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Review

Detecting SARS-CoV-2 in sludge samples: A systematic review

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HIGHLIGHTS

• The main method associated with increased SARS-CoV-2 load was the PEG + NaCl.
• The average positive rate for SARS-CoV-2 in sludge samples was 61 %.
• The highest rate of positive sludge samples for SARS-CoV-2 was detected in Turkey.
• A correlation was found between the sludge volume and the SARS-CoV-2 load.
• No correlation was found between the sample number and the SARS-CoV-2 load.

ABSTRACT

Aims: This paper aims to review the main sludge concentration methods used for SARS-CoV-2 detection in sewage sludge samples, discussing the main methods and sample volume related to increased viral load. In addition, we aim to evaluate the countries associated with increased positivity rates for SARS-CoV-2 in sludge samples.

Methods: This systematic methodology was registered in PROSPERO and followed the PRISMA guidelines. The search was carried out in the SciELO, PubMed/MEDLINE, Lilacs, and Google Scholar databases in January–March 2022. Quantitative studies with conclusive results were included in this review. Concentration methods (polyethylene glycol (PEG), PEG + NaCl, gravity thickening, skimmed milk flocculation, ultrafiltration, filtration using charged filters, primary sedimentation, and anaerobic digestion), as well as detection methods (RTqPCR and reverse transcription droplet digital PCR assay) were evaluated in this review. The SPSS v23 software program was used for statistical analysis.

Results: PEG (with or without NaCl addition) and gravity thickening were the most used sludge concentration methods to detect SARS-CoV-2. The main method associated with increased viral load (>2.02 × 10^4 copies/mL) was PEG + NaCl (p < 0.05, Mann-Whitney test). The average positivity rate for SARS-CoV-2 in sludge samples was 61 %, and a correlation was found between the sludge volume and the viral load (r = 0.559, p = 0.03, Spearman correlation).

Conclusion: The sludge volume may influence the SARS-CoV-2 load since the virus can adhere to solid particles in these samples. Other factors may be associated with SARS-CoV-2 load, including the methods used; especially PEG + NaCl may result in a high viral load detected in sludge, and may provide a suitable pH for SARS-CoV-2 recovery.
Coronavirus disease 2019 (COVID-19) is responsible for severe respiratory comorbidities (pneumonia and lung failure), in addition to gastrointestinal symptoms (Benvenuto et al., 2020). As COVID-19 was a global threat, understanding the ongoing situation and developing strategies to contain the virus spread are needed (Ahn et al., 2020). A study from China showed that most patients (80.9 %) had asymptomatic infection, but had a high viral load in the initial phase of infection and can eliminate the virus in the feces, thereby constituting a great challenge to contain the spread of COVID-19 (Helmy et al., 2020).

SARS-CoV-2 is a ribonucleic acid (RNA) virus containing about 30 different viral proteins, including the S glycoprotein and the viral nucleocapsid N protein (Ceraolo and Giorgi, 2020). The N protein regulates the viral replication process and is highly detected in the blood and feces of infected patients, while the S protein of SARS-CoV-2 is necessary for the viral interaction with ACE2 receptors in the host cell, allowing the SARS-CoV-2 to infect human cells and spread rapidly (Duan et al., 2020). Another protein associated with the RNA virus is the enzyme replicase (RNA polymerase), which is produced by the infected cell when the negative RNA molecule (RNA-) is synthesized from the positive RNA. Receptors related to the viral entry into the human cell can be found in gastrointestinal system organs, which justify viral detection in wastewater samples (Deidda et al., 2021).

The SARS-CoV-2 have affinity with the biosolids present in these samples (Balboa et al., 2021). In this context, municipal wastewater constitutes a complex matrix that includes suspended solid materials, nutrients, dissolved colloidal, and biodegradable organic matter, among others. Most of the solids are separated from the water to the sludge line in wastewater treatment plants (WWTP). Kocamemi et al. were one of the first to detect SARS-CoV-2 in primary sludge samples. Primary sludge is formed during gravity settling of wastewater in primary settling ponds, while activated sludge is the surplus part of the biomass growing in the secondary treatment process (Kocamemi et al., 2020).

The two sludge types (primary and secondary) can be concentrated in the thickener, from where they are sent to the sludge treatment unit (Balboa et al., 2021). Thickenened sludge, digested sludge, and digested sludge plus thermal hydrolysis constitute the treated sludge. Many WWTPs have a first solid separation stage (primary decanter), and then a secondary decanter separates activated sludge from clarified water (Samer, 2015). Primary and thickened sludge samples are considered adequate for detecting and quantifying SARS-CoV-2, as the virus can spread to the environment through aerosols generated in WWTPs. Although SARS-CoV-2 is not an airborne virus, it can attach to particles present in wastewater aerosols (Kitajima et al., 2020).
the high viral load detected on sludge samples. The study selection and data extraction were performed by analyzing the titles selected from the search strategy, analyzing the abstract, and finally, analyzing the full text.

The obtained data were: a) study identification; b) methods used; c) viral load; d) genes evaluated; e) city/country where sludge was obtained; f) sample number; g) sample volume; and h) primary sludge parameters. Only studies containing all essential data were selected for statistical analyses. We calculated the average of available values regarding: the viral load (number of copies per ml), positivity rate, Ct values, volume, and sample number. The viral load median was used (2.02 × 10^4) to verify which methods were associated with increased viral load, since we included non-parametric data in our analysis and the viral load values were available in all of the included studies. The average percentage of positive samples for SARS-CoV-2 in countries’ sludge was also calculated.

The ROBIS tool (Risk of Bias in Systematic Reviews) is used to assess the risk of bias in systematic reviews (Whiting et al., 2016). Almost all questions were answered as “Yes” or “Probably Yes”, and no potential risk of bias in specifying eligibility criteria was identified. Studies “without information” regarding the data used in graphs and tables were removed from this work. In addition, a sensitive and appropriate search strategy was used and the study’s characteristics were sufficient to interpret the results. Finally, the searches in databases were performed by three reviewers, and the obtained data were confirmed during a second analysis. After analyzing these data, the relevant information was presented descriptively through tables and discussed in the review. The quantitative data was compiled in the SPSS v. 26.0 program for statistical analysis, in which the Mann-Whitney test and Spearman’s correlation were used.

### 3. Results

The search method retrieved 5539 studies related to methods used for concentrating sludge samples to detect SARS-CoV-2. Among these studies, 1 study resulted from a search in the SciELO database, 376 were from PubMed/MEDLINE, 2 were from Lilacs, and 5160 articles were from Google Scholar. After the first evaluation, 2487 studies were excluded by analyzing the titles and abstracts, in both cases because they were not consistent with the review theme and aims. Next, 1868 articles were excluded for not meeting the inclusion criteria or meeting the exclusion criteria, and 1167 articles were excluded for being duplicates. In the end, 17 articles were included in this review (Table 1, Table 2).

#### 3.1. Main methodologies used for sludge processing/concentration and SARS-CoV-2 detection

Researchers have been using several methods for wastewater sludge concentration to detect and quantify SARS-CoV-2 in these samples. Polyethylene glycol (with or without NaCl addition) and gravity thickening were the most used concentration methods, corresponding to 8 out of 17 studies (Fig. 1). In addition, the main method associated with increased SARS-CoV-2 load (>2.02 × 10^4 copies/ml) was Polyethylene glycol + NaCl (p < 0.05 when compared with other methods; Fig. 2).

In addition, few studies (18 %) quantify the genetic material by spectrophotometry or fluorometry (QuBit) before RT-qPCR to normalize all samples to a comparable nucleic acid concentration. Only 2 studies performed RT-ddPCR analysis and the majority of studies performed RT-qPCR to detect SARS-CoV-2 in sludge samples from treatment plants.

#### 3.2. Positivity rate and SARS-CoV-2 load in sludge samples

The average positive rate for SARS-CoV-2 in sludge samples was 61 % (Table 3), and the average viral load detected in the studies was 2.56 × 10^5 (the highest viral load detected in the included studies was 2.06 × 10^6). The average Ct for the N1 primer in sludge samples was 32.41, while the average CT for the N2 primer was 32.7 (p > 0.05). Other genes, including RBD2, ORF1ab, OC43, MS2, E, S, and IP4 can be also detected in sludge samples. However, N genes were associated with higher viral load (>2.02 × 10^4 copies/ml) when compared with other primers (p < 0.05). Almost all studies quantified N primers, especially N1 primers (88 % for N1 and 76 % for N2). Although N2 values were consistently lower than N1, the N2 trend was similar to N1.

### Table 1

City/country of collected sludge, sample number, volume, and evaluated parameters in studies regarding SARS-CoV-2 detection on sludge samples. NA - not available.

| Reference                | City/country                  | Sample number (collected) | Volume (mL) | Parameters evaluated                             |
|--------------------------|-------------------------------|---------------------------|-------------|--------------------------------------------------|
| Peccia et al., 2020      | New Haven, Connecticut, USA.  | 73                        | 40          | Total solids                                     |
| Kocamemi et al., 2020    | Istanbul, Turkey              | 9                         | 250         | Suspended solids                                 |
| Serra-Compte et al., 2021| Barcelona, Ourense, Alicante, Sabadell, and Múrcia. | 107 (56 - non treated sludge; 51 - treated sludge) | 500         | NA                                               |
| Balboa et al., 2021      | Ourense, Northeast Spain      | 35                        | 250         | Total solids, suspended solids, and pH           |
| Bardi and Olaeeh, 2021   | Babol, Iran.                  | 7                         | 250         | Temperature, pH, dissolved oxygen, total solids, suspended solids, protein, lipids, carbohydrates, cellulose, Na, Ca, C, N, Zn, Co, Ni, Fe |
| Carrillo-Reyes et al., 2021| Querétaro, Mexico           | 22                        | 15          | Suspended solids                                 |
| Bhantarai et al., 2021   | Utah, USA.                   | 7                         | 250         | Suspended solids, temperature, pH               |
| Philo et al., 2021       | Seattle, Washington, USA      | 45                        | 1000        | pH, suspended solids                             |
| Khan et al., 2021        | Burlington, VT, USA          | NA                        | 2000        | Temperature                                     |
| D’Aoust et al., 2021a    | Ottawa, Ontario, Canada      | 23                        | NA          | NA                                               |
| D’Aoust et al., 2021b    | Ottawa, ON, and Gatineau city, QC, Canada | 24                  | 250         | Total solids and suspended solids                |
| Espinosa et al., 2022    | Belo Horizonte, Minas Gerais, Brazil | 13                  | NA          | pH, temperature, oxygen demand, total solids, suspended solids, total nitrogen, ammonium nitrogen, bacterial indicators analysis |
| Yanaç et al., 2022       | Winnipeg, Canada             | 45                        | 50          | Total solids                                     |
| Zulli et al., 2022       | Connecticut, USA             | 1698                      | 45          | NA                                               |
| Chakraborty et al., 2021 | Chennai, Tamil Nadu, India.  | 17                        | 250         | Dissolved oxygen, conductivity, pH, total solids, salinity, and biochemical oxygen demand |
| Zhao et al., 2022        | Wuhan, China                 | 39                        | 50          | Cytopathic effect                                |
| Pourakbar et al., 2022   | Maragheh, Azerbaijio Oriental, Iran | 16                  | 1500        | Retention time of solids in the aerobic section and in the anaerobic sludge digester |

Table 1 contains information regarding city/country in which sludge was collected, sample number collected, volume, and main evaluated parameters. Table 2 contains information about the studies’ methods, including the following concentration methods: PEG, PEG + NaCl, gravity thickening, skimmed milk flocculation, ultrafiltration, filtration using charged filters, primary sedimentation, and anaerobic digestion, as well as detection methods: RTqPCR and reverse transcription droplet digital PCR assay (RT-ddPCR).
Table 2
Main information regarding the methods used for sludge concentration; methods used for SARS-CoV-2 extraction and detection in sludge samples; viral load detected; positivity rate, Ct, and genes evaluated in studies regarding SARS-CoV-2 detection on sludge samples.

| Reference | Sludge processing/treatment and concentration | RNA extraction method | RNA detection and quantification | Ct, viral load (average), and positivity rate | Evaluated genes |
|-----------|---------------------------------------------|-----------------------|---------------------------------|---------------------------------------------|-----------------|
| Peccia et al., 2020 | Gravity thickener | Kit RNeasy PowerSoil Total RNA, Qiagen | -Spectrophotometry (NanoDrop, Thermo Fisher Scientific) | Ct: 34.6 for N1 primers and 34.5 for N2 primers. 2,31 × 10^5 copies/mL | N1 and N2 |
| Kocamemi et al., 2020 | Primary sedimentation, gravity thickening, mechanical dewatering, and PEG | Roche MagNA pure LC total nucleic acid isolation kit using Roche MagNA pure LC system (Penzberg, Germany) | -Thermo NanoDrop 2000c (Penzberg, Germany). | Ct: 34.8 2.11 × 10^5 copies/mL | RdRp |
| Serra-Compte et al., 2021 | Ultrafiltration and PEG 6000 precipitation for French samples | -NucliSENS® kit (BioMérieux) for French samples | -French: RNA UltraSense™ One-Step Quantitative RT-PCR System kit and SuperScript III One-Step RT-PCR System (Invitrogen). | -Murcia, Sabadell, and Alicante samples: 7,2 × 10^3 copies/mL-Bardia and Oliaei, 2021 Ultrafiltration and adsorption onto Amicon for Ourense samples. | IP4, E, N1, ORF1b, S and RdRp |
| Balboa et al., 2021 | Gravity thickening, thermal hydrolysis, anaerobic digestion, and polyethylene glycol (PEG) | MicroLab Starlet IVD and the STARMag Universal Cartridge Kit (Seegene, Seoul, South Korea) | -One-step multiplex RT-qPCR Allplex system™ | Ct: 32.1 for N primers -Primary Sludge: 10,775 copies/mL -Thickened sludge: 8,52 copies/mL | Genes E, N, ORF1b, RdRp, and S |
| Bardi and Oliaei, 2021 | Polyethylene glycol precipitation 9000 (80 g/L) using NaCl (17.5 g/L) | NucleoSpin® RNA Virus (Macherey-Nagel GmbH & Co. KG, Germany). | -Qubit Fluorometer (Invitrogen) | Ct: 35,34 for ORF1b and 35,51 for N primers 28,7 × 10^4 copies/mL | ORF1b, N, and S |
| Carrillo-Reyes et al., 2021 | Ultrafiltration and adsorption onto a membrane. (The concentration step was not applied for sludge samples.) | RNeasy Power Microbiome kit (Qiagen, Germany) | -Qubit Fluorometer (Invitrogen) | Ct: 34,8 2.11 × 10^5 copies/mL | RdRp, S, and N |
| Bhattarai et al., 2021 | Filtration methods using charged filters by Method A (sample acidification using 2 N HCl; pH 4) and Method B (supplement with MgCl2) | Kit AllPrep Power Viral DNA/RNA (Qiagen, Hidden, Germany). | -TaqPath Covid-19 RT-PCR Kit (Applied Biosystems) | Ct: 31.18 for N1 and 32.47 for N2 10,2 copies/mL | N1 and N2 |
| Philo et al., 2021 | Bag-mediated filtration system (BMFS) with and without Vertrel™ extraction, skimmed milk flocculation with and without Vertrel™ extraction, PEG, and ultrafiltration | QiAamp Viral RNA Mini Kit (QIAGEN, Germantown, MD, USA) | -TaqUniversal Probes OneStep Kit (Bio-Rad Laboratories, Hercules, CA, USA) | Ct: 25,82 for N primers 5,62 × 10^4 copies/mL | OC43, N1, N2, and N3 |
| Khan et al., 2021 | Filtration using a 2-μm membrane + PEG + NaCl | NucleoMag DNA/RNA kit (744220.1; Macherey-Nagel). | -Luna SARS-CoV-2 RT-qPCR Multiplex assay kit | Ct: 31.18 for N1 and 32.47 for N2 | N1 and N2 |
| | | | -One-Step RT-ddPCR Advanced Kit for Probes | RT-qPCR: 2.06 × 10^6 N1 gene copies/mL RT-qPCR: 72 % for N1 and 82 % for N2; RT-ddPCR: 55 % for N1 and 85 % for N2 | |
D’Aoust et al., 2021a  
PEG 9000 using NaCl  
RNeasy PowerMicrobiome Kit (Qiagen, Germantown, MD)  
Reliance One-Step Multiplex RT-qPCR Supermix (Bio-Rad, Hercules, CA)  
Ct: 35.9  
N1: 2.03 × 10^4 copies/mL  
N2: 2.01 × 10^4 copies/mL  
- PGS samples: 13.34 copies/mL (RT-ddPCR) and 16.71 copies/mL (RT-qPCR)  
- PCS samples: 3.35 copies/mL (RT-ddPCR) and 13.04 copies/mL (RT-qPCR)  
N1: 92.7 % positive samples for PCS and 79.2 % positive samples for PGS  
N2: 90.6 % positive samples for PCS and 82.3 % positive samples for PGS

D’Aoust et al., 2021b  
Filtration, PEG (80 g/L) + NaCl (0.3 M g/L)  
RNeasy PowerMicrobiome Kit (Qiagen, Germantown, MD)  
Singleplex, probe-based, one-step RT-qPCR (Reliance One-Step Multiplex RT-qPCR Supermix (Bio-Rad, Hercules, CA) using the 2019-nCoV Assay-RUO probe/primers mixes (IDT, Kanata, Canada) and RT-ddPCR)  
PCT: 13.34 copies/mL (RT-ddPCR) and 16.71 copies/mL (RT-qPCR)  
PCS: 3.35 copies/mL (RT-ddPCR) and 13.04 copies/mL (RT-qPCR)  
N1: 92.7 % positive samples for PCS and 79.2 % positive samples for PGS  
N2: 90.6 % positive samples for PCS and 82.3 % positive samples for PGS

Espinosa et al., 2022  
Adsortion-extraction method  
AllPrep PowerViral DNA/RNA kit (Qiagen®, Germany)  
RT-qPCR using MasterMix iTaq Universal Probes One Step Kit (Biorad)  
Ct: 28.82  
28 copies/mL  
4/6 positive samples (66.66 %)  
N1: 1.379.78 copies/mL  
N2: 113.67 copies/mL  
N1: 18/30 positive samples (60 %)  
N2: 16/30 positive samples (53.33 %)  
N1 and N2

Yanaç et al., 2022  
Ultratulation and organic flocculation  
RNeasy PowerMicrobiome Kit (Qiagen Sciences, Inc., Germantown, MD, USA)  
RTqPCR using TaqMan Fast Virus 1-Step Master Mix (Life Technologies, 199 Carlsbad, CA, USA),  
Ct: 28.82  
28 copies/mL  
4/6 positive samples (66.66 %)  
N1: 1.379.78 copies/mL  
N2: 113.67 copies/mL  
N1: 18/30 positive samples (60 %)  
N2: 16/30 positive samples (53.33 %)  
N1 and N2

Zulli et al., 2022  
Primary clarifier, gritting primary sludge with hydrocyclones, gravity thickening, and primary sedimentation  
RNeasy PowerSoil Total RNA kit (Qiagen) and Zymo, Quick-RNA Fecal/Soil Microbe Microprep, wastewater protocol  
Spectrophotometry, purity assessed by $\text{A}_{260/280}$ absorbance ratio and concentration adjusted to 200 ng μL$^{-1}$ (NanoDrop, Thermo Fisher Scientific) and one-step RT-qPCR kit (BioRad iTaq™ Universal Probes One-Step Kit)  
Ct: 31.21 for N1 primer and 31.95 for N2 primer (SED)  
1475 × 10^6 copies/mL  
3/4 positive samples (75 %)  
N1 and N2

Chakraborty et al., 2021  
Composite (COM), supernatant (SUP), sedment (SED), and syringe filtration (SYR).  
QIAamp Viral RNA mini k.it.  
PrimeScript II qRT-PCR mix (Takara Bio, USA, Cat no#RR600A). IDT 2019-nCoV CDC-USA kit (Integrated DNA Technologies, Coralville, IA, USA, Cat no#10006770)  
Ct: 31.21 for N1 primer and 31.95 for N2 primer (SED)  
1.475 × 10^6 copies/mL  
3/4 positive samples (75 %)  
N1 and N2

Zhao et al., 2022  
PEG 8000 (8 %, W/V, Millipore Sigma, USA) + NaCl (0.3 mol/L, Millipore Sigma, USA)  
Direct-zol RNA Kit (ZymoResearch, USA)  
CFX96 Touch Real-time PCR Detection System (Bio-Rad, USA) using a PrimeScript RT-qPCR Kit (Takara, China)  
1.33 × 10^5 copies/mL  
4/39 positive samples (10.25 %)  
RB02 and ORF1ab

Pourakbar et al., 2022  
Anaerobic digestion and PEG  
RN3a Virus Kit, (ROJE Technologies)  
COVID-19 ONE-STEP RT-PCR kit, PichInz Teb Diagnostics  
Ct: 33 for RdRp primer and 34.5 for N primer  
Primary sludge: (8.1 × 10^4 copies/mL)  
Secondary sludge: (1.9 × 10^5 gene copies/mL)  
8/16 positive samples (50 %)  
RdRp and N

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3.3. Influence of other factors on SARS-CoV-2 detection in sludge samples

Despite the methods used to concentrate samples, several factors can also affect SARS-CoV-2 detection in primary sludge, including the sample number, input volume, solids amount, pH, and temperature. The sample number varies between studies, with an average of 136 sludge samples included (treated and untreated sludge). On average, 446.6 mL of sludge was used in the experiments (Table 3) and a moderate and directly proportional correlation (\( r = 0.559, p = 0.03 \)) was found between the volume of sludge used in the treatment/concentration stages and the viral load detected (Table 3).

In this sense, the average volume of sludge samples with high viral load was 573.75 mL, while the average volume of samples with low viral load was 265.62 (\( p < 0.05, \text{Man-Whitney test, Fig. 3} \)). The concentrated sludge volume may influence the content of solid particles present in this sample since the virus can adhere to these particles. However, no correlation was found (\( r = -0.132, p = 0.654 \)) between the number of sludge samples used and viral load (Table 3). Moreover, the main parameters evaluated in sludge samples of selected studies were suspended solids (8 studies), total solids (7 studies), and pH (6 studies) (Fig. 4).

In addition, the number of cases where the samples are collected can also affect the SARS-CoV-2 positivity rate in sludge. Therefore, we evaluated the SARS-CoV-2 positivity rate in sludge obtained in 9 different countries to compare with the number of COVID-19 cases in these places. The highest rate of positive sludge samples for SARS-CoV-2 was detected in Turkey, India, USA, and Brazil (Fig. 5), corroborating with the epidemiological data observed in these countries (WLE, 2022). The Mann-Whitney test found a higher viral load in the “Turkey”, “India”, “USA”, and “Brazil” groups when compared with “China” sludge (\( p < 0.05; \text{Fig. 5} \)). The Mann-Whitney test did not find statistical differences when comparing other countries (\( p > 0.05 \)).

This result would be expected since Turkey, India, the United States, and Brazil showed a greater number of COVID-19 cases in the same period in which high concentrations of the virus were detected in the sludge (>15 million cases), while China had a significantly lower number of cases (<200,000 cases) when compared to countries with a high COVID-19 incidence (https://www.worldlifeexpectancy.com/world-coronavirus-report).

4. Discussion

4.1. Main methodologies used for sludge processing/concentration and SARS-CoV-2 detection

PEG precipitation is the most commonly used concentration method due to its simplicity, selectivity, and resistance to PCR inhibitors in

![Fig. 1. Main methodologies used for sludge sample processing and concentration included in studies regarding SARS-CoV-2 detection on sludge samples.](image)

![Fig. 2. Main methods associated with increased viral load (>2.02 × 10^4 copies/mL) included in studies regarding SARS-CoV-2 detection on sludge samples. Lines represent statistical differences between the “Polyethylene glycol + NaCl” group and other groups. *p < 0.05 (Man-Whitney test).](image)

| Table 3
| Main values (descriptive analysis) obtained in studies regarding the SARS-CoV-2 detection on sludge samples and correlation analysis (generated by SPSS v23) between viral load (copies/mL), sludge volume (mL), and sample number. |
|---|---|---|
| **Descriptive analysis** | **Bivariate analysis** | **(Spearman correlation)** |
| Viral load (average) | 2.56 × 10^5 copies/mL | Coefficient correlation |
| Viral load (median) | 2.02 × 10^4 copies/mL | Viral load and sample number |
| Positive samples (%) | 61 % | p value |
| Volume (average) | 446,6 mL | Viral load and sample number |
| Sludge sample number (average) | 136 | Coefficient correlation |
| * Represents p < 0.05. | | Viral load and volume |
wastewater (Kumar et al., 2021). PEG is also highly used for sludge sample concentration since it can reduce the processing time of the results in half, contributing to implement an early detection system for SARS-CoV-2 (Khan et al., 2021). RNA recovery using the PEG-NaCl method depends on several factors, including sample volume, percentage of PEG-NaCl addition, incubation period, and storage duration (usually at 4 °C after sample collection). Using sodium chloride (NaCl), the DNA fragments can be separated by their size according to the final PEG concentration variation (Khan et al., 2021; He et al., 2014). Therefore, the addition of NaCl may result in a higher detection rate when compared with common PEG (without NaCl), however, more studies are needed to validate this statement.

Thickening and dewatering (belt presses, centrifuges, filter presses) are highly used physical or mechanical units to reduce the moisture content of sludge. Sludge thickener was proposed as a suitable method for sampling sludge to detect SARS-CoV-2 aimed at WBE application (Foladori et al., 2022). Considering that enveloped viruses have an affinity toward biosolids, it was considered that the concentration of SARS-CoV-2 genetic material in the sludge can be higher than in wastewater. The concentration of SARS-CoV-2 may increase in the thickeners as a consequence of the relatively long retention time of about 24 h and the high solid content in the thickened sludge (Balboa et al., 2021). However, a long retention time in the thickener can affect the WBE due to: reduced amount of virus that can be found in the thickened sludge considering the low RNA stability over time; the delay in generating thickened sludge related to the influent flow rate; the lack of information on the daily loads (calculated considering 24-h samples); and the corresponding 24-h flow rate. Belt presses and filter presses are open devices which may cause direct exposure of operators to the viral particles during sludge management. Centrifuges instead are closed and minimize the production of aerosol and droplets. The dewatering unit could be another exposure route point for workers in WWTPs (Amoah et al., 2020).

Still regarding concentration methods, Reyes et al. verified the presence of SARS-CoV-2 RNA in influent, effluent, and activated sludge from two countries with increased proportions of positive sludge samples for SARS-CoV-2. Lines represent significant comparisons between the “Turkey”, “India”, “Brazil”, “USA” groups and “China”. ‘p < 0.05, Mann-Whitney test (Comparison Turkey and China: F = 3.49, p = 0.0002; Comparison India and China: F = 3.22, p = 0.0007; Comparison USA and China: F = 3.05, p = 0.003; Comparison Brazil and China: F = 2.96, p = 0.008).

![Fig. 3. Volume (mL) of sludge samples used for SARS-CoV-2 concentration in samples with high SARS-CoV-2 load (>2.02 × 10^4) and low SARS-CoV-2 load (<2.02 × 10^4). The boxes represent the interquartile range, the value inside the boxes represents the average volume for each group, and the bottom and top lines of the boxes are the first and third quartiles, respectively. The boundaries of the lines are the lowest and highest observation within 1.5 of the IQR of lower and upper quartiles. Asterisk represents p-value = 0.011 (Mann-Whitney test). Statistics and graphs were generated using SPSS.](image)

![Fig. 4. Main parameters evaluated in studies regarding the SARS-CoV-2 detection in sludge samples.](image)

![Fig. 5. Countries with increased proportions of positive sludge samples for SARS-CoV-2. Lines represent significant comparisons between the “Turkey”, “India”, “Brazil”, “USA” groups and “China”. ‘p < 0.05, Mann-Whitney test (Comparison Turkey and China: F = 3.49, p = 0.0002; Comparison India and China: F = 3.22, p = 0.0007; Comparison USA and China: F = 3.05, p = 0.003; Comparison Brazil and China: F = 2.96, p = 0.008).](image)
domestic sewage treatment plants located in Mexico using two different RNA concentration methods. The adsorption-based method resulted in higher RNA levels when compared with centrifugal ultrafiltration, which can be explained by the viral affinity with the sewage solid fraction (Carrillo-Reyes et al., 2021). Finally, Philo et al. evaluated different methods for concentration and recovery of SARS-CoV-2 in municipal primary wastewater and sludge from the Greater Seattle region between March and July 2020. Methods including polyethylene glycol precipitation (PEG), bag-mediated filtration (BMFS), skimmed milk flocculation, and ultrafiltration were evaluated, and curiously, skimmed milk flocculation resulted in higher positive samples (48.9 %) in undiluted assays (Philo et al., 2021).

Regarding treated samples, Serra-Compte et al. found 69 % of positivity for SARS-CoV-2 in thickened sludge, although only 1 % of digested sludge was positive. All samples obtained by anaerobic digestion followed by thermal hydrolysis tested negative. This can be explained by high temperatures applied during the treatment process, enabling the virus to be eliminated. Therefore, thermal hydrolysis can inactive SARS-CoV-2 in sludge. SARS-CoV-2 RNA was not detected in 63.6 % of samples after activated sludge treatment followed by clarification. After activated sludge treatment plus nutrient removal, SARS-CoV-2 was not detected in 81 % of samples. In addition, all sludge samples tested negative after membrane bioreactor treatment (Serra-Compte et al., 2021).

Pourakbar et al. evaluated the transfer route and inactivation of SARS-CoV-2 in activated sludge and sequential batch reactor, in which half of the residual sludge from the primary sedimentation tank was positive for SARS-CoV-2 (Pourakbar et al., 2022). In another study, the sludge from the primary clarifier had a higher number of SARS-CoV-2 copies when compared with other sludge samples from Utah, USA (Bhattarai et al., 2021). These data indicate that SARS-CoV-2 tends to be removed by sedimentation of primary clarifier sludge. The sedimentation by primary clarifiers can remove the SARS-CoV-2 from influents and effluents better than activated sludge systems and anaerobic digesters (Philo et al., 2021). The data indicate that sludge samples can have a higher positivity rate for the SARS-CoV-2 RNA when compared with influent samples. However, according to Philo, the high concentration of viral particles found in sludge samples is not comparable to virus concentration in wastewater samples since each WWTP has a different number of settling tanks, and the flow rate as well as the flocculants used can affect the SARS-CoV-2 concentration in these tanks (Philo et al., 2021).

Chakraborty et al. obtained primary sludge samples during August and September 2020 and evaluated four different concentration methods: composite (COM), supernatant (SUP), sediment (SED), and syringe filtration (SYR). The highest SARS-CoV-2 recovery rate (1.99 × 10^5 copies/mL) occurred when the sediment concentration method followed by the SUP process was used, providing comparable values to COVID-19 infection cases (Chakraborty et al., 2021).

RT-ddPCR analysis is not commonly used. Despite the high sensitivity of RT-ddPCR, Khan found that the RT-qPCR method always resulted in RNA concentrations at least one order of magnitude higher than RT-ddPCR (Khan et al., 2021). D’Aoust also found that the RT-qPCR method was associated with higher quantification of SARS-CoV-2 N1 and N2 genes in primary clarifier sludge compared with the RT-ddPCR method (D’Aoust et al., 2021a).

Both RT-qPCR and RT-ddPCR can produce false-negative results, which does not mean that RNA is absent in the sludge samples. On the other hand, these false negatives indicate that sludge samples likely need a pretreatment step, for example, sonication to release entrapped RNA into the surrounding bulk solution, which can then be enriched and recovered for analysis (Khan et al., 2021). Therefore, the appropriate uses of sludge concentration and detection methods are relevant for obtaining a consistent SARS-CoV-2 positivity rate.

4.2. Positivity rate and SARS-CoV-2 load in sludge samples

Measuring COVID-19 incidence in the population using sludge samples may result in greater sensitivity when compared with wastewater samples, since the primary decanter and sludge thickener act as concentrators of SARS-CoV-2 RNA (Balboa et al., 2021). Carrillo-Reyes observed that all effluent samples obtained in Mexico WWTPs were negative, indicating that SARS-CoV-2 RNA is removed by sewage treatment (Carrillo-Reyes et al., 2021). On the other hand, SARS-CoV-2 was detected in all sludge samples collected in Turkey in May 2020 (Kocamemi et al., 2020). It is important to highlight that the number of SARS-CoV-2 RNA copies might be higher in the primary and secondary sludge when compared with WWTPs wastewater effluents (Kocamemi et al., 2020).

This review found that N genes were associated with higher viral load compared with other SARS-CoV-2 genes. Interestingly, Zulli et al. showed that better RNA recoveries in terms of N1 and N2 were obtained with the following conditions: small sample volume (50 to 100 ml), 30 % (w/v) PEG-NaCl, short incubation time (≤ 12 h), and ≤ 24 h of storage duration (Zulli et al., 2022). On the other hand, variability in SARS-CoV-2 standard curves makes sample quantification difficult due to the long time in the sludge line, which can range from days to weeks; in addition, a certain inactivation of SARS-CoV-2 is expected before the final sludge disposal (Bardi and Oliacee, 2021). Peccia et al. suggest that viral load detected in sludge can be mainly affected by sludge handling in WWTPs. The authors found a high positivity (97 %) for SARS-CoV-2 in sludge 1–4 days before hospital admissions and 6–8 days before positive SARS-CoV-2 test results in New Haven, USA (Peccia et al., 2020).

Serra-Compte et al. evaluated the SARS-CoV-2 removal in samples processed from March to May 2020. SARS-CoV-2 was quantified in 16 WWTPs (8 in Spain and 8 in France) and treated sludge samples were used, including thickened sludge, digested sludge, and digested sludge plus thermal hydrolysis. In total, 83 % of samples containing primary, secondary, and mixed sludge tested positive for SARS-CoV-2. A small percentage of primary sludge samples were negative, which could be related to rainfall, while 57 % of secondary sludge was positive for SARS-CoV-2 (Serra-Compte et al., 2021). Due to pandemic restrictions, most industrial and commercial establishments were closed, therefore the WWTPs mainly received domestic wastewater and WWTPs industrial contamination was reduced, optimizing the detection of microorganisms in these environmental samples (Kocamemi et al., 2020).

D’Aoust et al. also analyzed primary sludge samples collected from June to August 2020 in Canada. The SARS-CoV-2 load was low (N1/N2 < 1 × 10–4 copies/PMMoV copies) at the beginning of the study. However, the N1/N2 signal increased 445 % between July 13–15 when compared with July 13–15, resulting in 2.03 × 10–4 and 3.01 × 10–4 copies of N1 and N2 genes, respectively. The normalized viral load declined after this peak; however, these data suggest strong correlations between viral load and COVID-19 surveillance measures (number of hospitalized cases, clinical positivity, and number of new daily cases). The authors found an increase in SARS-CoV-2 concentration in sludge 48 h before clinical trials and 96 h before hospitalizations due to COVID-19 (D’Aoust et al., 2021a).

In the Yañac study, primary sludge samples were directly submitted to viral RNA extraction, without a previous concentration method. Despite the low volume used in analysis, the N1 and N2 genes were found in most of the evaluated samples, indicating a high density of SARS-CoV-2 RNA in the primary sludge (Yañac et al., 2022). Sludge samples were also directly added to extraction kits in the study by Zulli to verify the relationship between SARS-CoV-2 RNA concentrations in primary sludge samples and the COVID-19 case rates in 18 cities served by 6 WWTPs over 10 months. The dynamics in reported case rates correlate with RNA concentrations in primary sludge, coinciding with the onset of October 2020 outbreaks for the corresponding cities (Zulli et al., 2022).

Lu Zhao and colleagues detected SARS-CoV-2 in wastewater, sludge, surface water, groundwater, sediment, and soil samples in Wuhan, China (April and May 2020). Samples of surplus sludge (SS), concentrated sludge (CS), and dehydrated sludge (DS) were collected at five different sites. However, a low positivity rate was observed and only one sample of dehydrated sludge tested positive. This result was consistent with the period the samples were tested, in which there was a low contamination risk in Wuhan. The authors also tried to isolate SARS-CoV-2 in positive samples, however the isolation was unsuccessful, suggesting that the
disinfection in wastewater treatment processes may effectively inactivate the SARS-CoV-2 (Zhao et al., 2022). Overall, the positivity rate of SARS-CoV-2 can vary from 10% to 100%, depending on factors to be discussed in the next topic (3.3).

4.3. Influence of other factors on SARS-CoV-2 detection in sludge samples

In this study, high volume of wastewater sludge was positively correlated with increased SARS-CoV-2 load. Corroborating with our study, Agrawal et al. suggested that the higher wastewater sample volume was (500 mL instead of 100 or 50 mL), the more uniform the concentrations of the viral genes (N, S, ORF1ab) were, especially when low incidence was reported (Agrawal et al., 2021). Low input volumes can cause biases in the detection and quantification of SARS-CoV-2 when the virus concentration in the sample is low, and the samples are not homogeneous in terms of total solids and SARS-CoV-2 distribution. Considering the high affinity of enveloped viruses to attach to solid particles (Ye et al., 2016), total solids concentration can be a determining factor for quantifying SARS-CoV-2 in primary sludge, in addition to the community infection dynamics (Yanac et al., 2022). D’Aoust et al. detected SARS-CoV-2 RNA in solids of primary clarified sludge and based on the experiments, sludge might be a preferable sample as a solids-rich medium when compared with samples from the sand chambers (D’Aoust et al., 2021a).

In this perspective, primary sludge contains high solids content with several human viruses, including new SARS-CoV-2 variants in circulation (Peccia et al., 2020). Moreover, the secondary sludge also contained increased RNA levels (10^8 higher than the influent), suggesting that the RNA migrates from liquid to the solid matrix in effluent treatment process (Carrillo-Reyes et al., 2021). Espinosa et al. evaluated the SARS-CoV-2 removal and reduction, as well as the sludge-liquid-solid partition in UASB (Upflow Anaerobic Sludge Blanket). As a result, almost 60% of the SARS-CoV-2 RNA was removed from UASB, not proceeding along with effluent for the remaining treatment, suggesting that SARS-CoV-2 has a high affinity for solids (Espinosa et al., 2022).

Balboa also pointed out the sludge as a potential site for SARS-CoV-2 detection due to its affinity for biosolids present in WWTWs. In this study, all primary sludge, secondary sludge, thickened mixed sludge, and digested sludge samples tested positive for the MS2 bacteriophage control and the viral RNA detected in the sludge were up to 20 copies/mL. This result suggests that the microorganisms were mostly retained in the sludge lines, mainly in the primary decanter, since a high content of solids can be found in these first sewage treatment stages (Balboa et al., 2021).

Although several studies evaluate solid amounts, fewer parameters are usually evaluated in sludge when compared with sewage samples, in which conductivity, ammonium, carbon, and phosphorus (among others) are commonly evaluated. This is due to the sludge treatment process in which many compounds can be easily removed. All samples treated to a pH of 10 resulted in a non-detect gene copy number for all tested samples, resulting in substantially lower recovery efficiency at a basic, rather than neutral or acidic, pH value (Balboa et al., 2021; Zulli et al., 2022). Moreover, complete inactivation of SARS-CoV-2 can be obtained at the high temperatures required for thermophilic digestion (55 °C) or thermal treatments (>100 °C) (Bardi and Oliaei, 2021). Therefore, standard handling procedures require that samples need to be heat-treated for 2 h at 65 °C, and traditional influent processing methods require that the sample be acidified to a pH of approximately 3.5 (Bhattarai et al., 2021).

It is unclear how different environmental conditions might affect the efficiency of the virus detection. However, results obtained from influent revealed that samples heated to 25 °C and 35 °C show similar SARS-CoV-2 copy numbers (1.05 × 10^4 and 1.14 × 10^4 copies/L, respectively). The samples heated to 65 °C exhibited significantly lower gene copies than samples at 25 °C and 35 °C (2.09 × 10^2 copies/L). Gene copy numbers were not detected at an incubation temperature of 75 °C, most likely due to the viral capsid’s denaturation from heat damage (Qiu, 2012; Bhattarai et al., 2021).

Bardi and Oliaei also evaluated the impacts of different temperatures and organic load (OL) on the SARS-CoV-2 concentration during anaerobic co-digestion of sewage sludge. The sludge was collected and filtered to remove impurities and coarse solids and the results showed that temperatures, as well as OL, had effects on SARS-CoV-2 viability. SARS-CoV-2 can survive up to 3.5 g of volatile solids per liter (gVS/L) at 20 °C, while viral genes are undetectable at 35 °C with the same OL. Furthermore, a low OL (1.5 gVS/L) and a high temperature (50 °C) are sufficient to reduce viral particles under undetectable concentration. The study also showed that intermediate metabolites accumulation (up to 2000 mg/L) and high temperatures can reduce the viral particle viability in the effluent (Bardi and Oliaei, 2021).

In addition, analysis of SARS-CoV-2 RNA suggests that the virus has high affinity for biological solids in activated sludge (Pourakbar et al., 2022; Graham et al., 2020; Bhattarai et al., 2021). Therefore, the solids might be a suitable site for viral RNA detection. The sludge from the treatment process is directed to the anaerobic digester, for which the solids retention time was about 30 days. It is worth noting that all samples from the digester solid phase were tested negative for SARS-CoV-2, suggesting that longer retention times and anaerobic biological processes are capable of destroying viral RNA (Pourakbar et al., 2022).

Higher RNA concentrations were observed in the secondary sludge (>0.71 × 10^4–3.1 × 10^4 copies/L) when compared to the primary sludge (0.32 × 10^4–1.3 × 10^4 copies/L) in the activation process. This result can be due to the long solids retention time in the treatment aerobic section, leading to viral RNA fixation to the biomass. High temperatures of the anaerobic digester (35–40 °C) can also provide thermal inactivation and prevent virus entry into solids. Thus, SARS-CoV-2 transmission to the environment through the liquid and solid phases can be reduced if these biological processes work correctly (Pourakbar et al., 2022).

Khan reported the effect of 4 factors on SARS-CoV-2 RNA recovery in primary sludge samples, namely: sample volume, PEG + NaCl percentage, incubation period, and storage duration at 4 °C. The study indicates that even with all factor variations, the detection protocol mainly results in false negatives for the N1 and N2 genes, suggesting that sludge samples need a pre-treatment step that releases the RNA associated with solids back into the solution before the RNA extraction and quantification (Khan et al., 2021).

Therefore, the SARS-CoV-2 dissemination within a population can be assessed by quantifying the virus in sludge samples of each country, and the detection of SARS-CoV-2 in sludge samples raises several questions about the dynamics of the virus spread in a specific country. In other words, SARS-CoV-2 detection in sludge can alert about the virus re-emergence, even when there is a decrease in symptomatic cases, functioning as an early warning system for preventive adoption of public health measures and being an epidemiological surveillance strategy for the country.

4.4. Main challenges regarding the SARS-CoV-2 detection on sludge samples

Several protocols on sludge analysis are not optimized for virus quantification, resulting sometimes in false-negative or false-positive errors. Therefore, coordinated guidelines on sampling, as well as good laboratorial practices and quality control need to be followed regarding RNA extraction, and RT-qPCR detection (Ahmed et al., 2022). It would be relevant to analyze more than one SARS-CoV-2 gene in WBE studies (i.e. N, S, ORF1ab) for better reliable results since variations in amplification of different target genes for different samples can be found. Moreover, SARS-CoV-2 RNA obtained in sludge and wastewater samples can contain both organic and inorganic inhibitors that could affect viral recovery efficiency and subsequently, virus detection (Agrawal et al., 2021).

The SARS-CoV-2 RNA concentration measured in the sludge samples may be dependent on excretion from the infected person, immunity level, city, and age. In addition, the persistence of the virus in these samples is influenced by factors such as temperature, population, drain flow rate, infected individuals, time and day of sampling, dilution effect, community...
socio-economic conditions, the extent of sanitation and facility, humidity, rainfall, among others (Venkata Mohan, 2021).

Finally, some considerations might be highlighted: The sampling station should be representative of the selected community to be studied; The sampling station should be selected at the downstream converging point of the discharge line if flowing water is being analyzed; Combined grab and composite sampling strategies can be adopted with a defined sampling frequency; Sampling frequency should be extended for not <24 h for hourly sampling and for not <7 days for daily sampling to get a true average representation of the community; For daily sampling, the time of sample should be carefully chosen to represent average values and estimation of the viral infection of the study area can be calculated based on the population discharge data (Venkata Mohan, 2021).

5. Conclusion

In summary, this review brings together recent studies regarding the main methods used for SARS-CoV-2 detection in sludge samples obtained in WWTPs. According to studies, the highest viral load (>2.02 × 10^4 copies/mL) could be detected with the PEG + NaCl method using high volumes of sludge samples (574 ml) in the concentration process. In addition, the main parameters evaluated in sludge samples were solids and pH, in which low pH and high solids concentrations can be related to increased viral detection. Fewer parameters are usually evaluated in sludge when compared with sewage samples since several substances can be easily removed during sludge treatment. However, more studies are necessary to evaluate the relationship between the SARS-CoV-2 load and other parameters which were not evaluated in this review.

CRediT authorship contribution statement

Author 1: Alice Barros Câmara
• Conceived and designed the analysis
• Collected the data
• Contributed data and analysis toll
• Performed the analysis (statistical analysis)
• Wrote the paper

Author 2: Júlia Bonfante
• Collected the data
• Contributed data

Author 3: Marília da Penha
• Designed the analysis
• Collected the data

Author 4: Sérvio Túlio Alves Cassini
• Conceived the analysis
• Contributed data
• Review the paper

Author 5: Regina Keller
• Conceived the analysis
• Contributed data
• Review the paper

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Data availability

The data that support the findings of this study are available from the corresponding author, on special request.

Declaration of competing interest

The authors declare no conflict of interest, financial or otherwise.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.160012.

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