1 Supplementary Methods

1.1 Sample Collection

Five Influent wastewater samples were collected as 24-hour composite samples over a period of 2 months with a 2-week interval in the spring of 2024, from the two Belgian wastewater treatment plants. Once collected, the wastewater samples were transported the same day at 4°C and the concentration and extraction methods were performed as soon as possible after arrival within 72 hours.

To evaluate the concentration and extraction methods two types of samples were used. First, 50 mL of raw wastewater was used. Secondly, 50 mL of spiked was prepared by adding at each time point (n = 5) clinical samples that contain Influenza A and B virus, RSV A and B virus and SARS-CoV-2 virus to 600 mL of raw wastewater sample to obtain a final concentration of either 50 or 100 cp/µL (Table 1). The spiking was done in order to ensure the presence of viral targets that are typically found at low concentrations in wastewater, thus allowing for a more robust assessment of method sensitivity and recovery efficiency.

Table 1: Clinical samples that were used to spike the raw wastewater samples.

Sample	Pathogen	Final Concentration (cp/µL)
H3N2_23-IS-00280	Influenza A H3N2	100
H1N1_23-IS-01030	Influenza A H1N1	50
RSVA_A2	RSV A	100
RSVB_WV/146	RSV B	50
B_VIC_23-IG-00115	Influenza B (VIC)	50
SC2_23-IS-00311	SARS-CoV-2	100

1.2 Concentration and Extraction methods

In this study various concentration and extraction methods were applied to all raw and spiked wastewater samples. Specifically, the following combination were tested: Innovaprep (1.2.1) followed by extraction with the QIAamp RNeasy PowerFecal Pro Kit (1.2.7), Nanotrap Magnetic Virus Particles (1.2.2) combined with the QIAamp RNeasy PowerFecal Pro Kit (1.2.7), and ultracentrifugation (1.2.3) paired with the QIAamp RNeasy PowerFecal Pro Kit (1.2.7). Moreover, PEG precipitation (1.2.4) was used in conjunction with both the QIAamp RNeasy PowerFecal Pro Kit (1.2.7) and the QIAamp Viral RNA Mini Kit (1.2.6). Finally, a commercial kit ZymoPURE Water DNA/RNA Kit (1.2.5) was used and included both concentration and extraction steps.

1.2.1 Concentration: InnovaPrep Concentrating Pipette Select

First, 5% Tween 20 solution was added to the raw wastewater samples (50 mL raw or spiked wastewater) at 1:100 and mixed by inversion. Next, the samples were centrifuged for 10 minutes at 7000g. Supernatant was then concentrated using a Ultrafilter Concentrating Pipette Tip on the InnovaPrep Concentrating Pipette Select (InnovaPrep, Drexel, MO, USA). Following options were used for the InnovaPrep: Valve open 770 ms; Pulse 1; Foam factor 10; Valve closed 100 ms; Flow start 3s; Flow end 10s; Flow min start 40s; Ext Delay 3s; Pump 25%; Ext pump delay 1s. After elution, the eluate was stored in -80°C freezer until nucleic acid extraction was completed.

1.2.2 Concentration: Nanotrap Magnetic Virus Particles

In this study, the Nanotrap Magnetic Virus Particles was combined with the QIAamp RNeasy PowerFecal Pro Kit. First, 100µL of Nanotrap Enhancement Reagent 2 (ER2) and 525 µL of Nanotrap Microbiome A Particles (Ceres Nanosciences) were added to each wastewater sample. The samples were incubated at room temperature for 30 minutes on a hulamixer. The beads were separated from the solution on a magnetic rack and washed with 1 mL of Nanotrap Buffer 2. Next the beads were separated from the solution and 650 µL of CD1 Buffer (QIAamp RNeasy PowerFecal Pro Kit) and 100 µL of phenol-chloroform-isoamyl alcohol was added and

incubated at room temperature for 10 minutes. Finally, the entire volume was transferred to a PowerBead Pro Tube (QIAamp RNeasy PowerFecal Pro Kit) and incubated at room temperature for 10 minutes while vortexing. The following steps follows the rest of the protocol for QIAamp RNeasy PowerFecal Pro Kit.

1.2.3 Concentration: Ultracentrifugation

The raw and spiked wastewater samples were first centrifuged for 10 minutes at 3000g at 4°C. Next, the samples were centrifuged at 150 000g for 60 minutes at 4°C on Optima XPN Ultracentrifuge (Beckman Coulter, Brea, CA, USA). The supernatant was removed without disturbing the pellet. The pellet was further resuspended in 200 µL phosphate buffered saline (PBS) and transferred into a new 1.5 mL micro-centrifugal tube and stored at -80°C until nucleic acid extraction.

1.2.4 Concentration: PEG Precipitation with salt addition

50 mL of each raw or spiked wastewater sample was centrifuged at 3000g for 10 minutes at 4°C. 5g Polyethylene Glycol 8000 (10%w/v) (Promega) and 1g NaCl (2% w/v) (Sigma Aldrich) were added in a 50 mL empty and sterile falcon tube and mixed with the supernatant of the wastewater sample. The sample was agitated with a hulamixer at 4°C for 30 minutes. After complete dissolvation, the sample was centrifuged at 12 000g for 2 hours at 4°C. The supernatant was discarded and 200 µL of PBS (Life Technologies) was added to the pellet. The solution was stored at -80°C until nucleic acid extraction.

1.2.5 Concentration + Extraction: ZymoPURE Water DNA/RNA Kit

The wastewater samples were concentrated and extracted according to the manual of the commercial kit. Briefly, 50 mL of wastewater was centrifuged at 4000g for 5 minutes at room temperature to pellet debris, after which the supernatant was carefully decanted into a new tube. Next, the sample was treated with 0.1 volume Wastewater Stabilization Buffer and incubated for 10 minutes before centrifuging at 10 000g for 20 minutes at room temperature. The supernatant was decanted and the pellet was resuspended in 250 µL DNA/RNA Shield.

Subsequently, the solution was mixed with 2 volumes of Viral RNA Buffer, processing it through Zymo-Spin IIICG Columns. Next, 400 µL of RNA Prep Buffer was added to the column and discarded through centrifugation and followed by adding 100µL of DNase/RNase Free Water and incubating it for 5 minutes on the column. To the elution, 200 µL of RNA Binding Buffer and 400 µL of 100% ethanol was added, put on a new Zymo-Spin IIICG Column and the flow-through was discarded. Next, 400 µL RNA Prep Buffer and 700 µL RNA Wash Buffer were each added to the column and the flow-through was each time discarded. Subsequently, 100 µL of DNase/RNase Free Water was added and incubated for 5 minutes on the column. For inhibitor removal, the eluate was treated with 600 µL Inhibitor Removal Binding Buffer and 30 µL MAGicBead cfDNA, incubated and processed using a magnetic stand to separate and wash the beads with 200 µL Removal Wash Buffer. Finally, 60µL Inhibitor Removal Elution Buffer was added the eluted nucleic acids were stored at -80°C for further analysis.

1.2.6 Extraction: QIAamp Viral RNA Mini Kit

The nucleic acids were extracted from each sample using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. As the precipitation eluate was 200 µL, the amount of Buffer AVL-carrier RNA and ethanol were increased proportionally. The sample was eluted with 60 µL of AVE elution buffer.

1.2.7 Extraction: QIAamp RNeasy PowerFecal Pro Kit

The nucleic acids were extracted from each sample using the RNeasy PowerFecal Pro Kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. The sample was eluted in 100 µL of RNase free water.

1.3 (RT-)ddPCR quantification

The concentration and extraction methods are evaluated in a first step for all samples with following RNA targets for RT-ddPCR: ORF1a (SARS-CoV-2) in duplex with RdRp_IP4 (SARS-CoV-2) [1], Influenza A and Influenza B in duplex, RSV A in duplex with RSV B, and Norovirus

GI and Norovirus GII in duplex; and for DNA targets ddPCR: the AMR genes blaCTX-M and tetM [2] in duplex (Table 2).

For the RT-ddPCR, the One-Step RT-ddPCR Advanced Kit for Probes from Bio-Rad (California, USA) was used. The kit components were thawed on ice for 30 minutes and thoroughly mixed by vortexing the tubes for 30 seconds at maximum speed. The master mix was made by mixing the reagents and subsequently aliquoted into individual reactions. The total volume of each reaction was 22 µL. For each reaction, 0.55 µL of primer at an initial concentration of 20 µM and 0.44 µL of each probe at an initial concentration of 10 µM were added. Additional components for all assays included 1.1 µL of 300 mM DTT, 5.5 µL One-Step Supermix, and 2.2 µL Reverse Transcriptase. For the SARS-CoV-2, influenza and RSV targets 10 µL of sample was added for the raw wastewater samples and 2 µL in case of the spiked wastewater samples. For the Norovirus targets, 1 µL of wastewater sample was added. Finally, the appropriate volume dH_2O to achieve a total volume of 22 µL was added.

For the ddPCR, the 2x ddPCR Supermix for probes (no dUTP) from Bio-Rad (California, USA) was used. The kit components were thawed on ice for 30 minutes and thoroughly mixed by vortexing the tubes for 30 seconds at maximum speed. The master mix was made by mixing the reagents and subsequently aliquoted into individual reactions. The total volume of each reaction was 22 µL. For each reaction, 0.55 µL of primer at an initial concentration of 20 µM and 0.44 µL of each probe at an initial concentration of 10 µM were added. Moreover, 10 µL of 2x ddPCR Supermix for Probes (No dUTP) was added in addition to 1 µL of wastewater sample and the appropriate volume dH_2O was added to achieve a total volume of 22 µL.

From both assays, 20 µL of the reaction mix and 70 µL of Droplet Generation Oil for Probes were loaded into a QX200TM droplet generator (Bio-Rad). Amplification for RT-ddPCR was carried out in a T100™ Thermal Cycler (Bio-Rad) with the following conditions for all assays: one cycle at 25 °C for 3 min, one cycle at 50 °C for 60 min (RT), one cycle at 95 °C for 10 min (Taq polymerase activation), 40 cycles at 95 °C for 30 s (denaturation) and 57.5 °C for 60 s (annealing), one cycle at 98 °C for 10 min (enzyme inactivation), and finally, one cycle at 4 °C

for 30 min (stabilization). Amplification for ddPCR was carried out in a T100™ Thermal Cycler (Bio-Rad) with the following conditions for all assays: one cycle at 25 °C for 3 min, one cycle at 95 °C for 10 min (Taq polymerase activation), 40 cycles at 95 °C for 30 s (denaturation) and 57.5 °C for 60 s (annealing), one cycle at 98 °C for 10 min (enzyme inactivation). Finally, the plate was transferred to the QX200 ™ reader (Bio-Rad), and the results were acquired using the HEX and FAM channels as instructed. The QuantaSoft software v1.7.4.0917 (Bio-Rad) was used for the interpretation of the results, and the threshold was set manually.

For each target, generalized linear models with a Gaussian error distribution were fitted to compare concentrations obtained with different concentration and extractions methods, using the precipitation method combined with the QIAamp RNeasy PowerFecal Pro Kit as reference. The concentrations were $\log_{10}$-transformed prior to analysis. For spiked targets, models were fitted separately from the raw wastewater. Estimated coefficient were exponentiated to obtain fold changes relative to the reference method, and 95% confidence intervals were calculated. P-values for method comparisons were extracted from the model summaries and adjusted for multiple testing using the false discovery rate method.

Table 2: Primers and probes used for the various assays in this study. A second, internal ZEN-quencher was added to the probes to obtain greater overall dye quenching in addition to the Iowa Black FQ (IABkFQ) quencher.

Target	Name	5' → 3' sequence	Reference
***bla*$_{CTX-M}$**	*bla*$_{CTX-M}$_FW	ACCAAYGATATYGCGGTKAT	[2]
	bla$_{CTX-M}$_RV	ACATCGCGRCGGCKYTCT	
	bla$_{CTX-M}$_PR	FAM/TCGTGCGCCGCTG/MGB-Eclipse	
tetM	*tetM*_FW	GGTTTCTCTTGGATACTTAAATCAATCR	[2]
	*tetM*_RV	CCAACCATAYAATCCTTGTTCRC	
	*tetM*_PR	HEX/ATGCAGTTATGGARGGGATACGCTATGGY/IABkFQ	
RSVA	RSVQA1	GCTCTTAGCAAAGTCAAGTTGAATGA	[3]
	RSVQA2	TGCTCCGTTGGATGGTGTATT	
	RSVQA_PR	FAM/ACACTCAAC-ZEN-AAAGATCAACTTCTGTCATCCAGC/IABkFQ	
RSVB	RSVQB1	GATGGCTCTTAGCAAAGTCAAGTTAA	[3]
	RSVQB2	TGTCAATATTATCTCCTGTACTACGTTGAA	
	RSVQB_PR	HEX/TGATACATT-ZEN-AAATAAGGATCAGCTGCTGTCATCCA/IABkFQ	
FluA	InfA_Forward	GACCRATCCTGTCACCTCTGAC	[4]
	InfA_Reverse	AGGGCATTYTGGACAAAKCGTCTA	
	Infa_Probe	FAM/TGCAGTCCT-ZEN-CGCTCACTGGGCACG/IABkFQ	
FluB	InflB_For	AAATACGGTGGATTAAATAAAAGCAA	[4]
	InflB Rev	CCAGCAATAGCTCCGAAGAAA	

	InflB_Probe	/HEX/CACCCATAT-ZEN-TGGGCAATTTCCTATGGC/3IABkFQ	
ORF1a	ORF1a-F	AGAAGATTGGTTAGATGATGATAGT	[1]
	ORF1a-R	TTCCATCTCTAATTGAGGTTGAACC	
	ORF1a-P	FAM/TCCTCACTG-ZEN-CCGTCTTGTTGACCA/IABkFQ	
RdRp	RdRp_IP4-F	GGTAACTGGTATGATTTCG	[1]
	RdRp_IP4-R	CTGGTCAAGGTTAATATAGG	
	RdRp_IP4-P	HEX/TCATACAAA-ZEN-CCACGCCAGG/IABkFQ	

1.4 Targeted Sequencing

To evaluate whether the routine sequencing protocol remained effective and whether PCR amplification was impacted by the presence of inhibitors, SARS-CoV-2 sequencing was performed on wastewater samples from two time points and from one Belgian WWTP. SARS-CoV-2 was selected for this assessment as it remains one of the most well-established pathogens currently monitored in wastewater surveillance using targeted sequencing. This sequencing protocol, detailed below, was applied on the various samples for which all concentration and extraction methods tested were used.

1.4.1 Library preparation and sequencing

The extracted RNA and all components were thawed on ice and the components were briefly mixed before use. In a PCR plate 2.4 µL LunaScript RT SuperMix (5x) and 9.6 µL template RNA were added and gently mixed by pipetting. To prevent contamination, a well was left empty between the samples. The RT plate was sealed and spun down. The reaction was incubated as follows: 25°C for 2 minutes, 55°C for 10 minutes, 95°C for 1 minute, and hold at 4°C indefinitely. For each sample, two PCR reactions were set up that included 12.5 µL of Q5 Hot Start High-Fidelity 2X Master Mix, 3.7 µL diluted ARTIC primer pool (ARTIC V.5.3.2), 3.8 µL nuclease free water and 5µL of cDNA. Following PCR conditions were used: 98°C for 30 seconds (1 cycle), 95°C for 15 seconds and 63°C for 5 minutes (35 cycles), and hold at 4°C indefinitely. After the PCR, pool A and B were combined and 50 µL of 1x ratio AMPure XP beads was added. After an incubation of 10 minutes, the beads were pelleted and washed twice with 80% ethanol. Finally, the pellet was resuspended in 15 µL nuclease-free water, incubated for 2 minutes at room temperature and the supernatant was retained for downstream

analysis. The purified RT-PCR products were used to prepare sequencing libraries using the Nextera XT DNA Sample Preparation Kit (Illumina, USA) following to the manufacturer's instructions with a 0.6x bead/DNA ratio for the PCR clean-up. All prepared libraries were sequenced on an Illumina MiSeq (Illumina, USA) using the MiSeq V3 chemistry according to the manufacturer's protocol. Paired-end reads of 2 x 250 base pairs (bp) were generated during the sequencing process.

1.4.2 Bioinformatic analysis

The FASTQ input datasets were first downsampled to 100,000x if coverage was estimated to be higher. Coverage was estimated based on the number of bases in the input FASTQ files and the length of the provided reference genome NC_045512_Hu-1. The 'sample' function of seqtk (v1.4) [5] was used for the downsampling. Reads were trimmed using fastp v0.23.4 with the following options: "--detect_adapter_for_pe", "--cut_front", "--cut_front_window_size" set to 1 , "--cut_front_mean_quality" set to 10, "--cut_tail", "--cut_tail_window_size" set to 1, "--cut_tail_mean_quality" set to 10, "--cut_right", "--cut_right_window_size" set to 4, "--cut_right_mean_quality" set to 20, and "--length_required" set to 40.. The trimmed reads were then mapped to the reference sequence using BWA-MEM (v0.7.17) [6] with the '-k' option set to 19 and the '-r' option set to 1.5. The paired reads and unpaired reads (after trimming) were mapped separately and the resulting SAM files were merged using samtools merge (v1.17) [7]. The median depth and percentage of the reference sequence that was covered were calculated using samtools depth v1.17 with the '-a' option enabled. For the evaluation of the targeted sequencing, the median depth and the covered rate of the genome were extracted for each sample.

Consensus sequences were generated using an iterative mapping approach, in which reads were first aligned to the reference sequence, and then to the consensus sequence obtained from the previous iteration. In each iteration, the reads are first mapped to the input sequence, followed by variant calling with BCFtools v1.17. The resulting variants were filtered and applied to update the input sequence. The reads were then remapped to this updated

sequence, and a second round of variant calling was performed using Clair3 v1.0.4. The sequence incorporating these variants was then used as the input for the next iteration. Iteration stopped when the consensus sequence did not change in the last two iterations, or after 15 iterations. Read mapping was performed using BWA-MEM, as described above. Variant calling with BCFtools was performed with the '--ploidy' parameter set to 1 and the max pileup depth ('-d') set to 8000. For Clair3 variant calling, the '--haploid_precise' and '--no_phasing_for_fa' options were used, and other options were left at default values. For both variant callers, variants with an allele frequency below 50%, a depth below 10x or a SNP quality below 5 were removed. An additional filter was applied to the variants detected by BCFtools to enforce a minimum mapping quality of 30.

1.5 Metagenomics

To characterize the communities within the sample and evaluate whether consistent communities were obtained across different methods, unbiased metagenomics was performed on wastewater samples to attempt to find both DNA and RNA targets collected on three time points from one Belgian. This metagenomics protocol, detailed below, was applied on the various samples that were concentrated and extracted with Nanotrap method in combination with the QIAamp RNeasy PowerFecal Pro Kit, the precipitation method in combination with the QIAamp RNeasy PowerFecal Pro Kit and the ZymoPURE Water DNA/RNA Kit. These methods were selected for metagenomics because they yielded the highest overall nucleic acid concentrations in (RT-)ddPCR assays and demonstrated reliable amplification performance in SARS-CoV-2 targeted sequencing.

1.5.1 Library preparation and ONT sequencing

The DNA concentration in the samples was quantified using Qubit, and samples were diluted with nuclease-free water to obtain 1 µg input DNA in a final volume of 48µL. If the DNA yield was below this threshold, the entire volume (48 µL) was used. For the DNA repair and end-prep, 3.5 µL of NEBNext FFPE DNA Repair Buffer, 2 µL of NEBNext FFPE DNA Repair Mix, 3.5 µL of Ultra II End-prep Reaction Buffer, and 3 µL of Ultra II End-prep Enzyme Mix

(NEBNext® Companion Module for ONT Ligation Sequencing, Bioké) were added to the DNA. he mixture was gently pipetted to ensure homogeneity and then incubated in a thermal cycler at 20°C for 5 minutes, followed by 65°C for another 5 minutes. Following incubation, AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used to purify the DNA. A 1:1 ratio of beads to DNA was used, with the beads resuspended by vortexing before being added to the DNA. The mixture was incubated on a rotator for 5 minutes at room temperature. After magnetic separation, the beads were washed twice with freshly prepared 80% ethanol and allowed to dry briefly before elution of the DNA in 61 µL of nuclease-free water.

For the adapter ligation, the sample was mixed with 25 µL of Ligation Buffer, 10 µL of NEBNext Quick T4 DNA Ligase (NEBNext® Companion Module for ONT Ligation Sequencing, Bioké), and 5 µL of Ligation Adapter (Ligation Sequencing Kit V14, Oxford Nanopore Technologies). The mixture was gently pipetted to mix and then incubated at room temperature for 10 minutes. AMPure XP beads were again used for clean-up with Short Fragment Buffer (SFB) (to ensure DNA fragments from all sizes are retained) to wash the beads and finally 15 µL of elution buffer was added for the elution. The prepared library was quantified with Qubit BR to adjust to final concentration of 35-50 fmol in preparation for flow cell priming and loading (Ligation Sequencing Kit V14 and MinION Flow Cells R10.4.1, Oxford Nanopore Technologies). Sequencing was performed on a R10.4.1 Flow cell run on a GridION device for 72h with MinKNOW 24.02.16 at room temperature.

1.5.2 Bioinformatics Analysis

After sequencing, raw data in POD5 file format was basecalled using dorado 0.7.0 sup and the sequencing data quality was assessed with Nanoplot v1.41.6 [8]. A Kruskall-Wallis Test was used to compare if there was an association across the different dates, methods or sample type (spiked vs non-spiked) for the parameters “median read quality”, “median read length” and “number of reads”. Moreover, to evaluate whether there are large differences between the samples, the taxonomic classification was performed on trimmed reads done with SeqKit v2.3.1 [9] (parameters: --min-qual 10 and –min-len 500) using Kraken2 v2.1.1 [10] with default

parameters and the results were visualized with Krona [11]. For Kraken2, the largest in-house Kraken2 database [12], named ‘full’, was used which includes reference and representative sequences from NCBI across several taxonomic groups that includes animals, archaea, bacteria, fungi, plants, protozoa and viruses (accessed on 13th of January 2024) along with the human reference genome (RefSeq accession GCF_000001405.40). To compare the different Kraken results of the samples, the results until depth level 5 were plotted on a radar chart that was constructed in RStudio 2022.02.2.485 (R-version 4.3.3) with package fmsb 0.7.6.