Supplemental Material

Supplemental Methods

A. Determination of preliminary RNA extraction conditions with magnetic beads using a factorial design

A screening statistical design was used to select conditions for the RNA extraction of ASCV-2 from wastewater and maximum recovery efficiency for the RT-qPCR. Thus, a full-factorial design of three factors was used to assess the magnetic bead-based extraction protocol in RNA extraction. A total of eight experiments were performed according to the experimental design shown in Table S2 which allowed evaluation of all possible combinations of the levels across the studied factors. Moreover, the eight experiments were carried out in a random order to limit the occurrence of potential bias. The three independent variables in this study were concentration of magnetic beads (µL mL^{-1} sample) (x_1), RNA elution temperature (°C) (x_2), and water matrix (DI water and wastewater) ($x3$). The dependent variable was evaluated as ASCV-2 recovery efficiency (%), calculated based on the number of copies quantified for each experimental run by RT-qPCR using Equation S1:

$$\text{Recovery efficiency} = \frac{\text{Total viral RNA gene copies recovered}}{\text{Total viral RNA gene copies spiked}} \text{x} 100 \qquad \text{Eq. } S1$$

The response variable in the full-factorial design was modeled as a linear combination of the main effects and two-way interactions, and residuals were approximately Gaussian and homoscedastic.

The eight samples needed for the experimental design were prepared by spiking ASCV-2 in either DI water or wastewater to a concentration of $1x10^3$ GU mL^{-1}. The 24-h composite wastewater sample used for these experiments was collected in a volume of 1 L on August 19, 2020, from the influent of the Halifax WWTF and transported to Dalhousie University on ice. Then, an aliquot of 45 mL was transferred to a 50-mL centrifuge tube and was immediately spiked with ASCV-2. The spiked wastewater aliquot was mixed thoroughly and incubated at 4 °C for 30 min prior to RNA extraction.

All RNA extractions were performed with reagents provided by LuminUltra Technologies Ltd (Fredericton, NB, CA). In a 15-mL centrifuge tube, 6.5 mL of Lysis buffer was added to 1 mL of spiked wastewater sample, vortexed for 30 sec, and immediately incubated at 30 °C for 10 min. A volume of 3.5 mL EtOH was added to the lysed sample; the tube was gently inverted five times to mix thoroughly and then spiked with 100 µL binding beads. The mixture was vortexed for 30 sec and incubated again at 30 °C for 10 min. The magnetic beads were precipitated by applying a magnet, and the supernatant was discarded. The magnetic beads were transferred to a 2-mL microcentrifuge tube and washed three times with 1 mL of Wash I solution. A second

wash step using 1 mL of Wash II solution was carried out three times. For each wash, the magnetic beads were vortexed for 30 sec, and the supernatant was discarded after magnet precipitation of the beads. Once washed, the tubes containing the magnetic beads were placed at room temperature with the caps off for approximately 1 h to evaporate residual EtOH. Then, 50 µL of elution buffer (preheated to 60 °C) was added to the magnetic beads. The tube was vortexed for 30 sec and incubated at 60 °C for 5 min. Finally, the magnet was applied to ensure separation, and the elution buffer was collected into a sterile tube for analysis.

B. Assessing preliminary RNA extraction conditions using wastewater spiked with gamma-irradiated SARS-CoV-2 and Human Coronavirus surrogates in wastewater

The conditions for RNA extraction using magnetic beads identified with the factorial design were evaluated for the recovery and quantification of SARS-CoV-2 surrogates spiked at different concentrations. Wastewater samples seeded with Gamma Inactivated SARS-CoV-2/Canada/ON/VIDO-01/2020 (GI-SCV-2) and Human Coronavirus 229E (HCV 229-E) were sourced from the National Microbiology Laboratory (Winnipeg, MB, Canada). GI-SCV-2 is a positive-sense single-stranded (+ssRNA) virus member of the subgenus *Sarbecovirus*, isolated from a patient infected with COVID-19 in January 2020 that was inactivated using gamma irradiation for laboratory use. HCV 229-E is classified as a human coronavirus in the strain designated 229E, enveloped, positive-sense, single-stranded RNA virus, member of the subgenus *Duvicorinavirus* isolated from a nasal and throat swab of a patient with mild upper respiratory illness and verified through whole genome sequencing (27,271 bp in 1 segment) (Human coronavirus 229E | ATCC®). A wastewater gab sample, post-grit, was collected from the Winnipeg WWT on August 31, 2020 (Winnipeg, MB, Canada), and aliquots of the well-mixed wastewater were individually spiked with either a high ($1.8x10^6 \pm 2.0\ x10^5$ GU L^{-1}) or low ($1.8x10^4 \pm 2.0\ x10^3$ GU L^{-1}) concentrations of GI-SCV-2, and high ($1.0x10^6$ infectious units L^{-1}) or low ($1.0x10^4$ infectious units L^{-1}) concentrations of HCV 229-E. A total of three 100-mL aliquots for each spike condition together with no-spike wastewater aliquots (blanks) were prepared at the National Microbiology Laboratory and shipped to Dalhousie University on ice to be analysed within 48 h of spiking.

Each of the three aliquots received for every surrogate spike condition (low-spike, high-spike, and no-spike) was processed in duplicate for RNA extraction with the best conditions identified by the statistical design of a sample volume of 1 mL, 100 µL of magnetic beads, and an RNA elution temperature of 60 °C. Following extraction, RNA was quantified by RT-qPCR and the recovery efficiency of each surrogate spike condition was calculated using equation S1. For HCV 229-E RT-qPCR analyses, a previously published Taq-Man based RT-qPCR assay was used. HCV 229E RT-qPCR was performed in 10-µL reaction mixtures using TaqMan™ Fast Virus one-step Master Mix (Thermo Fisher Scientific, CA). The reaction mixture contained 2.5 µL of Master Mix, 10 µM of forward primer, 10 µM of reverse primer, 10 µM of probe, 1.5 µL of PCR grade water, and 5 µL of template RNA. Thermal cycling conditions consisted of RT at 50 °C for 5 min, enzyme activation at 95 °C for 20 sec, and 45 cycles of 95 °C for 3 sec followed by 60 °C for 30 sec. Standard curves were not included for HCV 229E as the target was evaluated with the Ct values only. All RT-qPCR analyses for SARS-CoV-2 and HCV 229E

included no-template controls (NTCs), Positive Controls (PCs), and samples quantified in two technical replicates. In each assay the presence of RT-qPCR inhibitors was assessed by comparing the Ct values obtained for aliquots of undiluted and diluted (10- and 50-fold) RNA extracts.

Supplemental Tables and Figures

Table S1. Water quality parameters for each wastewater sampling location. Total suspended solids (TSS), temperature, flow, total biological oxygen demand (BOD), and ammoniacal nitrogen (NH 3-N).

Sampling location	**TSS (mg L^{-1})**	**Temperature (˚C)**	**Daily flow rate (m^3 per day)**	**Total BOD (mg L^{-1})**	**Ammoniacal nitrogen (NH 3-N)**
Ontario WWTF Sample (29-Mar-21)	103.7	3.9	N/A	N/A	N/A
Halifax WWTF (3-Mar-21)	67.2	9.3	135,616	Monthly average: 119	6.69
Halifax WWTF (10-Mar-21)	77.0	9.7	80,008	Monthly average: 119	16.09
Halifax WWTF (17-Mar-21)	332.0	11.0	67,857	Monthly average: 119	18.37
Halifax WWTF (24-Mar-21)	175.2	11.4	77,841	Monthly average: 119	12.95

N/A: Not available

Table S2. Experimental factors and their levels for the 2^3 full-factorial design.

Factor	**Level**	
	-1	**+1**
x_1: Magnetic bead concentration	15 µL mL^{-1} sample	100 µL mL^{-1} sample
x_2: Elution temperature	30 ºC	60 ºC
x_3: Water matrix	DI water	Wastewater

Table S3. Sequences for primers and probes of viral surrogates used in this study.

Organism	**Sequence type**	**Sequence (5' – 3')**
SARS-CoV-2 N2 gene	N2 Forward primer	TTACAAACATTGGCCGCAAA
	N2 Reverse primer	GCGCGACATTCCGAAGAA
	N2 Probe	FAM-ACAATTTGCCCCCAGCGCTTCAG–ZEN/3IABkFQ/
HCV 229E	Forward primer	TTCCGACGTGCTCGAACTTT
	Reverse primer	CCAACACGGTTGTGACAGTGA
	Probe	FAM-TCCTGAGGTCAATGCA-NFQ-MGB

Table S4. A summary of the linear model, which explained 99% of the variation in recovery. Effect sizes (other than the intercept) represent the change in recovery associated with an increase in the corresponding independent variable from the low level to the high level (Table 1). The effect of water matrix (x_3) represents the change in recovery associated with a switch from deionized water to wastewater.

Term	**Effect size (10^4 GU L^{-1})**	***p*-value**
Intercept	10.0	<0.001
Magnetic bead concentration (x_1)	7.9	<0.001
Elution temperature (x_2)	6.2	<0.001
Water matrix (x_3)	-7.6	<0.001
x_1:x_2	3.0	0.002
x_1:x_3	-3.0	0.002
x_2:x_3	-2.0	0.016
x_1:x_2:x_3	-1.7	0.032

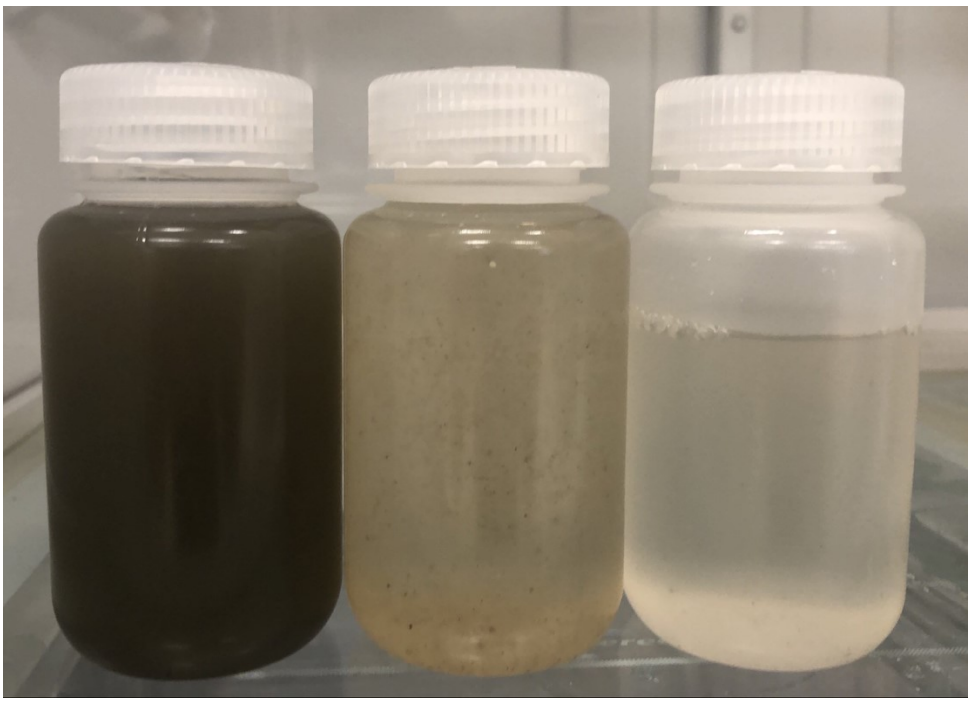

Figure S1. Visual representation of the three wastewater samples used to assess the impact of suspended solids in the recovery of HI-SCV-2 from wastewater using the Pellet Tween method. TSS concentrations of the wastewater were 332mg L^{-1} (Left); 175 mg L^{-1} (Middle); and 77 mg L^{-1} (Right).

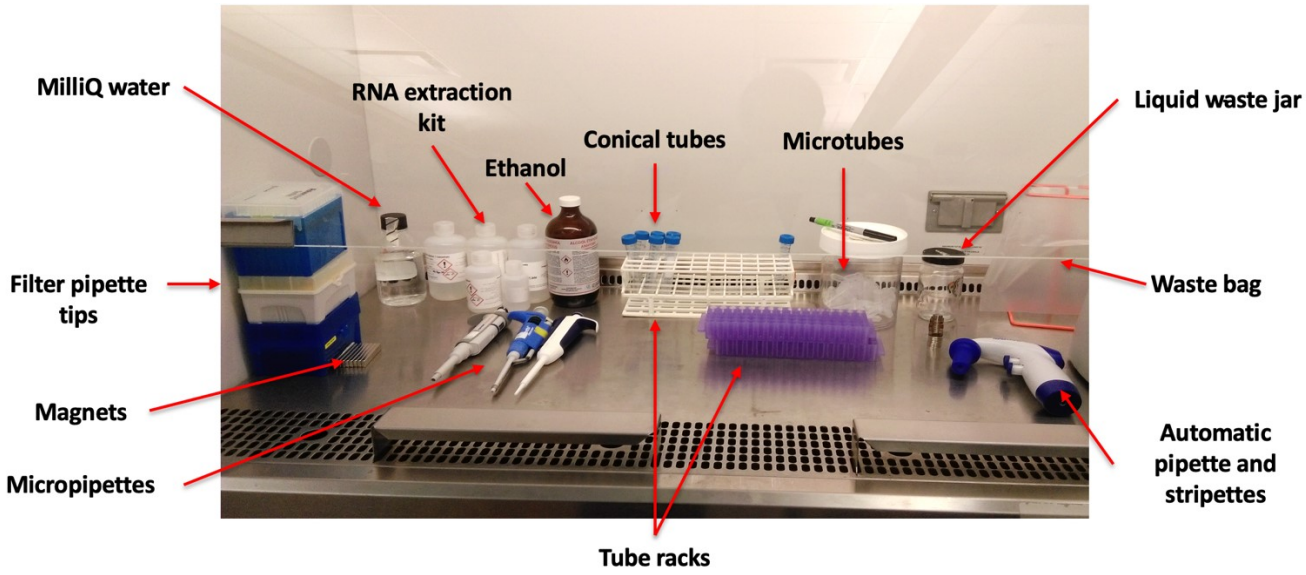

Figure S2. Material utilized to carry out the magnetic beads-based RNA extraction method.

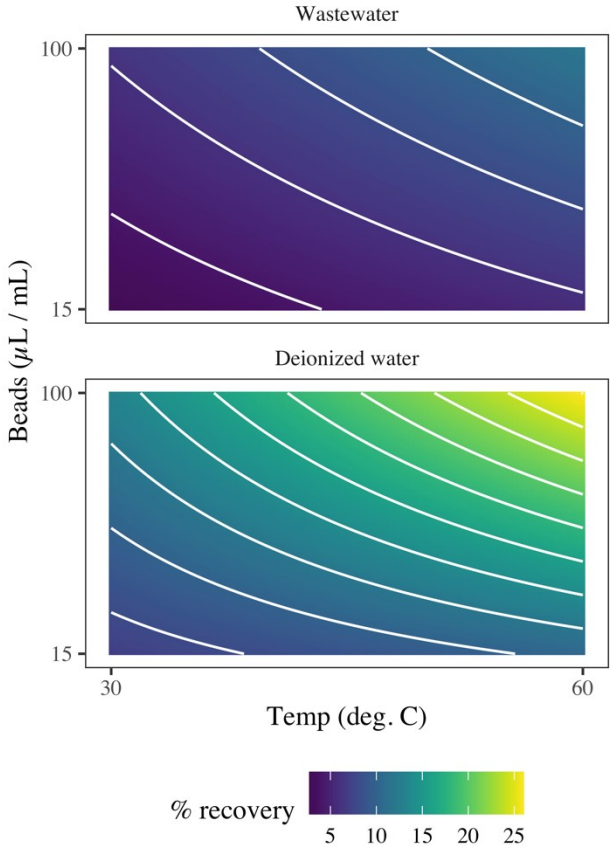

Figure S3. Response surface contour plot (i.e., the plane predicted by the linear regression model) illustrating the effect of bead mix concentration, elution temperature, and water matrix (and their two-way interactions) on percent recovery.

Supplemental Discussion

A. Effects of RNA extraction parameters on recovery efficiency

The effects of concentration of magnetic beads and elution temperature on ASCV-2 RNA recovery in DI water and municipal wastewater were assessed through a factorial experimental design (Table S2). The combination of method parameters from the full-factorial design that resulted in the highest RNA recovery from 1 mL of sample in both DI water (26.0 ± 0.8%) and wastewater (11.8 ± 1.4%) was a magnetic beads concentration of 100 μL mL^{-1} sample and an elution temperature of 60 ºC. Overall, ASCV-2 recoveries were clearly influenced by the water matrix with values ranging from 1.3 to 11.8% for wastewater and 6.3 to 26.0% for DI water. To better visualize the influence of the factors on RNA recovery, a response surface contour plot illustrating the relationship among the dependent (bead mix concentration, elution temperature, and water matrix) and independent (% recovery) variables was generated (Figure S3). The response surface contour plot was generated by predicting from the linear model over a grid of input values spanning the extremes of the factor settings. SARS-CoV-2 was not detected in any of the unseeded wastewater samples.

In recent comparable wastewater studies, SARS-CoV-2 RNA was concentrated from considerably larger sample volumes. For instance, SARS-CoV-2 RNA was detected in all of 54 untreated Detroit wastewater samples following RNA concentration from 45-L sample volumes.[11] Ahmed et al., (2020) reported "small volumes" of wastewater for RNA extraction as samples consisting of 50 mL,[1] which is 50-fold greater than the sample volume used in this study. Working with smaller wastewater sample volumes is advantageous, as they are easier and safer to handle, store, and transport. While this method performs at low sample volumes (1 mL), it does not incorporate a concentration step, indicating that it is suitable for wastewater samples that contain SARS-CoV-2 in concentrations present above its method detection limit.

The concentration of magnetic beads had the largest impact on RNA recovery, as the beads play a primary role in the binding of target nucleic acids. Certainly, a greater concentration of magnetic beads provides additional surface area, allowing for more binding sites for nucleic acids.[12] A magnetic beads concentration of 100 μL mL^{-1} sample resulted in significantly higher RNA recovery efficiencies (about 60 and 70% increases in recovery from wastewater and DI water, respectively) than did a concentration of 15 μL mL^{-1} sample. Furthermore, when magnetic beads were dosed at a concentration of 100 μL mL^{-1} sample, the recovery efficiency was maximized. The effect of this variable calculated from the linear model showed that increasing the concentration of magnetic beads from 15 to 100 μL mL^{-1} sample increased recovery by $7.9x10^4$ GU L^{-1}.

In both matrices, increasing the elution temperature from 30 to 60 °C resulted in a 60% greater RNA recovery. The effect of elution temperature calculated from the linear model showed that an increase from 30 to 60 °C increased recovery by $6.2x10^4$ GU L^{-1}. Previous studies have outlined the important role of temperature for the desorption of nucleic acids.[2,8,9] Nonetheless, there is a lack of consensus as many methods are performed at room temperature,[4,6,7] whereas

other studies have demonstrated that more RNA is released at increased temperatures. For example, high temperatures have been reported to weaken the hydrogen bonds formed between nucleic acids-adsorption materials, therefore, serving as an ideal tool to enhance the elution process. However, it should be considered that high elution temperatures (>60 ºC) may increase the risk of RNA denaturation. In our preliminary experiments, recovery efficiency increased considerably when RNA was eluted at 55 ºC in comparison to room temperature (data not shown), and it was further improved by incubating at 60 ºC. It is likely that the short incubation periods used in this work in combination with the pre-heated elution buffer promoted the solubilization of extracted RNA while minimizing RNA degradation.

Of the three variables assessed in the factorial design recovery experiments, the matrix showed a large impact on RNA recovery. The effect of the matrix calculated from the ANOVA showed that extracting RNA from wastewater (rather than DI water) decreased recovery by $7.6x10^4$ GU L^{-1}. The higher recovery yields obtained with DI water demonstrated the effect of the complex composition of wastewater on the performance of the magnetic bead-based extraction protocol. RNA recovery efficiency can be influenced by the matrix when the particulate and dissolved components in the wastewater are carried along with the target virus during isolation.[10] As RNA extraction was performed for raw wastewater without any pre-treatment, it is likely that debris and solids present in the matrix competed with nucleic acids for binding sites on the magnetic beads.[5] Due to this reduced surface area, lower quantities of RNA were able to bind to the magnetic beads in the wastewater than in DI water. Further research is necessary to elucidate the effect of the wastewater matrix on the performance of magnetic beads to enhance the recovery of viral RNA fragments.

B. Recovery of gamma-irradiated SARS-CoV-2 and HCV 229E surrogates in wastewater with high and low viral concentrations

The magnetic bead-based RNA extraction protocol obtained with the statistical design was evaluated for recovery of GI-SCV-2 and HCV 229E as SARS-CoV-2 surrogates in wastewater. Overall, the method allowed differentiation between high and low concentrations, and no-spike (blank) wastewater samples for both surrogates. Neither of the two surrogates were detected in the blank samples. Mean concentrations of $8.33x10^4 \pm 7.8x10^3$ GU L^{-1} and $1.55x10^4 \pm 3.6x10^3$ GU L^{-1} were recovered for aliquots spiked at high and low concentrations of GI-SCV-2.

Initially, mean GI-SCV-2 recovery was 86.1% for the low spike concentration, whereas at the high spike concentration, mean recovery dropped to 4.6%. A potential cause for the decrease in GI-SCV-2 recovery at the high spike concentration may be PCR inhibition. Although the use of magnetic beads has been expected to counter inhibitory effects better than other column-based extraction methods,[3] hey are still affected by inhibitory compounds. To investigate inhibition as a potential cause of the GI-SCV-2 recovery loss, RNA extracts from the wastewater samples spiked with $1.8x10^4$ and $1.8x10^6$ GU L^{-1} were diluted 10- and 50-fold, respectively (n=3). For the low spike concentration, the RNA was diluted beyond the detection limit and could not be enumerated. However, mean recoveries from the diluted RNA extracts for the high spike

concentration samples increased from 4.6 to approximately 100.1%. This observation supports the hypothesis that the wastewater samples contained PCR inhibitors that were co-extracted with surrogate RNA.

For the recovery of HCV 229-E, Ct values from the qPCR were used to provide an indication of the copy number of target RNA as logarithmically Ct values are inversely proportional to viral load. The Ct values for HCV 229E ranged from 32.90 to 35.21 for the wastewater spiked with $1.0x10^6$ infectious units L^{-1} and from 37.42 to 38.22 for samples seeded with $1.0x10^4$ infectious units L^{-1}. The ability of this new magnetic beads-based RNA extraction method to consistently distinguish low from high concentrations suggests the potential applicability of the method to detect temporal trends of SARS-CoV-2 in a community monitoring program.

References

1. Ahmed W, Bertsch PM, Bibby K, Haramoto E, Hewitt J, Huygens F, Gyawali P, Korajkic A, Riddell S, Sherchan SP, Simpson SL. Decay of SARS-CoV-2 and surrogate murine hepatitis virus RNA in untreated wastewater to inform application in wastewater-based epidemiology. Environmental Research. 2020 Dec 1;191:110092.
2. Elaissari A, Holt L, Meunier F, Voisset C, Pichot C, Mandrand B, Mabilat C. Hydrophilic and cationic latex particles for the specific extraction of nucleic acids. Journal of Biomaterials Science, Polymer Edition. 1999 Jan 1;10(4):403-20.
3. Hata A, Katayama H, Kitajima M, Visvanathan C, Nol C, Furumai H. Validation of internal controls for extraction and amplification of nucleic acids from enteric viruses in water samples. Applied and environmental microbiology. 2011 Jul 1;77(13):4336-43.
4. He H, Li R, Chen Y, Pan P, Tong W, Dong X, Chen Y, Yu D. Integrated DNA and RNA extraction using magnetic beads from viral pathogens causing acute respiratory infections. Scientific reports. 2017 Mar 23;7(1):1-8.
5. Huggett JF, Novak T, Garson JA, Green C, Morris-Jones SD, Miller RF, Zumla A. Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. BMC research notes. 2008 Dec;1(1):1-9.
6. La Rosa G, Iaconelli M, Mancini P, Ferraro GB, Veneri C, Bonadonna L, Lucentini L, Suffredini E. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Science of The Total Environment. 2020 Sep 20;736:139652.
7. La Rosa G, Pourshaban M, Iaconelli M, Muscillo M. Quantitative real-time PCR of enteric viruses in influent and effluent samples from wastewater treatment plants in Italy. Annali dell'Istituto superiore di sanita. 2010;46:266-73.
8. Li X, Zhang J, Gu H. Adsorption and desorption behaviors of DNA with magnetic mesoporous silica nanoparticles. Langmuir. 2011 May 17;27(10):6099-106.
9. Li Y, Yoshida H, Wang L, Tao Z, Wang H, Lin X, Xu A. An optimized method for elution of enteroviral RNA from a cellulose-based substrate. Journal of virological methods. 2012 Dec 1;186(1-2):62-7.
10. Michael-Kordatou I, Karaolia P, Fatta-Kassinos D. Sewage analysis as a tool for the COVID-19 pandemic response and management: the urgent need for optimised protocols for SARS-CoV-2 detection and quantification. Journal of environmental chemical engineering. 2020 Oct 1;8(5):104306.
11. Miyani B, Fonoll X, Norton J, Mehrotra A, Xagoraraki I. SARS-CoV-2 in Detroit wastewater. Journal of Environmental Engineering. 2020 Nov 1;146(11):06020004.
12. Yuan QB, Huang YM, Wu WB, Zuo P, Hu N, Zhou YZ, Alvarez PJ. Redistribution of intracellular and extracellular free & adsorbed antibiotic resistance genes through a wastewater treatment plant by an enhanced extracellular DNA extraction method with magnetic beads. Environment international. 2019 Oct 1;131:104986.