhang**	Nelson et al., 2012* Prado et al., 2019; Fumian et al., 2010; Bofill-Mas et al., 2010	**	**	**
PMMV (310 nm; non-enveloped, linear, non-segmented, positive-sense ssRNA virus)	Municipal Ponds	3.16×10^6 to 1.00×10^9	6.31×10^4 to 1.52×10^8	**	Symonds et al., 2014; Schmitz et al., 2016; Kitajima et al., 2018*	Effluent	Infectivity confirmed by inoculating effluent with test plants	Bačnik et al., 2020
HPyV (40–50 nm; non-enveloped, circular, non-segmented dsDNA virus)	Municipal	1.83×10^7 to 4.17×10^7	1.20×10^3 to 3.70×10^5	7.40×10^7 to 2.5010^8 (Anaerobically digested sludge)	De Gascun & Carr, 2013* McQuaig et al., 2009; Wong et al., 2010	**	**	**
Sapovirus (41–46 nm; non-enveloped, linear, non-segmented positive-sense, ssRNA virus)	Municipal	5.01×10^3 to 1.00×10^5	2.50×10^2 to 2.51×10^3	**	D'Souza, 2015* Schmitz et al., 2016	**	**	**
BK Polyomavirus (40–44 nm; non-enveloped, circular, non-segmented dsDNA virus)	Municipal	3.16×10^4 to 1.00×10^7	0.40×10^3 to 2.51×10^4	**	Ambalathingal et al., 2017* Schmitz et al., 2016	**	**	**
AiV (30 nm; non-enveloped, linear, segmented, positive-sense ssRNA virus)	Municipal	7.90×10^4 to 1.58×10^7	1.00×10^3 to 2.51×10^5	**	D'Souza, 2015* Schmitz et al., 2016; Kitajima et al. (2014)	**	**	**
Parechovirus (28 nm; non-enveloped, linear, non-segmented, positive-sense ssRNA virus)	Municipal	**	**	2.51×10^7 to 2.51×10^8 (Anaerobically digested sludge)	Olijve et al., 2018; * Bibby and Peccia, 2013	Sludge	1/307	Hamparian et al., 1985
Reovirus (85 nm; non-enveloped, linear, segmented, positive-sense dsRNA virus)	Municipal	2.10×10^3 to 3.00×10^4 (in titer per litre of sewage)	1.70×10^6 to 3.72×10^6 0.00 to 6.03×10^{6c}	**	Betancourt & Gerba, 2016* Ridinger et al., 1982	Effluent pre-UV Effluent post-UV Raw Sludge Anaerobically digested sludge	47/51 24/50 12/15 1/9	Qiu et al., 2018 Qiu et al., 2018 Gallagher and Margolin, 2007 Gallagher and Margolin, 2007

SARS-CoV-1 (150–200 nm; enveloped, linear, non-segmented positive-sense, ssRNA virus)	Hospital sewage	**	**	**	Wang et al., 2005b	Hospital sewage seeded with virus	1/1	Wang et al., 2005b
						Hospital sewage before disinfection with chlorine	0/10	
SARS-CoV-2 (60–140 nm; enveloped, linear, non-segmented positive-sense, ssRNA virus)	Municipal	1.90×10^1 to 3.00×10^6	2.40×10^3 to 2.51×10^{5a}	1.25×10^4 to 4.6×10^8 (Primary Sludge)	Zhu et al., 2020; * See Table 4 for complete list of references	Influent	0/8	Rimoldi et al., 2020
				1.17×10^4 to 4.02×10^4 (Waste Activated Sludge)		Effluent	0/4	
	Hospital	0.00 (after primary disinfection tank before septic tank)	0.50×10^0 to 1.87×10^4 (after septic tank with disinfection)		Zhang et al., 2020	**	**	**

Notes:
dsDNA, double-stranded DNA
ssRNA, single-stranded DNA
* Reference for virus size and structure.
** No available quantitative (concentration) or viability data of virus in specified sample matrix in previous studies.
[a] Secondary Treatment Effluent.
[b] Expressed in genome copies/L.
[c] Before UV Treatment.

transmission have been postulated to be important, notably via fomites (van Doremalen et al., 2020), ocular surface (Lu et al., 2020), and fecal–oral route (Wu et al., 2020a; Xiao et al., 2020; Yeo et al., 2020). SARS-CoV-2 has been reported to affect the human gastroenteric tract (Ding and Liang, 2020) and the presence of the viral RNA was detected in fecal samples (Wu et al., 2020a; Xiao et al., 2020) and wastewater conveyed to WWTPs (Ahmed et al., 2020; La Rosa et al., 2020; Lodder and de Roda Husman, 2020; Medema et al., 2020; Wu et al., 2020b; Wurtzer et al., 2020).

Therefore, the analysis of wastewater constitutes a powerful tool for surveillance of the propagation of diseases associated with pathogenic viruses. The presence of these different pathways highlights the importance of the control and removal of viruses in wastewater treatment. Correspondingly, the efficiency of treatment systems to remove viruses must be determined based on their quantification and identification. The presence of human pathogenic viruses in wastewater not only poses a specific sanitary risk, but it also provides a reliable indicator of the extent of circulation of the viruses in the population.

3. Methods of pre-treatment of wastewater samples

The accuracy of detection of viruses depends on the sample volume, nucleic acid extraction yield (for nucleic acid-based methods) and purity (Bofill-Mas and Rusiñol, 2020; Haramoto et al., 2018; Hryniszyn et al., 2013; Sidhu et al., 2013). A study conducted by Hjelmsø et al. (2017) showed that both the concentration of nucleic acids and the nature of the methods used for the extraction significantly influence the results of viral metagenomic analyses, particularly those of viral community composition, viral specificity, and viral pathogen detection. This means that the methods for concentration, nucleic acid extraction, and detection must be chosen appropriately.

This section presents a summary of concentration methods of wastewater samples, pre-treatment methods for sludge samples, and nucleic acid extraction methods.

3.1. Concentration methods

The processing of a sample collected for detection and quantification of viruses depends on the type of sample matrix. Influents generally have higher concentration of viruses than other environmental samples (Haramoto et al., 2018). Raw wastewater samples have higher turbidity (Falman et al., 2019; Qiu et al., 2016; Sidhu et al., 2013), higher suspended solids (Prado et al., 2019) and higher organic matter concentrations (Falman et al., 2019) than other environmental water samples. In addition, influent wastewaters have high concentrations of humic acids and heavy metals, that may interfere with the molecular methods of assaying for viruses (Prado et al., 2019). On the other hand, sludges are very heterogeneous sample matrices, in which viruses tend to be adsorbed on the surface of the flocs (Symonds et al., 2014). These characteristics affect the accuracy of the detection of viruses in these samples. This necessitates the use of concentration steps, some of which consist of primary and secondary concentration methods. It should be noted that molecular methods for detection and quantification of viruses do not provide complete information on the infectivity of viruses present in water media. Those methods determine the presence of molecular fragments (DNA or RNA) of the viruses. The viability of a virus can be determined through the cytopathogenic effect of the infected sample in suitable cell lineages that act as a host species for the virus. Therefore, for the reliable determination of this parameter it is important to preserve the viability of the virus during the sampling, the handling and the treatment of the wastewater sample. A summary and comparison of different types of concentration methods used in processing samples for detection of viruses from wastewater treatment plants are presented in the following discussion and summarized in Table 2.

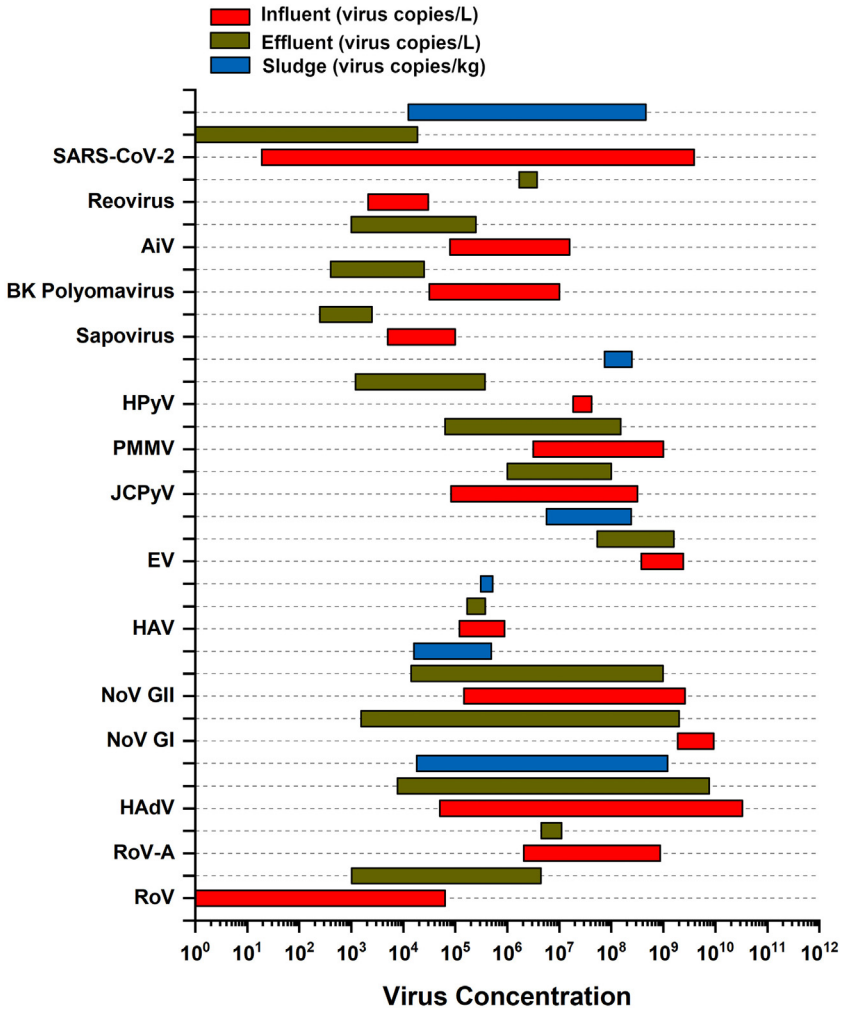

Fig. 1. Occurrence of representative viruses in wastewater.

3.2. Virus adsorption-elution method

Viruses can be removed from water matrices through adsorption onto a membrane filter and subsequent recovery by elution: a process named as virus adsorption-elution (VIRADEL). Metcalf (1961) first reported the recovery of influenza virus from aqueous suspensions through adsorption on a membrane filter. Wallis and Melnick (1967) examined the concentration of enteroviruses by dilution in salt solution to allow adsorption to membrane filters, and subsequent elution. In the current VIRADEL method, the samples pass through a membrane filter

Table 2
Methods of concentration of viruses in wastewater samples.

Method	Sample Volume (L)	Advantages	Disadvantages	References
VIRADEL	0.50–400	Reduces amount of PCR inhibitors	Higher sample volume needed Requires pre-conditioning of samples (when using electronegative membranes) Multiple steps lead to viral loss	Falman et al., 2019; Kuo et al., 2010; Masclaux et al., 2013; O'Brien et al., 2017; Osuolale and Okoh, 2017; Prado et al., 2011; Soto-Beltran et al., 2013
Ultrafiltration	1–10	May be used for simultaneous concentration of viruses and other microbes	Clogging of filters when sample is of high turbidity (except for tangential ultrafiltration) Slow filtration rate	Cashdollar and Wymer, 2013; Jahne et al., 2020; Morales-Morales et al., 2003; Sidhu et al., 2018
Centrifugal ultrafiltration	0.01–0.10	Lower sample volume needed Reduces amount of PCR inhibitors Rapid and simpler method Easier processing of multiple samples	Small pore size may result to clogging of filters High turbidity samples may need pre-filtration	Nordgren et al., 2009; Sidhu et al., 2013
Precipitation with PEG	0.40–1	Higher efficiency in concentrating RNA viruses	Concentrates enzymatic inhibitors (PCR inhibitors)	Amdiouni et al., 2012; Ibrahim et al., 2017; Masclaux et al., 2013; Strubbia et al., 2019

that may have an electronegative or electropositive charge. The viruses are retained on the surface of the filter due to electrostatic attraction between viral particle and membrane surface (Farkas et al., 2018). The VIRADEL method was developed for water samples with low turbidity (Farkas et al., 2018). Modifications to this method are necessary for samples with high turbidity, such as influent wastewaters. Prado et al. (2011) pre-filtered influent hospital wastewater samples before electronegative membrane filtration. VIRADEL method has been widely used for large volumes of sample to be processed, notably river samples (Hamza et al., 2009; Maunula et al., 2012), tap water samples (Hill et al., 2009; Polaczyk et al., 2007), groundwater samples (Lee et al., 2011), and coastal waters (Fong et al., 2005; Goyal and Gerba, 1983; Katayama et al., 2002). Wastewater sample volumes ranging from 0.5 to 400 L were used in recent studies that applied VIRADEL as primary concentration method (Falman et al., 2019; Kuo et al., 2010; Masclaux et al., 2013; Osuolale and Okoh, 2017; Prado et al., 2011; Soto-Beltran et al., 2013). However, the processing of large volumes of samples leads to the concentration of PCR inhibitors. The volume of sample to be treated must be adequate to ensure a final high viral content, keeping low that of PCR inhibitors, in order to facilitate the subsequent molecular amplification techniques of the nucleic material of the virus (Qiu et al., 2016). This consequently improves the quality of viral DNA or RNA that will be extracted in the downstream processes. Aside from the difficulties associated to the handling of large volumes of samples, another disadvantage of VIRADEL is the number of steps, including: *i*) pre-filtration, *ii*) membrane adsorption, *iii*) elution, resulting to prolonged processing time and loss in yield of recovered viruses.(Qiu et al., 2016)

3.2.1. Electronegative filtration

The outer surface of non-enveloped viruses is composed of capsid proteins whose isoelectric point is typically below the pH of wastewater. Therefore, this class of viruses has a net surface negative electrical charge (Hamza et al., 2009; Mayer et al., 2015). The use of electronegative membranes, common in VIRADEL methods, requires the lowering of the pH prior to filtration in order to induce the protonation of the protein capsid and the resultant inversion of the electrical charge of the viral surface (Hamza et al., 2009). The positively charged viruses can then be adsorbed onto the surface of a negatively charged membrane (Cashdollar and Wymer, 2013; Hamza et al., 2009). The addition of salts, such as magnesium or aluminum chlorides improves the adsorption of the viruses on the negatively charged-membrane (Hamza et al., 2009; Haramoto et al., 2009; Masclaux et al., 2013; Osuolale and Okoh, 2017). The first report on the adjustment of pH of suspension medium to 5.0 for optimum adsorption of poliovirus (PV) to membrane filters was made by Wallis and Melnick (1967). Hamza et al. (2014) used a negatively charged membrane of 0.45 μm pore size for adsorption of human enteric viruses from sewage. The sample's pH was adjusted to 3.5 to modify the charge of the viruses and enhance the attraction to the electronegative membrane. Prado et al. (2011) concentrated effluent and pre-filtered influent wastewater samples with gastroenteric and HAV using a negatively charged membrane.

The recovery of the viruses through elution is done after the adsorption to the electronegative membrane. In some methods, the elution step is eliminated, and the nucleic acids of the viruses are directly extracted from the filter (Ahmed et al., 2015). This latter method has been used to concentrate and directly extract nucleic acids of JC Polyomavirus (JCPyV) and BK Polyomavirus (McQuaig et al., 2009) and other enteric viruses (Ahmed et al., 2015) in environmental water samples.

3.2.2. Electropositive filtration

Electropositive membrane filters can be alternatively used in VIRADEL methods. On the basis of the electrical charge of virus suitable for adsorption on membrane filters, the adjustment of the sample pH to reverse the virus surface charge is not necessary when using electropositive membranes (Cashdollar and Wymer, 2013). Soto-Beltran et al. (2013) examined the effectiveness of two electropositive filters in the concentration of PV from a WWTP's effluent (prior to chlorination stage). In that study, two methods of concentration using NanoCeram filter and 1MDS filter were compared in terms of virus recovery. Overall efficiencies of these methods were 57% for NanoCeram and 23% for 1MDS filter methods. The Virosorb 1MDS filter is made of an electropositive glass and cellulose medium (Polaczyk et al., 2007; Soto-Beltran et al., 2013). The NanoCeram filter is made of nanoalumina fibers, which makes it electropositive (Karim et al., 2009; Soto-Beltran et al., 2013).

3.2.3. Virus elution from electronegative or electropositive filters

After being retained by the filters, the viruses must be recovered through elution from the collection substrate (Pepper and Gerba, 2015). For negative filter membranes, a common elution solution is an alkaline solution of beef extract in glycine buffer solution (Hamza et al., 2009). This solution is passed through the filter and results in the desorption of the retained viruses from the filter. The alkaline pH restores the native negative charge of the virus, resulting in an increased repulsion and subsequent desorption of the viruses from the filter membrane. In addition, the additive organic matter present in the buffer solution competes with the viruses for adsorption to the filter facilitating its removal (Pepper and Gerba, 2015).

Other elution buffer solutions used include glycine/NaOH solution (Di Bonito et al., 2017; Miura et al., 2011), sodium polyphosphate (Soto-Beltran et al., 2013) and skimmed milk (Cupples et al., 2010) to recover viruses from environmental samples. These elution buffer solutions are in the range of 70 mL to 1000 mL in volume (Falman et al., 2019; Hamza et al., 2009; Kuo et al., 2010; Prado et al., 2011; Soto-Beltran et al., 2013).

3.2.4. Secondary concentration

After elution of the viruses mobilized from the retaining membrane, the eluate frequently needs to undergo further re-concentration. The aim of this step is to reduce as much as possible the sample volume prior to nucleic acid extraction steps (Pepper and Gerba, 2015). In a study by Falman et al. (2019), the efficiency in recovery of PVs in wastewater was evaluated for several secondary concentration methods. Adsorption using an electropositive filter and elution with beef extract was used as a primary concentration method. The reported results showed that optimized flocculation with skimmed milk resulted in the efficient recovery of poliovirus type 1 (PV1) with yield of 106 ± 25% while the yield with Polyethylene glycol (PEG) precipitation was 59 ± 19%.

3.3. Ultrafiltration methods

This method relies on the principle of size-exclusion using membrane filters that have pore sizes that are smaller than the viral particles (Morales-Morales et al., 2003; Olszewski et al., 2005).

In a study conducted by Sidhu et al. (2018), the concentration of influent and effluent wastewater treatment samples was done using a hollow fibre ultrafiltration system (HFUFS), followed by another filtration step with a 100 K molecular weight cut-off filter. Results showed that the recovery of the HAdV was 37% from influent samples and 67% from effluent samples. A study by Prado et al. (2019) on the detection of enteric viruses from wastewater treatment plant samples used ultrafiltration as a primary concentration step, followed by celite-based secondary concentration. One disadvantage of this method is the small pore size of filters, leading to clogging of filters when samples have high turbidity. Grassi et al. (2010) concentrated raw and treated wastewater samples to detect RoV using tangential flow ultrafiltration. The tangential flow ultrafiltration method is generally suitable for high turbidity samples since clogging is prevented because the water flow is parallel to the membrane surface (Farkas et al., 2018).

3.4. Centrifugal ultrafiltration method

Qiu et al. (2016) developed a one-step centrifugal ultrafiltration method to concentrate human enteric viruses from wastewater. This

concentration technique makes use of size exclusion through a centrifugal filter, Centricon Plus-70, with a molecular weight cut-off (MWCO) of 30 kDa. Compared to the 10 L volume of sample needed in VIRADEL, this rapid concentration method used a lower input volume of 100 mL. The reported results showed that the centrifugal ultrafiltration method resulted in an efficient recovery that was comparable to that of the VIRADEL method. In another study by Sidhu et al. (2013), centrifugal ultrafiltration was used to concentrate HAdV from 10 mL of primary wastewater samples.

These latter studies utilized the input sample volume that was smaller compared to the amount needed using the VIRADEL technique. This allows for easier collection of samples, simpler processing, and shorter filtration time. This also allows processing of multiple samples simultaneously.(Qiu et al., 2016)

Centrifugal ultrafiltration does not require any pH adjustments before filtration and after elution, which is necessary in the electronegative filtration methods, thus maintaining the stability of viruses sensitive to pH variations.

3.5. Direct precipitation with polyethylene glycol

Polyethylene glycol (PEG) is an inert and biocompatible polymer frequently applied for precipitation of proteins. PEG acts as an "*inert solvent sponge*" (Atha and Ingham, 1981) sequestrating water molecules from the solvation layer surrounding the proteins of the viral capsid, enhancing the virus-virus interactions and resulting in the precipitation. Early applications of precipitation with PEG method to recover viruses from environmental samples include release of solids-associated viruses from wastewater and sludge samples (Wellings et al., 1976) and their use to concentrate viruses from shellfish, natural fresh and estuarine water, and freshwater sediment samples (Lewis and Metcalf, 1988). Masclaux et al. (2013) utilized precipitation with PEG to concentrate Hepatitis E viruses in influent and effluent samples from municipal WWTPs. Another study by Strubbia et al. (2019) applied PEG to concentrate NoV from raw sewage samples. In the same study a modified PEG method (Pyro-PEG) was utilized, applying elution with sodium pyrophosphate and sonication prior the PEG precipitation step. An increased number of norovirus GI (NoV GI) and norovirus GII (NoV GII) concentrations in the treated wastewater samples was observed using Pyro-PEG in comparison to conventional PEG method. This increase in detection is attributed to the sonication step which released viral particles from organic matter and reduced the number of bacteria in the sample.

The application of PEG in the study of Amdiouni et al. (2012) resulted to a high efficiency in the recovery of RNA viruses. This is useful for the concentration of viruses from wastewater samples since several RNA viruses are present in wastewater (Adriaenssens et al., 2018; Ng et al., 2012).

As a drawback, PEG may result to non-selective precipitation because it induces the precipitation of various proteins, such as enzymes, that can interfere and even inhibit the subsequent detection of viral genome by PCR amplification methods (Masclaux et al., 2013; Shieh et al., 1995).

3.6. Skimmed milk flocculation

Skimmed milk flocculation, a concentration method first developed to recover adenoviruses from seawater Calgua et al. (2008) is also used to recover viruses from wastewater samples. This method relies on the physical processes including *i*) adsorption of viruses to pre-flocculated skimmed milk proteins, *ii*) sedimentation of the flocs with adsorbed viruses, and *iii*) dissolution of sediments using a phosphate buffer solution. The combination of elution with glycine buffer and the skimmed milk flocculation was successfully used by Calgua et al. (2013) to recover HAdV, JCPyV, and NoVGII from raw urban sewage samples. Assis et al. (2018) applied the skimmed milk flocculation for the recovery of HAdV and RoV from WWTP effluent samples. The latter study revealed that higher recovery rates of HAdV and RoV were obtained by eliminating the initial centrifugation step and by doubling the concentration of skimmed milk. The elimination of centrifugal step was done because of low solids in the treated effluent.

An advantage of this concentration method is that it does not require special equipment and reduced number of processing steps, allowing simultaneous concentration of a large number of samples (Calgua et al., 2008).

3.7. Pre-treatment of sludge samples

Viruses tend to be adsorbed on sludge flocs via electrostatic and hydrophobic interactions. Because sludge is a highly aggregated matrix, viruses must first be desorbed from the sludge through pre-treatment methods.(Pepper and Gerba, 2015)

The traditional method used for pre-treatment of sludge samples is based on the American Society for Testing Materials (ASTM) Standard 4994-19. It involves the concentration of viruses to sludge flocs by adsorption and subsequent elution of viruses. In this method, $AlCl_3$ solution is added to the sample and the pH is adjusted to the value of 3.5 to achieve the optimum adsorption of viruses on the sludge flocs. The mixture is then subjected to centrifugation for the separation of the liquid phase. Solids are washed with an eluent solution to desorb the viruses, and the eluate is subjected to another centrifugation step. The pelleted solids are discarded and the supernatant is passed through a 0.22 μm filter to remove bacteria and other particles (ASTM D449-19, 2019; Pepper and Gerba, 2015; Xagoraraki et al., 2014).

Wu and Liu (2009) used a modified method where sonication (50 kHz, 100 W) of the sample was conducted before the last centrifugation step. This pre-treatment step was conducted prior to the direct counting of viral particles by epifluorescence microscopy (EFM). The effect of sonication on the recovery was examined by varying the duration of sonication. The viral count after one minute of sonication was found to be significantly higher than that obtained without sonication. The sonication enhanced the desorption of viruses from the solid sludge and the release of viruses from their host cells. However, sonication carried out for longer than a minute led to lower viral counts, which may be attributed to possible damage to viral particles. Ultrasonication of activated sludge samples was applied by Ma et al. (2013) to separate viruses from the sample matrix, and subsequently increased the efficiency of flow cytometry (FCM), which requires a sample free of aggregated flocs. Aside from sonication, Brown et al. (2015) used Tween 80, a non-ionic surfactant and emulsifier, and sodium pyrophosphate, an ionic dispersant, to enhance the dislodgement of viruses from the sludge matrix, demonstrating an increased efficiency of virus counting with FCM.

Ultracentrifugation was also examined and compared to the beef extract elution method in recovering enteric viruses from primary sewage sludge samples. However, both methods resulted in less than 7.5% mean recovery of HAdV, Rotavirus A (RoV-A), NoV GII and HAV from the sludge. These results suggested that the applied concentration methods were unable to reduce the presence of the inhibitors that affected the subsequent PCR-based detections (Prado et al., 2014).

Enzymatic virus elution (EVE), a pre-treatment step that minimizes the content of PCR inhibitors in sludge samples, was studied by Sano et al. (2003). Instead of the commonly used 10% beef extract as eluant, a solution containing hydrolytic enzymes (10 g/L) and a cation exchange resin (CER), at a concentration of 10 milliequivalents per liter (meq/L), was added to the sample pellets after the first centrifugation step. Hydrolytic enzymes used by Sano et al. (2003) were lysozyme, carboxylesterase, chymotrypsin, and papain. Multivalent cations present in the sample can enhance the adsorption of viruses to sludge flocs. The addition of CER sequestrates these cations, preventing the re-sorption of the viruses onto particle of sludge samples. In the study by Sano et al. (2003), the efficiency of RT-PCR was increased by using the EVE method instead of the conventional elution with 10% beef extract. The overall increase in virus recovery efficiency (from 7% to

31%) resulting from the combined EVE method and RT-PCR was ascribed to: *i*) the reduction of the concentration of PCR inhibitors from the sludge, i.e. peptides decomposed by the additive hydrolytic enzymes, *ii*) the capture of multivalent cations by the cation exchange resin; *iii*) the elimination of beef extract eluant, which is a source of further components which inhibit PCR amplification of viral genome (Hata et al., 2011; Rock et al., 2010).

Elution with beef extract and elution with glycine were compared by Rock et al. (2010) in terms of the reduction of PCR inhibitors present in biosolids or treated sewage sludges. Excitation-emission spectroscopy results showed that the natural organic matter levels were higher in the samples eluted with beef extract than in samples eluted with glycine. Lower organic matter levels led to higher amplification efficiency of PV genome by RT-qPCR.

3.8. Nucleic acid extraction methods

In the PCR-based methods, the target sequence of the genome material of the virus is amplified. Viruses have genome materials that are either RNA or DNA (Artika et al., 2020). The viral genome materials may be classified as single-stranded or double-stranded. Nucleic acid strands have different polarities, whether positive (+) or negative (−). The viral genome structures are either linear or circular. Viruses also have segmented or complete genomes (Guttman, 2013; Murphy, 1988; O'Carroll and Rein, 2016).

As previously discussed, the PCR-based amplification starts with template DNA sample. In the case of RNA viruses, RNA is reverse transcribed to complementary DNA (cDNA), which becomes the starting material. The quality and purity of these biomacromolecules affect the efficiency of the amplification and quantification methods.

The isolation and purification of DNA/RNA proceeds in these steps: lysis, purification, and recovery. Different types of DNA extraction methods include boiling, column method, magnetic beads, and use of FTA cards (Barbosa et al., 2016).

In the recent studies investigating presence of viruses in wastewater samples, commercially available DNA and RNA kits have been extensively used. The most common DNA extraction kits rely on the use of a column that has silica-based membranes (Barbosa et al., 2016). The binding of DNA to silica in the presence of chaotropic salt sodium iodide (NaI) was first examined in the study of Vogelstein and Gillespie (1979) to extract DNA from agarose gel. Boom et al. (1990) also developed the method that made of use of silica, in the presence of the chaotropic agent guanidinium thiocyanate, to purify nucleic acids from human serum and urine.

The column extraction method is classified as a solid phase-DNA extraction method. In this method, the sample undergoes lysis prior to column loading. Lysate is loaded to the column in the presence of chaotropic salts such as guanidine hydrochloride, guanidine isothiocyanate, sodium iodide, and sodium perchlorate. These salts allow the nucleic acids to selectively adsorb to the silica support, through disrupting their affinity to water. Purification comes after adsorption through washing with a reagent. The last step involves elution of the nucleic acids from the column using a buffer solution. (Barbosa et al., 2016; Butler, 2010)

Examples of the kits with silica-based membranes that were used to extract viral nucleic acids from wastewater samples include QIAamp DNA Blood Mini Kit (Hamza et al., 2014), QIAamp DNA Blood Maxi Extraction Kit (Jahne et al., 2020), QIAamp Viral RNA Mini Kit (Calgua et al., 2011; Masclaux et al., 2013; Prado et al., 2011, 2019), Qiagen DNeasy Blood and Tissue kit (Sidhu et al., 2018), and AllPrep DNA/RNA Mini Kit (Symonds et al., 2014).

Automatic extractors have been used by Ibrahim et al. (2017) and Di Bonito et al. (2017) to extract nucleic acids of viruses from influent and effluent wastewater samples. Most of the automated extractors rely on the use of magnetic beads which bind the nucleic acids, leaving impurities in the solution. Elution is conducted to recover the DNA bound on the beads (Barbosa et al., 2016). An advantage of using automatic extractors is their high throughput and lower variability of analytical results (Dundas et al., 2008).

4. Detection methods of viruses in wastewater

There have been numerous studies on the detection of viruses in different water matrices, including: surface water treated for drinking water purposes (Wang et al., 2020b), bottled drinking water (Martin-Latil et al., 2012), well water (Emelko et al., 2019; Ji et al., 2020), seawater (Canh et al., 2019), irrigation water (Rusiñol et al., 2020), surface water (Canh et al., 2019; Guo et al., 2018; Hata et al., 2014; Khalenkov et al., 2008) and wastewater (Di Bonito et al., 2017; Ibrahim et al., 2017; Jahne et al., 2020; Masclaux et al., 2013; Prado et al., 2019; Qiu et al., 2016; Symonds et al., 2014).

Different sampling points in the examined treatment plants have been used in these studies, as shown in Table 3. Samples were taken from influents, primary settling tanks, secondary and tertiary treatment effluents, and final effluents. Some of these studies examined the fate of viruses inside WWTPs by examining samples originating from secondary treatment steps (Ibrahim et al., 2017; Prado et al., 2011, 2019; Symonds et al., 2014). Other studies collected samples prior to and after tertiary treatment to determine its effectiveness in the context of the removal of viruses (Prado et al., 2011; Qiu et al., 2018). Aside from the presence of viruses in wastewater, the occurrence of viruses in the sludges produced in the examined treatment operations has been studied (Brown et al., 2015; Symonds et al., 2014; Wu and Liu, 2009). Few studies have investigated the presence and abundance of viruses in the ambient air from WWTPs (Brisebois et al., 2018; Masclaux et al., 2014; Pasalari et al., 2019).

As discussed, the sample processing methods affect the efficiency of subsequent detection methods. The choice of a specific method for detection or quantification also depends on the ease of cultivation of the viruses in laboratory conditions (Ibrahim et al., 2017). Specific characteristics of the viruses affect the concentration and detection methods to be applied to the samples being examined. Wastewater and sludge samples have both a variety of DNA or RNA viruses (Bibby and Peccia, 2013; Ng et al., 2012). Viruses are also classified according to their structures as enveloped and non-enveloped viruses. Non-enveloped viruses are characterized by the encapsulation of their nucleic acids by capsid proteins. Enveloped viruses have an additional lipid bilayer membrane that surrounds the capsid proteins (Lucas and Knipe, 2002; Ye et al., 2016a). Disruption of the lipid bilayers by sample processing methods can lead to lower recoveries, affecting subsequent detection (Ye et al., 2016a). This is of particular interest for investigations related to coronaviruses (CoVs) (Casanova et al., 2009; Geller et al., 2012; La Rosa et al., 2012; Wang et al., 2005b).

Methods for the detection and quantification of viruses found in wastewater include epifluorescence microscopy, transmission electronic microscopy, pulsed-field gel electrophoresis, immunofluorescence assay, flow cytometry, traditional cell-culture, and molecular methods. Main features of these methods are summarized in this section.

It is noted that these detection methods provide different information regarding the presence of viruses in wastewater and sludge samples. As presented in the following discussions, some methods provide qualitative data and others quantitative data. Molecular methods for the quantification of a virus are based on the determination of the number of selected segments of the genetic material of the virus. Thus, the virus can be detected even if inactivated, *i.e.* when the viral capsid or envelope is compromised, and even when the genetic material is fragmented. Cell-culture based methods and immunological methods are used for analysis of viability of viruses.

4.1. Transmission electron microscopy (TEM)

Viruses are typically too small for detection using optical microscopy. Electron microscopy enables the inspection of these small particles (Laue,

Table 3
Detection and quantification of viruses in wastewater and sludge samples.[a]

Wastewater sample	Viruses	Concentration/pre-treatment method	Nucleic acid extraction	Virus detection/quantification	Reference
Wastewater treatment samples: (a) Effluent of natural oxidizing pond (b) Effluent of rotating biodisk	AiV Genotype B	Beef extract and $AlCl_3$ method followed by precipitation with PEG	Automatic extractor NucliSENS® EasyMag™	RT-PCR	Ibrahim et al., 2017
Raw sewage	Oncogenic viruses: HPV HPyV HHV MMTV	Elution with glycine	Automatic extractor NucliSENS® EasyMag™	Combined multiplex PCR and bead-based Luminex technology	Di Bonito et al., 2017
Influent wastewater	HAdV EV RoV NoV GI NoV GII	VIRADEL	QIAamp DNA Blood Mini Kit	Combined real time PCR (qPCR) and multiplex Luminex xMAP assay	Hamza et al., 2014
Combined wastewater; Graywater	HAdV NoV GI NoV GII	Ultrafiltration followed by elution with sodium polyphosphate solution	QIAamp DNA Blood Maxi Extraction Kit	(a) For HAdV: qPCR and TaqMan assay; Digital droplet PCR (ddPCR) (b) For Nov GI and NoV GII: RT-qPCR; RT-ddPCR	Jahne et al., 2020
Influent wastewater	PV1	Primary: Filtration with electropositive filter and elution with beef extract and glycine Secondary: (a) beef extract-Celite (b) ViroCap flat disc filter (c) concentrating Pipette (d) PEG/NaCl precipitation (e) skimmed-milk flocculation	–	–	Falman et al., 2019
(a) Influent municipal wastewater; (b) Effluent municipal wastewater	HEV HAdV 40 Nov GII Porcine Adenovirus	(a) VIRADEL (b) Precipitation with PEG	QIAamp Viral RNA Mini Kit	RT-PCR	Masclaux et al., 2013
Effluent	EV RoV	Adsorption-elution method (Mg-method and Al-method)	ZR Viral RNA kit	RT-qPCR	Osuolale and Okoh, 2017
Hospital wastewater treatment plant samples: (a) Influent (b) Effluent of sedimentation tank (c) Final effluent (after chlorination)	RoV-A HAdV NoV GI NoV GII HAV	Adsorption-elution using electronegative membrane	QIAamp Viral RNA Kit	(a) Conventional PCR, RT-PCR (b) qPCR	Prado et al., 2011
Municipal wastewater treatment samples: (a) Pre-UV treatment (b) Post-UV treatment	NoV RoV Sapovirus Astrovirus JC Virus HAdV EV Reovirus	Adsorption-elution using NanoCeram disc filters	MagaZorb total RNA Prep kit	qPCR	Qiu et al., 2018
Municipal wastewater treatment samples: (a) Influent (b) Effluent of Secondary Treatment (c) Reclaimed water	HAdV JCPyV RoV-A	(a) For Influent and Secondary Effluent: Concentration with Celite (b) Reclaimed water: Ultrafiltration	QIAmp Viral RNA Mini Kit	RT-qPCR	Prado et al., 2019
Wastewater treatment samples: (a) Influent (b) Effluent	HAdV HPyV HTtV	Ultrafiltration	Qiagen DNeasy Blood and Tissue kit	qPCR	Sidhu et al., 2018
Wastewater treatment samples: (a) Influent (b) Effluent of water treatment ponds	NoV RoV PMMV EV	Sludge samples: ASTM 4994-19 Wastewater samples: (a) For EV: Dilution with beef extract solution, centrifugation and filtration through protein-treated filter	AllPrep DNA/RNA Mini Kit	(a) For NoV, RoV, PMMV: RT-qPCR (b) For EV: Cell Culture	Symonds et al., 2014

Table 3 (*continued*)

Wastewater sample	Viruses	Concentration/pre-treatment method	Nucleic acid extraction	Virus detection/quantification	Reference
(c) Effluent of UASB reactor (d) Sludge from water treatment pond (e) Sludge from UASB reactor		(b) For q-PCR: Adsorption- elution			
Municipal wastewater treatment samples: (a) Influent (b) Sludge	Virus type not determined (quantification only)	Elution with solutions (a) Beef extract (b) Sodium pyrophosphate (c) Glycine (d) Potassium citrate (e) Lysozyme	–	(a) Epifluorescence Microscopy (EFM) using SYBR Green I as stain (b) Transmission Electronic Microscopy (TEM) (a) Pulsed-field Gel Electrophoresis	Wu and Liu, 2009
Influent wastewater	HAdV JCPyV	Concentration with Phosphate-buffered saline (PBS) solution	For qPCR: QIAamp Viral RNA mini Kit	(a) Immunofluorescence assay (b) Plaque assay (c) Tissue culture infectious dose-50 (d) qPCR	Calgua et al., 2011
Influent wastewater Primary settled wastewater Secondary settled wastewater Activated sludge Effluent	Virus type not determined (quantification only)	Ultrasonication	–	Flow cytometry (FCM)	Ma et al., 2013
Activated sludge	Virus type not determined (quantification only)	Addition of dispersants Tween 80, sodium pyrophosphate, and sodium cholate; Ultrasonication	–	Flow cytometry	Brown et al., 2015
Activated sludge mixed liquor	Virus type not determined (quantification and determination of viral size distribution only)	Sonication	PFGE Method	PFGE	Otawa et al., 2007
Sludge samples (influent and effluent of anaerobic digesters)	Enteroviruses Coronavirus HKU1, Klassevirus, Cosavirus Parechovirus	Precipitation with PEG	Qiagen Viral RNA extraction kit	PCR; High-throughput Sequencing	Bibby and Peccia, 2013
Sludge sewage samples	Enteric viruses	EVE	Sepa-gene RV-R	RT-PCR	Sano et al., 2003
Sewage sludge samples	HAdV RoV-A NoV GII HAV	Ultracentrifugation; Beef Extract Elution	QIAamp Viral RNA Kit	RT-qPCR qPCR	Prado et al., 2014
Primary sludge samples Activated sludge samples Thickened sludge samples	EV	Beef Extract elution with sonication; Precipitation with PEG	RNeasy plant mini kit	RT-PCR	Monpoeho et al., 2000
Raw sludge samples Treated sludge samples	Mammalian orthoreovirus	Adsorption elution; Organic Flocculation	QIAamp Viral RNA Kit	ICC-RT-qPCR; Plaque Assay	Gallagher and Margolin, 2007

Note: [a]Data for SARS-CoV-2 are reported in Table 4.

2010). One of the earliest methods used to quantify viruses is TEM, an imaging technique with nanometer-scale resolution. This method allows the quantification, and identification and classification of viruses according to morphology.(Roingeard et al., 2019)

A basic methodology of TEM is negative staining. In negative staining, the virus particles are adsorbed on a pre-treated specimen support. After adsorption, the particles are stained with heavy metals, commonly uranyl acetate and phosphotungstic acid (PTA). After the staining and drying steps, the samples are analyzed under the electron microscope. Information from negative staining includes virus count, and virus sizes and structures (Laue, 2010).

Wu and Liu (2009) examined the morphology of viruses present in activated sludge and anaerobic digestion sludge samples from a municipal wastewater treatment plant using TEM. The observed morphologies were then used to identify viral particles according to a protocol by the International Committee on Taxonomy of Virus guidelines. TEM results showed that the sludge samples had numerous morphological types, indicating a diverse viral community.

TEM may be applied to identify viruses in emerging infectious diseases since viruses' morphologies are known to be stable even after the mutation of their nucleic acids (Laue, 2010). However, a limitation of the TEM is that it is highly selective to host-specific infectious virus, leading to a viral count result lower than the actual population (Brown et al., 2015). The evaluation of very large number of samples using TEM is difficult since it requires the expertise of highly trained personnel and sophisticated equipment while TEM analyses cannot be automated at present (Barreto-Vieira and Barth, 2015).

4.2. Nucleic acid staining with fluorescent dyes

This virus quantification method makes use of highly fluorescent nucleic acid dyes (Wen et al., 2004). Water samples are passed through a filter, commonly with a pore size of 0.22 μm (Otawa et al., 2007; Wu and Liu, 2009). The nucleic acids in the virus particles are then stained by a fluorescent dye. Staining with this type of dye allows the formation of fluorescent dots with dimensions larger than actual virus particles.

The formation of fluorescent dots occurs when the fluorescent dye bound with nucleic acids are put under excitation. When the sample is viewed under an epifluorescence microscope, the fluorescent dots can be counted as the virus particles (Ortmann and Suttle, 2009).

One of the common dyes used in the recent relevant studies is SYBR Green I whose blue emission is observed using an epifluorescence microscope (Otawa et al., 2007). Counting of viruses from influent, effluent, and sludge samples was done by Wu and Liu (2009) using epifluorescence microscopy with SYBR Green I. Otawa et al. (2007) also employed this direct count method, using SYBR Green I as staining agent, to enumerate viruses in mixed liquor activated sludge samples.

One advantage of this method is that the stained virus particles can be counted even at lower magnifications thus obviating the need to use TEM instrumentation (Ortmann and Suttle, 2009). Another advantage of EFM is that it can be used to count viruses that are not cultivable in laboratories. Aside from this, through the use DNase treatment, the EFM method can differentiate virus particles with nucleic acids from virus-like particles without nucleic acids. However, a limitation of this method is that it has a low efficiency in detecting RNA viruses or single-stranded DNA viruses (Forterre et al., 2013).

4.3. Flow cytometry (FCM)

The combination of the use of fluorescent nucleic acid-specific dyes and flow cytometry has been applied for the quantification of viruses in wastewater samples.

In FCM, samples containing viruses are diluted with buffer solutions and stained with fluorescent dyes. These samples are then injected into the flow cytometer. The hydrodynamics effect of the surrounding sheath fluid allows the virus particles to enter a stream in a single file. The individual particles intersect with a beam of monochromatic light, commonly from an argon-ion laser. The scattering and fluorescence produced by interactions of each particle with the incident laser beam is collected by detectors and analysed as the scatter and fluorescence intensity, respectively (Brown and Wittwer, 2000).

In the FCM approach, the samples are stained with a fluorescent dye, which binds selectively to DNA or RNA. The intensity of fluorescence of the DNA/dye and RNA/dye complexes is therefore correlated with the DNA/RNA content of the sample (Adan et al., 2017; Brown and Wittwer, 2000). Advantages of FCM are its notably high accuracy and high speed of quantification (Ma et al., 2013).

Ma et al. (2013) utilized FCM to determine the abundance of viruses in influent, primary and secondary settled water, activated sludge and effluent samples from a municipal wastewater plant. Comparing viral counts determined by FCM and EFM showed a higher sensitivity and higher speed of quantification using FCM. Brown et al. (2015) quantified viruses from activated sludge samples using FCM. In that study, the FCM method was shown to have a higher sensitivity than that of TEM by a factor of 2.7.

4.4. In situ fluorescence

The fluorescence-based analyses discussed in the preceding paragraphs can only be performed in the specialized laboratories. Pollard (2012) presented the conceptual design of a fluorescence instrument for in-situ and/or online monitoring of abundance of viruses in varying water matrices including effluent wastewater. The proposed portable instrument has an inline filter to remove bacteria from the water sample. A mixing coil is positioned after the filter where DNase and the fluorescent probe SYBR-Gold are added to the sample to form the DNA/RNA-SYBR viral complex. This mixture is directed to a reverse osmosis (RO) filter that concentrates the viruses. The RO concentrate, which contains stained viral particles, passes through a unit that measures the fluorescence signal. On the other hand, the permeate becomes the "background fluorescence", which is subtracted from the sample viral fluorescence. Although the proposed instrument is still at a conceptual stage, tests of this method showed that the EEM fluorescence intensity has a linear correlation ($r^2 = 0.97$) with the viral count.

4.5. Immunofluorescence assay (IFA)

One advantage of IFA over other quantification methods is that it can be used to analyse the infectivity of viruses (Calgua et al., 2011). In this method, a sample from an infected cell culture is adsorbed on a microscope slide. The viral protein antigen is detected by sequentially incubating the sample fixed on the slide with a specific antibody and a fluorescent chemical-conjugated secondary antibody that recognizes the former. Under optical excitation, the fluorophore-conjugated antibody fluoresces. When viewed under a fluorescent microscope, the antigen-antibody complex appears as a fluorescent particle (Dowd et al., 2009; Im et al., 2019).

This method was successfully used by Calgua et al. (2011) to quantify HAdV and JCPyV in raw sewage entering wastewater treatment plants. The reported results showed that the sensitivity of IFA is an order of magnitude higher than those of the other cell culture methods used in the study. The viability of the human adenovirus 2 (HAdV 2) found in activated sludge samples and effluent samples from a wastewater treatment plant was also verified by Schlindwein et al. (2010) using IFA.

4.6. Enzyme linked immunosorbent assay (ELISA)

ELISA is a method that detects the presence of microbial antigens in various matrices (Boonham et al., 2014). It relies on the principle of antigen binding to its specific antibody and eliciting a change in color or fluorescence due to the resultant enzyme activity. The first step of the process is the binding of antigen at a specific antibody immobilized on a surface, commonly in a set of 96-well microtiter plates. A second enzyme-linked antibody, specific for the same antigen, is utilized to form an antibody-antigen-antibody sandwich. The enzyme-coupled antibody reacts with a substrate that changes color when modified by the enzyme. The change in color or fluorescence is correlated with the concentration of the probed antigens in the sample.(Gan and Patel, 2013)

Kargar et al. (2013) utilized ELISA to detect RoV-A in influent and effluent samples of urban and hospital sewage disposal systems. This detection allowed determining the efficiencies of removal of RoV from urban and hospital wastewater treatment systems.

4.7. Pulsed-field gel electrophoresis (PFGE)

The PFGE method makes use of a pulsating electric field to enable the separation of high molecular weight DNA fragments according to their molecular sizes (Nassonova, 2008; Díez et al., 2000).

In this method, alternating electric fields generated between two separate electrodes cause the molecules to re-orient periodically to align to the imposed electric field (Le Tang et al., 2017). The ability of DNA molecules to re-orient themselves and respond to the imposed modulated electric field depends on their molecular sizes and charges. It takes a shorter time for the smaller molecules to re-orient by migrating through the pores of a gel matrix towards the new anodes. On the other hand, larger DNA molecules take a longer time to re-orient. The larger DNA molecules that migrate slower than the set pulse time tend to migrate as one band through the gel matrix (Le Tang et al., 2017; Lopez-Canovas et al., 2019).

The band pattern formed by a viral community serves as its fingerprint. The number of the formed bands gives an estimate of the number of different viruses (diversity) in a sample (Díez et al., 2000).

Otawa et al. (2007) applied the PFGE method to determine the diversity of viruses in activated sludge samples from 14 wastewater treatment plants. The method revealed that the prevailing sizes of viral DNAs in the samples were in the range of 40 to 70 kb. Based on the similarity of the band patterns observed in the PFGE data, the PFGE method was

able to demonstrate that the detected viral species were common to the different activated sludge wastewater treatment plants.

Wu and Liu (2009) used PFGE in their study concerned with the diversity of the viruses in influent, primary settling tank, effluent, and sludge samples of a municipal wastewater treatment plant. Their experimental results showed that the activated sludge, anaerobic digestion sludge, and effluent had the highest number of bands. Similar band patterns were observed between the influent and primary settling tank samples. The PFGE method revealed that the dominant sizes of viral DNAs were in the ranges of 30 to 80 kb and 200–350 kb.

4.8. Molecular methods

Molecular methods for the detection and quantification of viruses are based on nucleic acid amplification. This section discusses the different methods of viral nucleic acid amplification, and subsequent detection and analysis.

4.8.1. Conventional polymerase chain reaction (cPCR)

PCR is an amplification method which relies on the principle of cloning a DNA fragment from a DNA extract through *in vitro* replication (Kadri, 2020). In the case of detection of viruses, this method amplifies or clones a fragment of the viral genome (Rodriguez et al., 2009). A pair of oligonucleotides (primers), which define the starting site of DNA *polymerase*, is used to flank the DNA fragment that is to be replicated. Each of the oligonucleotide is designed to become attached to a certain edge of the DNA target, depending on its given sequence (Kadri, 2020). Amplification through PCR makes use of an thermostable enzyme, commonly the Taq Polymerase, to synthesize new DNA strands from existing strands in the DNA extract (Lo and Allen, 2006). PCR proceeds in three steps: denaturation or separation of double strands of DNA at temperature >90 °C, annealing of primers at temperature close to their melting temperature (Tm) , and elongation at ≥72 °C (Kadri, 2020; Mackay, 2002). Repeating these three steps produces multiple copies of DNA sequences (Lo and Allen, 2006). Two-step PCR, which combines annealing and extension, has also been used leading to reduction of time of amplification (Girones et al., 1993; Jin et al., 2014).

After the amplification step, the PCR products are subjected to agarose gel electrophoresis (Mackay, 2002; Rodriguez et al., 2009) for their qualitative analysis.

4.8.2. Reverse transcription-polymerase chain reaction (RT-PCR)

In the case of RNA viruses, the amplification of the nucleic acid starts with the production of DNA from RNA using a reverse transcriptase enzyme (Cobo, 2012). In this method, the produced DNA is the cDNA which becomes the new template that is amplified using the PCR method described in Section 3.8.1.

Reverse Transcription allows creating cDNAs from RNA viruses (Schinazi et al., 2013). RNA viruses present in wastewater include, among many others, NoV, Aichi virus (AiV), parechovirus, HAV, hepatitis E virus (HEV), astrovirus, RoV and CoVs (Hellmér et al., 2014b).

Ibrahim et al. (2017) utilized the RT-PCR technique to amplify nucleic acids of AiVs, which have RNA genomes, in wastewater samples. Amplification with RT-PCR enabled the analysis of AiVs which are not easily cultivable in the laboratory. The RT-PCR method was also used to amplify the nucleic acids of HEVs, which are positive-strand RNA viruses, in influent and effluent samples of wastewater treatment plants (Di Profio et al., 2019; Masclaux et al., 2013). Osuolale and Okoh (2017) successfully used RT-PCR to amplify the nucleic acids of the RNA viruses enterovirus (EV) and RoV, in wastewater effluent samples. In this study, the RoVs were subjected to heating prior RT-PCR to separate its double-stranded RNA.

RT-PCR was used by Monpoeho et al. (2000) to determine the abundance of EV in primary sludge, activated sludge and thickened sludge samples. The results showed that the largest viral concentration was present in the primary sludge samples. Smaller concentrations were found in the activated sludge samples, followed by the thickened sludge samples.

RT-PCRs are conducted using random, oligo (dT) or specific primers (Newby et al., 2009; Rodriguez et al., 2009). The use random primers will result in non-specific production of cDNA from the existing RNA array in the sample. Oligo (dT) primers hybridize the polyadenine tails in mRNAs (Jalali et al., 2017; Rodriguez et al., 2009). On the other hand, specific primers amplify a region of interest in the viral genome (Rodriguez et al., 2009). The choice of primers affects the result of the amplification method. This is relevant to the detection of emerging viruses in wastewater.

4.8.3. Real-time polymerase chain reaction (qPCR)

The qPCR is a method that allows simultaneous PCR amplification of nucleic acids and detection of the products (Cobo, 2012). It is classified as a quantitative method because it allows quantification of the target sequences, in contrast to the conventional PCR method, which provides qualitative data through gel electrophoresis (Kadri, 2020). Aside from providing quantitative data, qPCR also displays high sensitivity and is not affected by limiting concentrations of reagents (Watzinger et al., 2006).

In the qPCR method, PCR products are labelled by binding with fluorescent dyes or fluorogenic probes (Arya et al., 2005). A Real-Time thermocycler is used to monitor the fluorescence emission during the PCR amplification (Arya et al., 2005; Cobo, 2012). The principle of quantification is based on the correlation between the intensity of fluorescence emission and the amount of amplification of the product of PCR after each cycle (Kadri, 2020).

RT-qPCR uses the same principle as qPCR except that it starts with RNA material instead of DNA, requiring a reverse transcription step (RT) prior to that of qPCR. Another advantage of the qPCR or RT-qPCR over the conventional method is that it eliminates the step of the agarose gel electrophoresis after the amplification step.

Qiu et al. (2018) utilized qPCR to detect and quantify HAdV, JCPyV, and astrovirus in samples collected before and after UV treatment in a wastewater treatment plant. In the same study, RT-qPCR was used to detect and quantify NoV GII, reovirus, EV, RoV, and sapovirus.

Prado et al. (2011) used qPCR and RT-qPCR to detect viruses in hospital wastewater effluents. The latter study demonstrated that the use of RT-qPCR in detecting NoV was more efficient than that of the conventional RT-PCR. This was attributed to the possibility of amplifying shorter fragments in RT-qPCR than was achieved in conventional RT-PCR. Kong et al., also reported that shorter PCR products, or amplicons, are generally amplified more effectively than long ones. However, this hypothesis contrasts with results of other studies, in which there were no sensitivity differences between conventional RT-PCR and RT-qPCR based on the length of the amplicons (Bastien et al., 2008). Therefore, other factors could contribute to the variations, for instance the specificity of the primers, their accessibility to the region to be amplified, and the protocols used (Ferreira et al., 2009).

In a study by Wong et al. (2010), quantification by RT-qPCR showed no significant difference between enteric virus levels in dewatered sludge and sludge after mesophilic anaerobic digestion treatment. This showed that anaerobic digestion may not be efficient for the inactivation of viruses in wastewater sludge.

RT-qPCR was used to detect and quantify SARS-CoV-2 in wastewater samples of Massachusetts (Wu et al., 2020b). Results of these measurements gave a value of 1.04×10^3 genome copies per liter of sample. While additional WWTPs in other areas should be tested further, the results showed that RT-qPCR data from wastewater samples could be used to estimate the viral burden in the affected population. RT-qPCR was also applied to detect and quantify SARS-CoV-2 in raw wastewater samples of Paris, France (Wurtzer et al., 2020). Although the infectivity of the viruses in the examined samples was not yet ascertained by this

method, these RT-qPCR results are highly important because they can be interpreted to indicate that contamination of the wastewaters in Paris had existed even before the onset of the rapid increase of the Coronavirus disease 2019 (COVID-19) cases in the affected population. These studies demonstrate the potential role of RT-qPCR in the development of the Wastewater Based Epidemiology (WBE) approach that can be applied to investigate viral disease outbreaks in communities (Ahmed et al., 2020; Xagoraraki et al., 2014).

It should be noted that a limitation of the qPCR and RT-qPCR methods, as in the conventional PCR and RT-PCR, is that they detect both infectious and non-infectious viruses. Still, the data of these methods are useful in evaluating the efficiency of disinfection treatments, since they detect viruses that cannot be effectively detected in cell culture-based methods. These PCR-based methods may be used to detect viruses, such as RoV and NoV, that are difficult to propagate in cell cultures (Qiu et al., 2018).

Recently, combined azo dye pre-treatment and qPCR has been applied even for the quantification of damaged viruses, overcoming the difficulties associated to the culturing of some viruses for the determination of the viability (Leifels et al., 2020).

4.8.4. Multiplex PCR

Multiplex PCR allows simultaneous detection of different viruses present in a single sample using more than one set of primers in one reaction (Newby et al., 2009). Multiplex qPCR also allows detection of different specific viruses using more than one fluorescent reporter (Haramoto et al., 2018).

Hamza et al. (2014) used Luminex assay, a novel multiplex PCR-based assay, to simultaneously detect human enteric viruses present in wastewater influents. This approach makes use of the Luminex XMap technology with microspheres that are uniquely labelled with variable amounts of 2–3 different fluorophores to obtain up to 500 spectrally distinct microsphere sets. In this method oligonucleotide probes specific for desired DNA sequences are coupled to the beads and the PCR products obtained using biotin-labeled primers are hybridized with probes. Addition of phycoerytrin-streptovidin complex that interacts with biotinylated sequences allows detecting the presence of amplicons on the beads by fluorescence emission. The microspheres enter an analyzer that makes use of the principle of flow cytometry. The beads are identified after they pass through lasers of different wavelengths and the spectral response from each bead is associated with a single oligonucleotide probe. The Luminex analyzer also measures the fluorescence emitted by the phycoerythrin to quantify the amount of bound nucleic acids (Dunbar and Hoffmeyer, 2013; Hamza et al., 2014).

The results reported in Hamza et al. (2014) showed that the multiplex assay was as sensitive as qPCR in the detection of several enteric viruses in wastewater samples. Di Bonito et al. (2017) also successfully used the multiplex PCR bead-based technology (Luminex) to detect oncogenic viruses in untreated wastewater. This class of viruses, that are related to human cancer, include mucosal and cutaneous human papillomavirus (HPV), human polyomavirus (HPyV), human herpesvirus (HHV) and mouse mammary tumor virus (MMTV).

The multiplex qPCR has been shown to have a similar sensitivity to singleplex PCR in the detection of RoV-A in raw sewage samples (Fumian et al., 2010).

Aside from the multiplex assay's sensitivity, another advantage of this method is its speed associated with the simultaneous detection principle that it employs. It also requires lower reagent cost and labor compared to singleplex PCR-based assays.(Dunbar and Hoffmeyer, 2013; Fumian et al., 2010; Hamza et al., 2014)

4.8.5. Sequencing

Aside from quantification, PCR products may also be subjected to DNA sequencing. DNA sequencing is the process of determining the specific order of nucleotides for a segment of DNA (Artika et al., 2020).

The most common sequencing method is based on the dideoxy or chain-termination method, or the Sanger method (Clark et al., 2019; Hoy, 2019). In this method, DNA polymerase randomly produces multiple partial copies of the DNA template that have varying lengths. The lengths of each subset DNA copies differ by one nucleotide, resulting to each copy ending in a different nucleotide position. Each final nucleotide is labelled with a fluorescent tag, with a different color for ddATP, ddGTP, ddCTP and ddTTP. The DNA copies undergo capillary electrophoresis where they are separated by size, with the smaller fragments forming the fastest moving bands. A fluorescent detector records the fluorescence emitted by the final nucleotides of each fragment. The sequence of the template DNA is revealed by the diagram of fluorescent peaks produced (Clark et al., 2019; Hoy, 2019).

Recent studies concerned with the detection of viruses in wastewater have applied the Sanger method using commercially available sequencing kits such as the BigDye Terminator Cycle (Di Profio et al., 2019; Hellmér et al., 2014b; Ibrahim et al., 2017; Montazeri et al., 2015; Prado et al., 2011). Most of the sequencing analyses used in these and similar studies are now automated to increase speed and sensitivity of the process (Artika et al., 2020).

Direct sequencing was conducted by Ibrahim et al. (2017) to identify the genotype of AiVs found in the secondary effluent of a pilot wastewater treatment plant. The sequence analysis revealed that the strains of AiV found in the wastewater samples were the same strains found in patients with acute gastroenteritis.

Hellmér et al. (2014a, 2014b) used the sequencing analysis to investigate whether the identification of pathogenic viruses in sewage can be indicative of an epidemic outbreak. Wastewater samples from Gothenburg, Sweden were subjected to analysis by RT-qPCR and sequence analysis. The reported results showed that two strains of the HAV detected in sewage samples were the same strains found in fecal samples of infected patients in Gothenburg. The strains were also found to be the same strains associated to an outbreak in the region at the time the sewage samples were collected.

In a similar study (Di Profio et al., 2019), wastewater samples from Abruzzo Region in Southern Italy were collected to monitor the prevalence of HEV. Results of sequencing analyses showed the presence of the HEV Genotype 3 strains, which were previously detected in animal and human hosts in the region. Although further exploration of these findings is needed, the result of this study suggests that the high number of people infected with HEV in the region may be due to transmission from animals to humans.

Partial sequencing was conducted by Prado et al. (2011) to determine the diversity of viruses in samples from a hospital wastewater treatment plant located in Rio de Janeiro, Brazil. Results of this study showed that HAdV serotypes 40 and 41 detected in the effluent samples were serotypes that are associated to gastroenteritis cases in children in Rio de Janeiro and Salvador, Brazil.

High-throughput sequencing has been recently applied in studies investigating the prevalence and diversity of viruses in sewage samples. The Next Generation Sequencing (NGS) technique allows a larger number of samples to be analyzed in a single run (Hoy, 2019; Martínez-Puchol et al., 2020). In this type of sequencing, the following steps are involved: producing millions of copies of DNA fragments, and conducting and recording millions of sequences simultaneously (Clark et al., 2019; Hoy, 2019). This parallel sequencing of a large number of samples reduces turnaround time and cost of sequencing (Hoy, 2019; Martínez-Puchol et al., 2020).

Fumian et al. (2019) successfully used NGS to determine Norovirus GII genotypes found in influent, primary effluent, and secondary effluent from a wastewater treatment plant in Rio de Janeiro, Brazil. Results demonstrated the prevalence of 6 major genotypes of NoV GII in the wastewater samples collected for a period of one year. These genetic data were compared to the corresponding clinical data as part of a surveillance of the circulation of pandemic and epidemic norovirus strains in the examined area. Two strains of noroviruses, GII.5 and GII.7, were

found to be prevalent in the wastewater samples but were not detected in the clinical samples. This indicates that these strains may lead to asymptomatic or mild infections, which are not reported.

NGS techniques were used by Martínez-Puchol et al. (2020) to determine the distribution of human pathogenic viruses in three wastewater treatment plants in Barcelona City, Spain. Sequencing analyses showed that the three wastewater treatment plants had similar distribution of types of HAdV, HPV and HEV types despite the differences in the location, and volume of wastewater treated.

Raw and digested sludge samples from wastewater treatment plants in the United States of America were tested using DNA sequencing to assess infectious risks related to reuse of sludge as agricultural soil amendment (Bibby and Peccia, 2013). NGS showed that aside from the viruses normally associated with gastrointestinal infection, the examined sludge samples had an abundance of viruses associated with respiratory diseases. These viruses include HAdV Types B and C, Bocavirus, and Coronavirus HKU1, which may be transmitted by airborne route (droplet spread) or by fomites. This poses an additional concern since aerosols are known to be released during application of stabilized sludge on agricultural land. This indicates the presence of another possible pathway of transmission of viruses related to human disease by exposure to sludge from wastewater treatment. Current regulations of pathogens in sludge include the monitoring of levels of enteroviruses and fecal coliforms. The abundance of emerging viruses other than EV in sludge may indicate a need to review the regulations.

Recent metagenomic analyses of samples from WWTPs have revealed that a large number of viral genomes cannot be recognized using information from public sequence databases. Tamaki et al. (2012) found that 85–90% of viral sequences derived from tropical WWTP samples were unclassified. Similar results were obtained by Alhamlan et al. (2013) during the analysis of the viral community of a dairy lagoon wastewater, where 81–83% of derived sequences were unrecognizable according to the GenBank database. Around 37% of sequences derived from different WWTP samples studied by Ng et al. (2012) were unclassifiable by BLASTx against the GenBank viral database. These results show that further work using sequencing is needed to fully identify novel viral species in WWTP systems.

The latter studies show the role of sequencing analysis in the ongoing effort to identify the diversity of strains of viruses in wastewater and wastewater sludge samples. The use of this approach to monitor wastewater systems may serve as an early warning method to detect possible disease outbreaks (Hellmér et al., 2014b) and as a tool for public health surveillance (Fernandez-Cassi et al., 2018). It may also be used as a basis for re-evaluation of regulatory requirements related to pathogens in wastewater systems (Bibby and Peccia, 2013; Montazeri et al., 2015).

4.8.6. Integrated cell culture- polymerase chain reaction (ICC-PCR)

4.8.6.1. Cell culture. Traditional cell culture methods use cytopathic effects (CPE) for measuring infectious viruses (Qiu et al., 2018). In this approach, cells are cultivated under controlled conditions and infected with viruses, which need host cells to replicate. The identification of presence of viruses is observed through CPEs that are visible under light microscopy. CPEs are manifested as deterioration of monolayer cells that have been infected by a virus (Dilnessa and Zeleke, 2017).

The observed CPEs and the rates of CPE appearance are used to identify viruses. Destruction of the monolayer cell, in which the infected cells shrink easily and detach from the glass, is typically associated to EV. Focal degeneration of infected cells is typical of herpesviruses. Enlargement and formation of grape-like clusters are associated with HAdV. The appearance of altered areas, referred to as inclusion bodies, in stained cells is also a characteristic type of cytopathic effects observed for HAdV and herpesviruses (Suchman and Blair, 2007).

Aside from the long period needed to obtain results, another limitation of the traditional cell culture is that some viruses do not grow in cell cultures (Dilnessa and Zeleke, 2017; Reynolds, 2004). Another complication is that some viruses such as RoV and HAV do not give visible cytopathogenic results and are therefore not detected by conventional cell culture method. It is also not a highly specific method since multiple types of viruses can be supported by a single cell line (Reynolds, 2004).

4.8.6.2. ICC-PCR. The combination of cell culture and PCR was developed to address the limitations of each method when used individually (Rodriguez et al., 2009). In the ICC-PCR method, the samples with viruses are introduced to cell culture media and incubated for 2–3 days. Samples of cell culture with viruses are subjected to freezing, which causes lysis of the cells. The virus particles are released from their host cells when lysis occurs. After this, PCR-based amplification is applied to the lysate (Newby et al., 2009).

The approach of integrated cell culture-real time PCR (ICC-qPCR) was used by Qiu et al. (2018) to evaluate the efficiency of UV disinfection in a wastewater treatment plant. The results showed that the ICC-qPCR method was more efficient than qPCR in detecting reovirus. This study also demonstrated that RoV was rarely detected by the ICC-qPCR method. This was attributed to the insufficient rate of RoV propagation in cell culture in the study.

Chowdhary et al. (2005) utilized ICC-PCR to detect PVs in wastewater from sewage treatment plants. Since PVs are RNA viruses, the extracted viral RNAs were subjected to reverse transcription before amplification. ICC-PCR provided results in 4–5 days while traditional cell culture took 18 days.

The viability of HAdV in raw sewage, secondary effluent, and reclaimed water was assessed by Prado et al. (2019) using ICC-qPCR. Using this method showed that a significant amount of HAdV was viable even after undergoing the activated sludge wastewater treatment.

Integrated cell culture- real time RT-PCR (ICC-RT-qPCR) was shown to be more efficient than traditional plaque assay in detecting the presence of mammalian orthoreovirus in raw and treated sludge samples (Gallagher and Margolin, 2007).

The latter studies showed that in terms of virus inactivation, the ICC-PCR method is useful in assessing the efficiency of different stages of wastewater treatment in virus removal. However, ICC-PCR has its limitations such as the non-detection of some viruses that are not cultivable in cell culture. It should also be noted that it is designed to detect specific types of viruses depending on the PCR primers used. It is also therefore prone to false negatives in the detection of other viruses present in the sample. Because of this, some studies still use the traditional cell culture along with ICC-PCR (Qiu et al., 2018).

4.9. Process control viruses

The efficiency of the methods used to concentrate, detect and quantify viruses is monitored through process controls (Blanco Fernández et al., 2017; Hennechart-Collette et al., 2015). Process control viruses are either added to samples prior to their concentration, to concentrated samples prior to nucleic acid extraction, or to nucleic acid extracts prior to the amplification and detection (Haramoto et al., 2018). The chosen process control viruses must have similar morphology as the target viruses, must not be naturally found in the samples and must be easily quantified by nucleic acid-based assays and infectivity assays (Blanco Fernández et al., 2017; Diez-Valcarce et al., 2011). Murine norovirus has been used as a process control for NoV because of the former's similarity in the morphology and genetic structure to the target virus (Hennechart-Collette et al., 2015). Murine norovirus was used by Kitajima et al. (2014) as a process control virus to determine the efficiencies of viral nucleic acid extraction and RT-qPCR in a study on the detection and quantification of viruses, including NoVs, in wastewater. Bacteriophage PP7 was utilized by Fumian et al. (2010) as a process control to determine the efficiency of concentration of RoV-A from wastewater samples. Mengovirus strain vMC0 has been used as a process control virus to assess the efficiency of concentration, nucleic acid extraction, and detection of enteric viruses from water samples (Coudray-

Meunier et al., 2015; Teixeira et al., 2020). Farkas et al. (2018) utilized mengovirus strain vMC0 as a process control to assess the efficiency of different concentration methods in the recovery of enteric viruses from wastewater samples. Mengovirus has a similar structure as HAV (Coudray-Meunier et al., 2015), and was utilized as a process control to asses viral RNA extraction and PCR efficiencies in a study on the detection of HAV from WWTPs (Ouardani et al., 2016). However, the study of Petterson et al. (2015) showed that there is a low correlation between the recoveries of spiked mengovirus, and the enteric viruses HAdV, NoV GI, and NoV GII from surface water samples. This result indicated that there is a need for further exploration of process control viruses that would be as close as possible to the structure and behavior of the target viruses.

5. Challenges and perspectives

5.1. Concentration methods of wastewater for assay of emerging viruses: coronaviruses and SARS-CoV-2

Since the identification of coronaviruses in the mid-1960s, several novel viruses, including SARS-CoV-1 (2002, China), Middle East respiratory syndrome–CoV (MERS-CoV, 2012, Saudi Arabia), and SARS-CoV-2 (2019, China), have emerged. Up to now, seven human CoVs have been identified. CoVs are enveloped single-stranded RNA viruses with sizes ranging from 60 to 220 nm. The viral protein capsid is covered by a lipid bilayer membrane that contains proteins or glycoproteins and crown-like spikes on the surface. Contrarily to "*simple*" non-enveloped RNA viruses, CoVs are less stable in wastewater. SARS-CoV-1 has been shown to persist up to 2 days at room temperature, both in domestic and hospital sewages, and more than 14 days at 4 °C (Wang et al., 2005b, 2005c). These considerations pertinent to the stability of CoVs, should be taken into account during the sampling and storage of wastewater samples, as well as during the treatment of wastewater for the detection, quantification and particularly in the determination of the viability of these viruses.

Methods for concentration of CoVs in water media were recently examined by La Rosa et al. (2020). Enveloped viruses such as CoVs are sensitive to pH variations, with their optimal stability at slight acidic pH of 6–6.5 (Sattar et al., 2009). Nevertheless, SARS-CoV-2 demonstrated high stability in a wide range of pH values (Chin et al., 2020). These should be considered in the case of VIRADEL recovery of CoVs where virus elution from electronegative or electropositive membranes is performed at strong alkaline or acid pH, respectively. Five studies have examined the efficiency of recovery of CoVs: the recovery of bovine enteric CoV (Collomb et al., 1986), SARS-CoV-1 (Wang et al., 2005a), murine hepatitis virus (MHV)(Ye et al., 2016b), bovine CoV(Abd-Elmaksoud et al., 2014), and transmissible gastroenteritis virus (TGEV) (Blanco et al., 2019). Adsorption on glass wool and glass powder (Abd-Elmaksoud et al., 2014; Blanco et al., 2019; Collomb et al., 1986) or on silica gel compounded with aluminum hydroxide (Wang et al., 2005a), followed by elution with neutral (Wang et al., 2005a) or alkaline (Abd-Elmaksoud et al., 2014; Blanco et al., 2019; Collomb et al., 1986) buffers were some of the techniques adopted for the concentration of the above mentioned CoVs. PEG precipitation, ultracentrifugation and ultrafiltration were compared by Ye et al. (2016b) in the context of the recovery efficiency of MHV. Ultrafiltration was demonstrated the most efficient method with 25.1% of recovery of MHV. Blanco et al. (2019) applying virus adsorption onto glass wool with subsequent elution with alkaline buffer and PEG precipitation, explored on the recovery of TGEV, effects of pH, contact time and composition of eluent. An adsorption of 42.7% and the complete removal of virus from the glass wool adsorbent was obtained by overnight elution with glycine/beef extract buffer at pH of 11.0 in presence of TWEEN 80 (0.3%).

Recent results of the investigation of the detection and quantification of SARS-CoV-2 are summarized in Table 4. Most of those studies have been published as pre-print articles and not yet subjected to peer-review process at the moment of writing of this review. Therefore, the information reported in these publications should be considered with caution. In absence of a general protocol for recovery of CoVs and of SARS-CoV-2 from wastewater, different procedures of sampling, sample storage and concentration, and virus detection have been applied (Tables 4 and 5).

Currently, scarce information is available on the efficiency of recovery of SARS-CoV-2 from wastewater. This requires investigations on the determination of the concentration of SARS-CoV-2 in wastewater model samples spiked with a known number of virus copies. As most of the existing concentration methods have been used to recover non-enveloped human enteric viruses, further research must be conducted to evaluate the efficiency of existing concentration methods in recovering enveloped viruses, such as the SARS-CoV-2 (Ahmed et al., 2020). As previously discussed, enveloped viruses have a structure that is different from non-enveloped viruses. Enveloped viruses tend to be sensitive to some organic solvents used in concentration methods (La Rosa et al., 2020; Ye et al., 2016a). A related study by Ye et al. (2016a, 2016b) compared recovery efficiencies of different methods used to concentrate enveloped viruses from wastewater. The latter study showed that using ultracentrifugation at 100,000*g* for an hour resulted in lower recoveries of murine hepatitis virus (MHV) and Pseudomonas phage (φ6). The lower recovery efficiencies were attributed to the strong centrifugal forces that may have disrupted the lipid bilayer membranes of the enveloped viruses. In the same study, an optimized ultrafiltration method, with a pre-filtration step using a 0.22 μm filter, resulted in relatively higher recoveries of enveloped viruses. On the other hand, PEG precipitation showed low recoveries of the model enveloped viruses. Percentage recoveries of SARS-CoV-2 have not been determined due to handling and facility restrictions related to biosafety (Ahmed et al., 2020). Further studies need to be carried out to determine the efficiencies of alternative concentration methods applicable to recover SARS-CoV-2 viruses from wastewater and in sludge samples. Control experiments on the efficiency of recovery of viruses, from SARS-CoV-2 infected wastewater, were performed by determining the concentration of F-specific RNA phages according to ISO 10705 (Medema et al., 2020), or by spiking mengovirus vMC0 (University of Valencia, Spanish Type Culture Collection, CECT 100000) in wastewater samples according to ISO 15216-2:2017 (Randazzo et al., 2020). Haramoto et al. (2020) utilized the electronegative membrane-vortex method to concentrate SARS-CoV-2 from wastewater samples and evaluated the efficiency of recovery using pepper mild mottle virus (PMMV). Medema et al. (2020) suggested the spiking of other human CoVs, such as CoV 229E, to the sewage samples as a reliable internal standard for the determination of the efficiency of recovery of CoVs from wastewater. SARS-CoV-1 is most proximal to the novel SARS-CoV-2, among the viruses investigated in wastewater. Wang et al. (2005a) reported an average SARS-CoV-1 recovery efficiency of 1% by applying adsorption of virus on silica gel compounded with aluminum hydroxide and elution with a buffer at pH of 7.4.

Modes of sampling and sample storage can affect the detection and viability of CoVs in wastewater. As discussed, SARS-CoV-1 infectivity persisted in municipal and hospital wastewater for 2 days at room temperature and for more than 14 days at 4 °C. On the other hand, to the best of the authors' knowledge, information is lacking on the effect of freezing on viability and efficiency of recovery of CoVs from wastewater. Composite and grab samples from WWTPs were analyzed for detection of SARS-CoV-2 (Table 4). In some investigations, debris and particulate were removed by centrifugation or filtration or by a combination of these techniques prior to concentration of the wastewater samples. The nature of these treatments can affect the final virus concentration since CoVs tend to be adsorbed onto organic matter and can even be protected by suspended solids (Gundy et al., 2008). For safe handling of samples, pasteurization at 57 or 60 °C can be applied for inactivation of viruses and other pathogens present in wastewater (La Rosa et al., 2020; Wu et al., 2020b). Several techniques for secondary concentration

Table 4
Detection and quantification of SARS-CoV-2 in wastewater and sludge.

Reference; Location of WWTPs	Sample type; Sampling mode; Volume; Storage temperature	Sample pre-treatment	Concentration method	PCR inhibitor treatment	RNA extraction	Concentration in genome copies/L		Viability test results, number samples with infectious virus/total number of samples	Detection/quantization/viability analysis methods
						Influent	Effluent		
Medema et al., 2020; The Netherlands	Municipal wastewater; Composite sampling (24 h); 250 mL; 4 °C	Centrifugation	Centrifugal filtration (Centricon Plus-70, MWCO of 10 kDa)	–	RNeasy PowerMicrobe (Qiagen) and Biomerieux Nulisens Kit (Biomerieux) in combination with semi-automated KingFisher mL purification system (Thermo Scientific)	[a]	[a]	[a]	RT-PCR: (one step) EvoScript RNA Probes Master (Roche); Indirect evaluation by F-specific RNA phages assay
(Wu et al., 2020b, 2020a) Massachusetts, USA	Municipal wastewater; Composite sampling (24 h); n.r.; 4 °C	Pasteurization (60 °C, 90 min) and filtration (0.2 μm pore size)	PEG/NaCl, centrifugation	–	TRIzol	1.04×10^3	[a]	[a]	RT-PCR Reverse transcriptase, NEB RT-qPCR; TaqMan fast advanced master mix, Thermofisher
Nemudryi et al., 2020; Montana, USA	Municipal wastewater; Composite sampling (24 h); 500 mL; n.r.	Filtration (5 μm and 0.45 μm pore size)	Centrifugal filtration (Corning Spin-X UF, MWCO of 100 kDa)	–	RNeasy Mini Kit (Qiagen)	10^4	[a]	[a]	RT-qPCR (one-step) TaqPath™ 1-Step RT-qPCR Master Mix, CG (ThermoFisher); RT-PCR SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific); Q5 High-Fidelity DNA Polymerase (New England Biolabs)
Ahmed et al., 2020 Australia	Municipal wastewater; Composite sampling (24 h) and grab sampling; 100–200 mL; 4 °C	Filtration (0.45 μm pore size)	a. Electronegative membrane filtration (HAWP09000, Merk; pore size of 0.45 μm) b. Centrifugal filtration (Centricon Plus-70, MWCO of 10 kDa)	–	RNeasy PowerWater Kit; RNeasy PowerMicrobiome (Qiagen)	1.90×10^1 to 1.2×10^2	[a]	[a]	RT-qPCR (one-step) iTaq™ Universal Probes One-Step Reaction Mix (Bio-Rad)
Wurtzer et al., 2020; France	Municipal wastewater; n.r.; 11 mL; n.r.	–	Ultracentrifugation	PCR Inhibitor removal resin (Zymo Research)	PowerFecal Pro kit with a QIAsymphony automated extractor (QIAGEN)	5.00×10^4 to 3.00×10^6	[a]	[a]	RT-qPCR
Wang et al., 2020b, 2020a; China	Hospital sewage; n.r.; n.r.; n.r.	–	–	–	SARS-CoV-2 nucleic acid detection kit (Shanghai Berger Medical Technology Co., China)	[a]	[a]	0/6	RT-PCR; Viability in Vero-E6 cells
(Randazzo et al., 2020); Spain	Municipal wastewater; Grab sampling 200 mL; 4 °C	pH Adjustment at 6;	Precipitation with $AlCl_3$, centrifugation; elution with beef extract (3%, pH 7.4), centrifugation and resuspension in PBS	–	Nucleospin RNA virus Kit (Macherey-Nagel)	1.48×10^5 to 3.90×10^5	Secondary Effluent: 2.51×10^5 Tertiary Effluent: No detection	[a]	PrimeScript™ One Step RT-PCR Kit; RT-qPCR diagnostic panel assays (US CDC, 2019-nCoV RUO Kit and the positive control 2019-nCoV_N_Positive Control by Integrated DNA Technologies).
La Rosa et al., 2020; Italy	Municipal wastewater; Grab sampling; 250 mL; −20 °C	Pasteurization (57 °C, 30 min)	PEG-Dextran precipitation	One step PCR Inhibitor removal kit (Zymo Research)	NucliSENS miniMAG semi-automated extraction system (bioMerieux,)	[a]	[a]	[a]	RT-PCR SuperScript III/IV Reverse Transcriptase (ThermoFisher Scientific); Kit Platinum™ SuperFi™ Green PCR Master Mix, Thermo),

(continued on next page)

Table 4 (*continued*)

Reference; Location of WWTPs	Sample type; Sampling mode; Volume; Storage temperature	Sample pre-treatment	Concentration method	PCR inhibitor treatment	RNA extraction	Concentration in genome copies/L		Viability test results, number samples with infectious virus/total number of samples	Detection/quantization/viability analysis methods
						Influent	Effluent		
Rimoldi et al., 2020; Italy	Municipal wastewater; Grab sampling; 500 mL; Specific temperature not reported (samples were under refrigeration)	Filtration (0.7 μm and 0.2 μm nominal pore size)	Not carried out	–	QIAMP VIRAL RNA mini kit (Qiagen, Hilden, Germany)	[a]	[a]	Influent: 0/8 Effluent: 0/4	Real-Time RT-PCR Viability in Vero E6 cells
Bar Or et al., 2020; Israel	Municipal wastewater; Composite sampling (24 h); 0.25–1 L; −20 °C or − 80 °C	Centrifugation	a. PEG or alum precipitation, centrifugation, b. Centrifugal filtration (Amicon, MWCO of 30 kDa)	–	RNA extraction kit (RNeasy mini kit- QIAGEN and EasyMAG-bioMerieux, France)	Only Cycle Treshold (Ct) numbers were given (i.e. number of cycles required for the fluorescent signal to cross the threshold)	Only Cycle Treshold (Ct) numbers were given (i.e. number of cycles required for the fluorescent signal to cross the threshold)	[a]	RT-qPCR
Haramoto et al., 2020	Municipal wastewater; Grab sampling; Influent: 200 mL Secondary Effluent: 5000 mL; n.r.	–	Electronegative membrane-vortex method; Adsorption direct RNA extraction method	–	RNeasy PowerWater Kit (Qiagen)	Not detected	2.4×10^3	[a]	RT-qPCR Nested PCR
Zhang et al., 2020	Municipal wastewater; Grab sampling; 2.0 L; 4 °C	Centrifugation	PEG 6000/NaCl precipitation		EZ1 virus Mini kit (Qiagen, Germany)	Not detected (After primary disinfection tank before septic tank)	0.5×10^3 to 18.7×10^3 (After septic tank with disinfection with sodium hypochlorite)	[a]	RT-qPCR
(Alpaslan Kocamemi et al., 2020); Turkey	Municipal wastewater sludge; Grab sampling; n.r.; n.r.	Centrifugation; filtration (0.45 and 0.2 μm nominal pore size); pH Adjustment at 7.0–7.2	PEG 8000/centrifugation	n.r.	Roche MagNA pure LC total nucleic acid isolation kit using Roche MagNA pure LC system (Penzberg, Germany)	Primary Sludge: 1.25×10^4 Waste Activated Sludge: 1.17×10^4 to 4.02×10^4		[a]	RT-qPCR
Peccia et al., 2020	Municipal wastewater sludge; Grab sampling; 2.5 mL; -80 °C	Not carried out	Not carried out (Direct addition of sludge to RNA extraction kit)	n.r.	RNeasey PowerSoil Total RNA kit, Qiagen	Primary Sludge: 1.7×10^6 to 4.6×10^8		[a]	RT-qPCR

Note: n.r. Not reported

[a] No available quantitative (concentration) or viability data of virus in wastewater or sludge

of the water sample, or combination of them, were used, including: *i*) centrifugal filtration (Ahmed et al., 2020; Bar Or et al., 2020; Medema et al., 2020; Wu et al., 2020b), *ii*) ultracentrifugation (Wurtzer et al., 2020), *iii*) precipitation with PEG (Bar Or et al., 2020; La Rosa et al., 2020; Wu et al., 2020b; Zhang et al., 2020), aluminum chloride (Randazzo et al., 2020) or alum (Bar Or et al., 2020); *iv*) electronegative membrane filtration (Ahmed et al., 2020; Haramoto et al., 2020).

5.2. Detection and quantification of SARS-CoV-2 in wastewater and sludge

The investigations on SARS-CoV-2 in wastewater and WWTPs exploit the methodological principles applied to other pathogenic and non-pathogenic viruses, applying some modifications necessary for adaption to the characteristics of this new strain of coronavirus.

Here, we briefly describe the molecular materials and procedures adopted so far by different authors for the study of SARS-CoV-2 in wastewater samples. Table 5 reports the main sequences of primers and fluorescent probes used.

Medema et al. (2020) used either of two different commercially systems, RNeasy Power Microbiome Kit (Qiagen) and Nuclisens kit (Biomerieux), in combination with the semi-automated KingFisher mL purification system (ThermoScientific) to extract RNA from viral particles in samples from WWTPs in The Netherlands. For identification, one-step RT-qPCR method (EvoScript RNA Probes Master, Roche) was used with primers specific for three regions (N1—N3) of the nucleocapsid protein gene (N) and envelope protein gene (E). A quantitative culture assay for F-specific RNA phage was also conducted in this study to indirectly evaluate the efficiency of recovery of SARS-CoV-2 by the purification and concentration treatment steps. The effect of these sample processing steps to the viability of the viruses was also examined.

Ahmed et al. (2020) extracted RNA, using the RNeasy PowerWater Kit and RNeasy PowerMicrobiome Kit (Qiagen), from the ultra-filtered sample or from the electronegative membrane. For specific detection and quantification, RT-qPCR was used with the primers N_Sarbeco and NIID_2019-nCOV_N, both of which are specific for the nucleocapsid protein gene. One-step kit RT-qPCR was adopted, where reverse transcription and qPCR occurred in the same reaction well.

La Rosa et al. (2020) assessed the presence of SARS-CoV-2 in Italian WWTP by means of RT-PCR, using two groups of specific primers for SARS-CoV-2: a newly developed set targeting ORF1ab (La Rosa et al., 2020) and a published set, designed for the pharyngeal swab, specific for the spike (S) protein gene. In this study, broad-range primers, which were designed before the detection of the new strain of coronavirus, were also tested, amplifying a conserved region of the ORF1ab of Coronavirinae members. However, these primers did not give specific signals due to some nucleotide differences in this region found with subsequent sequencing of SARS-CoV-2.

Wurtzer et al. (2020) investigated the presence of viruses in wastewater samples collected from 3 major WWTPs in Paris, using the RT-qPCR with primers targeting the envelope protein gene (E) and RNA-dependent RNA polymerase gene (RdRP). After concentration by ultracentrifugation, the viral genome was extracted using an optimized protocol from the commercial PowerFecal Pro kit on a QIAsymphony extractor (Qiagen).

Nemudryi et al. (2020) detected SARS-CoV-2 in concentrated wastewater samples from WWTP of Bozeman (USA) by one step RT-qPCR. The primers, supplied by the commercially 2019-nCoV EUC CDC kit, were specific for two regions (N1, N2) of the nucleocapsid protein gene. RNA extraction was carried out via the commercially RNeasy mini kit (Qiagen). Nemudryi et al. (2020) also evaluated the phylogenetic relationship of the isolated SARS-CoV-2 genome with the other sequenced worldwide, using 10 published primer pairs (Table 5) to amplify with non-quantitative RT-PCR and sequence some polymorphic regions dispersed in the genome.

Wu et al. (2020b) preliminarily detected the presence of SARS-CoV-2 in PEG-precipitated wastewater samples from a WWTP of Massachusetts (USA) with classical PCR and published primers directed towards the (S) protein gene spike. After detection, RT-qPCR was used to quantify the viral titer, with primers targeting N1, N2 and N3 loci of the nucleocapsid protein gene (N) and published by US, CDC. Viral RNA was extracted with Trizol (Thermofisher).

The concentration of SARS-CoV-2 in raw wastewater obtained by recent studies varies from 10^1 to 10^6 genome copies/L (Table 4). Concentrations of SARS-CoV-2 in secondary effluent range from 10^3 to 10^5 genome copies/L (Haramoto et al., 2020; Randazzo et al., 2020). SARS-CoV-2 was also present at a concentration of 10^3 to 10^4 genome copies/L in the effluent from the septic tanks of Fangcang hospital in Wuhan, Hubei, China (Zhang et al., 2020). The obtained concentration of the virus in primary sludge samples is 10^4 to 10^8 genome copies/L (Alpaslan Kocamemi et al., 2020; Peccia et al., 2020). This concentration in primary sludge samples is slightly higher than that in the wastewater samples. These results are in accordance with the tendency of enveloped viruses to adsorb to the solid-fraction of wastewater (Ye et al., 2016a), resulting to their accumulation in the wastewater sludge. This is important in the study of the emerging viruses, such as SARS-CoV-2, since the concentrated sludge sample can provide information on the viruses present in wastewater and in corresponding affected communities.

As of this time of writing of the review, only two studies have performed analysis of viability of SARS-CoV-2 in wastewater. Wang et al. (2020a) conducted the analysis by inoculation of the virus on Vero-E6 cells. No viable virus was detected in all of the influent and effluent hospital wastewater samples. Rimoldi et al. (2020) performed the same viability test of SARS-CoV-2 in municipal wastewater samples, in which no viable virus was also detected. The study of Zhang et al. (2020) revealed that SARS-CoV-2 genomes were not detected in the influent of a hospital septic tank (after primary disinfection tank) but were detected in the effluent at a concentration of 0.50×10^3 to 1.87×10^4 genome copies/L after disinfection with sodium hypochlorite. The viral genomes in the septic tank effluent were detected when the free chlorine concentration has declined to non-detectable level. The absence in the influent and presence in the effluent suggested the release of embedded viruses in the stool particles, which provided protection from the treatment process. Although the latter study did not perform a viability analysis, the results indicate the need for further studies to confirm that the sewage or wastewater treatment systems are not SARS-CoV-2 transmission pathways.

5.3. Detection of viruses from aerosols in wastewater systems

A limited number of studies have shown that wastewater systems emit bioaerosols that have significant concentrations of viruses such as RoV, NoV, HAdV, HAV, and Herpes Simplex Virus Type 1 (HSV1) (Brisebois et al., 2018; Masclaux et al., 2014; Pasalari et al., 2019). Viruses in aerosols pose a risk to the WWTP or sewage workers who are exposed to these viruses via the inhalation or contamination by contact with deposited aerosols on surfaces (Masclaux et al., 2014). Highly resistant viruses, such as RoV and NoV, that are present in the local atmosphere of a WWTP can also pose risks to the residents living near the facility (Pasalari et al., 2019).

Although no evidence has so far emerged concerning the emission of SARS-CoV-2 to the ambient air at WWTPs or sewage systems, future studies on this potential exposure pathway need to be conducted to assess possible risks to workers and affected residents. The study of van Doremalen et al. (2020) examined the stability of SARS-CoV-2 in aerosols. Although the experimental condition used in that study had only a 3 h duration, it showed that SARS-CoV-2 remained viable in aerosols. The study also compared the viability of SARS-CoV-2 and SARS-CoV-1 in aerosols. It showed that both viruses have similar stabilities. SARS-CoV-1 virus was previously reported to be transmitted through aerosols in sewage systems of a residential building in Hong Kong, leading to a disease outbreak in the examined area (Chim et al., 2003; Hung, 2003).

Table 5
Main primers and probes used in molecular detection and quantification of SARS-CoV-2 in wastewater.

Study	Oligonucleotide name	Oligonucleotide Sequence[a] (5′ to 3′)	Target genes	Reference
Ahmed et al., 2020;	N_Sarbeco_F	CACATTGGCACCCGCAATC	Nucleocapsid protein (N)	(Corman et al., 2020)
April 18, 2020	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG		
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ		
	NIID_2019-nCOV_N_F2	AAATTTTGGGGACCAGGAAC	Nucleocapsid protein (N)	(Shirato et al., 2020)
	NIID_2019-nCOV_N_R2	TGGCAGCTGTGTAGGTCAAC		
	NIID_2019-nCOV_N_R2ver3	TGGCACCTGTGTAGGTCAAC		
	NIID_2019-nCOV_N_P2	FAM-ATGTCGCGCATTGGCATGGA-BHQ		
La Rosa et al., 2020;	2274 - CO-FW1	GTGCTAAACCACCGCCTG	ORF1ab	(La Rosa et al., 2020)
May 7, 2020	2275 - CO-REV1	CAGATCATGGTTGCTTTGTAGGT		
	2276 - CO-FW2	CGCCTGGAGATCAATTTAAACAC		
	2277 - CO-REV2	ACCTGTAAAACCCCATTGTTGA		
	WuhanCoV-spk1-f	TTGGCAAAATTCAAGACTCACTTT	Spike protein gene (S).	(Shirato et al., 2020)
	WuhanCoV-spk2-r	TGTGGTTCATAAAAATTCCTTTGTG		
	NIID_WH-1_F24381	TCAAGACTCACTTTCTTCCAC		
	NIID_WH-1_R24873	ATTTGAAACAAAGACACCTTCAC		
	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	RdRP gene	(Corman et al., 2020)
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA		
	RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGCBHQ1		
Wurtzer et al., 2020;	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCG	RNA-dependent RNA	(Corman et al., 2020)
April 17, 2020	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	polymerase gene (RdRp)	
Alpaslan Kocamemi et al., 2020	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ		(Corman et al., 2020)
May 16, 2020	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ		
Medema et al., 2020	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	Nucleocapsid protein (N)	Centers for Disease Control and
March 30, 2020	2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG		Prevention, CDC (US 2020)
Wu et al., 2020	2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1		
April 7, 2020	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA		
Randazzo et al., 2020	2019-nCoV_N2-F	GCGCGACATTCCGAAGAA		
April 28, 2020	2019-nCoV_N2-F	FAM-ACAATTTGCCCCCAGCGCTTCAG- BHQ1		
Peccia et al., 2020[a]	2019-nCoV_N3-F	GGGAGCCTTGAATACACCAAAA		
June 12, 2020	2019-nCoV_N3-F	TGTAGCACGATTGCAGCATTG		
	2019-nCoV_N3-F	FAM-AYCACATTGGCACCCGCAATCCTG- BHQ1		
Medema et al., 2020	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Envelope protein gene (E)	(Corman et al., 2020)
March 30, 2020	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA		
Wurtzer et al., 2020	E_Sarbeco_P	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ		
April 17, 2020				
F. Wu et al., 2020	S-RPA-Forward_v1	GAAATTAATACGACTCACTATAGGGAGGT	Spike protein gene (S)	(Zhang et al., n.d.)
April 7, 2020		TTCAAACTTTACTTGCTTTACATAGA		
	S-RPA-Reverse_v1	TCCTAGGTTGAAGATAACCCACATAATAAG		
Nemudryi et al., 2020	N1 forward and reverse	sequences not available	Nucleocapsid protein (N)	2019-nCoV CDC EUA
April 20, 2020	N2 forward and reverse	sequences not available	Nucleocapsid protein (N)	Kit-IDT#10006606
Zhang et al., 2020	CCDC-ORF1, forward primer	CCCTGTGGGTTTTACACTTAA	ORFlab	Not indicated
May 14,2020	Reverse primer	ACGATTGTGCATCAGCTGA		
	Fluorescence probe	FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1		
	CCDC-N, forward primer	GGGGAACTTCTCCTGCTAGAAT	Nucleocapsid protein (N)	Not indicated
	Reverse primer	CAGACATTTTGCTCTCAAGCTG		
	Fluorescence probe	FAM-TTGCTGCTGCTTGACAGATT-TAMRA		
Haramoto et al., 2020	N_Sarbeco_F1	CACATTGGCACCCGCAATC	Nucleocapsid protein (N)	(Corman et al., 2020)
20 June 2020	N_Sarbeco_R1	GAGGAACGAGAAGAGGCTTG		
	N_Sarbeco_P1	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ		
	NIID_2019-nCOV_N_F2	AAATTTTGGGGACCAGGAAC	Nucleocapsid protein (N)	(Shirato et al., 2020)
	NIID_2019-nCOV_N_R2ver3	TGGCACCTGTGTAGGTCAAC		
	NIID_2019-nCOV_N_P2	FAM-ATGTCGCGCATTGGCATGGA-BHQ1		
	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	Nucleocapsid protein (N)	Centers for Disease Control and
	2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG		Prevention, CDC (US 2020)
	2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1		
	2019-nCoV_N2-F	TTACAAACATTGGCCGCAAA		
	2019-nCoV_N2-F	GCGCGACATTCCGAAGAA		

Note: FAM: 6-carboxyfluorescein; BBQ: blackberry quencher; BHQ (and BHQ1): Black Hole Quencher 1
[a] N1 and N2 primer sets only

5.4. Development of rapid detection methods

Regulations related to wastewater treatment do not commonly require routine monitoring of viruses before wastewater discharge to receiving bodies of water. However, several studies have shown that pathogenic viruses remain abundant in wastewater and sludge even after treatment. Monitoring of viruses is not widely conducted in wastewater treatment plants because of the cost, time and complexity involved in conducting detection methods. The need for rapid, highly accurate, and sensitive methods at optimum cost arises.

The possibility of use of *in situ* monitoring tools is to be further explored in this context. The use of biosensors to detect viruses in environmental samples has been previously studied but there is only a limited number of studies on application of this approach to detect viruses in wastewater. Chung et al. (2019) utilized a microfluidic paper analytic device (μPAD) to detect NoV in environmental water samples including reclaimed wastewater. In the latter study, NoV viruses were detected without prior sample concentration and nucleic acid amplifications. The NoV were captured by the μPAD, where antibody-conjugated fluorescent submicron particles were also added. The use of μPAD relies on the correlation between the virus concentration and the number of aggregated particles formed by antibody-antigen binding. Additional visualization methods were developed in the latter study to make the detection method suitable to field

applications. The quantity of NoV in the samples was determined using a smart-phone fluorescence microscope and a mobile graphical user interface (GUI) application. However, this method can only be used to detect specific viruses whose detection depends on the antibody used. The use of biosensors such as μPAD is a potential tool for rapid in detection of viruses in environmental water samples such as wastewater and is still in the nascent stage.

Along with the molecular detection methods, tools such as paper-based devices for rapid, *in situ*, and sensitive detection can possibly provide timely information about the circulation of emerging pathogenic viruses such as SARS-CoV-2 in wastewater and in affected communities (Mao et al., 2020).

6. Conclusions

There is a wide range of detection and quantification methods that have been used to detect and quantify viruses in wastewater and sludge samples. Molecular methods have been extensively used in the recent studies for specific and sensitive detection of viruses in wastewater. However, it is notable that there are instances when the use of at least two detection methods proved helpful in monitoring the abundance and diversity of viruses in wastewater and sludge. This is to ensure that wider range of viruses found in samples are detected and quantified. Analysis of viability of viruses is also needed for a complete information regarding their occurrence in wastewater and sludge samples.

Pre-treatment methods must be chosen according to the characteristics of the wastewater or sludge sample matrices. In applying these techniques, methods to reduce effects of inhibitors that are originally present in the wastewater or sludge samples, or in the reagents must be considered. Recent developments that resulted in increasing efficiency of detection of viruses in sludge samples focused on their release from the highly aggregated sludge matrix. Addition of optimized ultrasonication step to the traditional adsorption-elution method, and the use of dispersants and enzymes to break down sludge flocs are the current developments in the sludge sample processing methods.

High-throughput sequencing has been used in the comprehensive studies of wastewater treatment systems, allowing for a large number of samples to be processed simultaneously. This approach has been shown to be important in studying the fate of viruses during and after wastewater and sludge treatment processes, in evaluating efficiencies of the treatment systems, and in identifying paths of transmission of human diseases through exposure to wastewater or sludge.

Recent studies have illustrated the importance of data of molecular methods for studying the previous, ongoing and possible disease outbreaks. Detection and quantification methods developed to ascertain the occurrence and identities of viruses in wastewater clearly play an important role in using the WBE approach in studying the circulation of infectious viruses such as SARS-CoV-2 in exposed communities.

Research needs identified in this review include further validation of the processing methods for wastewater samples, with a specific focus on ascertaining concentrations of emerging viruses such as SARS-CoV-2 using seeded samples. Further validation of assays that use primers specific to SARS-CoV-2 in wastewater matrices should also be conducted. Further studies should also include SARS-CoV-2 infectivity assays of influent, effluent and sludge samples. Future research on the presence of viruses in aerosols emitted from wastewater or sewage systems must also be conducted.

The development of rapid, and sensitive methods could also be considered to complement the molecular methods to provide timely information about presence and abundance of viruses in wastewater samples, as part of routine monitoring or as part of a WBE approach to investigate ongoing or emerging disease outbreaks.

CRediT authorship contribution statement

Mary Vermi Aizza Corpuz: Data curation, Writing - original draft, Writing - review & editing. **Antonio Buonerba:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing. **Giovanni Vigliotta:** Data curation, Writing - original draft, Writing - review & editing. **Tiziano Zarra:** Writing - review & editing. **Florencio Ballesteros:** Writing - review & editing. **Pietro Campiglia:** Writing - review & editing. **Vincenzo Belgiorno:** Writing – original draft, Writing - review & editing, Funding acquisition. **Gregory Korshin:** Writing - original draft, Writing - review & editing. **Vincenzo Naddeo:** Conceptualization, Project administration, Supervision, Funding acquisition, Validation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

On behalf of all authors, I declare that there is no conflict of interest. All authors have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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