CORONASTEP Report 01 September 2020
SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

Monitoring of SARS-CoV-2 in wastewater has been established on a weekly basis in Luxembourg from 31st March 2020 for a total of 188 samples so far. **Last week, a new wastewater treatment plant (Wiltz) was added to the twelve (WWTP) already monitored for SARS-CoV-2 in the inlet pipe** (Table 1). For the WWTP of Schifflange and Pétange, archived frozen samples have been analysed back to October 2019.

In general, since the beginning of the sampling carried out within the framework of the CORONASTEP project, the dynamics of SARS-CoV-2 RNA copies in influents of WWTPs has followed the dynamics of active COVID-19 cases observed at the national level (Table 2), whatever the WWTP studied. In the present report, t**he dynamics of SARS-CoV-2 in wastewater in presented in link with the weekly cases of infection in the contributory area each wastewater treatment plant**.

In addition, in Figures 2, 3 and 4 of this report, the data are presented in **SARS-CoV-2 standardized fluxes** (RNA copies / day / 10,000 population equivalents), using the daily flow of each treatment plant and the maximum capacity of each. Three distinct profiles can be distinguished:

- A first group comprising the major WWTPs of the country (Beggen, Schifflange, Bettembourg, Pétange) always presents the highest viral fluxes compared to the other plants. These fluxes closely follow the dynamics of the contaminations in the population of the contributory area of the wastewater treatment plant (Figure 2).
- In the second group, comprising the treatment plants of Hesperange, Mersch, Bleesbruck, Grevenmacker, Uebersyren and Echternach, the detected SARS-CoV-2 RNA fluxes are generally lower (between 5.10^9 and 10^{10} RNA copies/ day/10 000 eq. inh.) but nevertheless follow the dynamics of the second wave. For some of them, the viral dynamics may appear fuzzier (Grevenmacker, Uebersyren, Echternach, Bleesbruck), but this is partly due to a lower number of samples analysed. Furthermore, it is important to remember that most of these WWTPs could not be sampled during the first wave of contamination.
- The third group is the Troisvierges treatment plant. No virus had been detected in the wastewaters of this plant until the middle of the second wave, with a suddenly very high value. Two other samples have tested positive to date, but this plant certainly needs to be analysed at a higher rate in order to draw more accurate conclusions.

The both newly analysed wastewater treatment plants cannot be classified in one of these groups yet, due to a too limited number of samples. No SARS-CoV-2 signal was observed in Wiltz and Boevange wastewaters last week.

The last samples analysed (week 35) confirmed the decrease in SARS-CoV-2 RNA concentrations observed the previous week (week 34) for all treatment plants analysed. The SARS-CoV-2 signal is no longer detectable in four wastewater treatment plants.

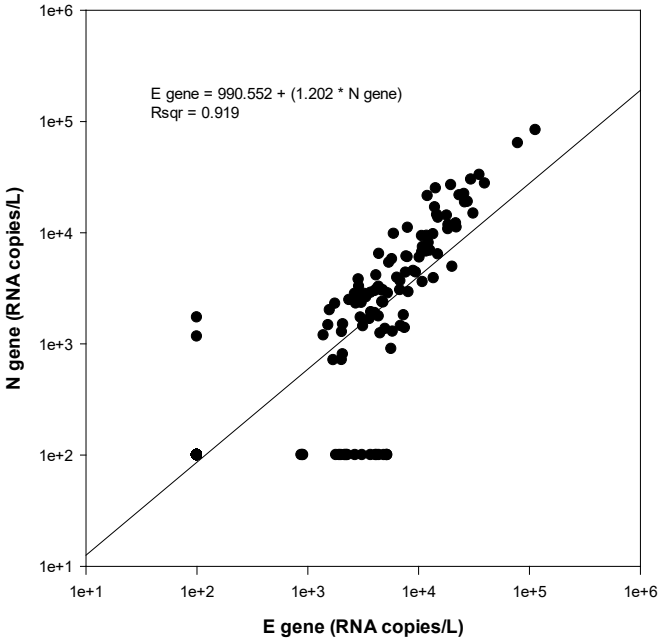
Table 1- Timing of sewage sampling

			2019				2020																										
Wastewater Treatment Plant	**Max capacity (eq. inhabitants)**	**Inhabitants connected**	Week 41	Week 43	Week 46	Week 51	Week 3	Week 7	Week 9	Week 11	Week 14	Week 15	Week 16	Week 17	Week 18	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Week 25	Week 26	Week 27	Week 28	Week 29	Week 30	Week 31	Week 32	Week 33	Week 34	Week 35	**Total samples**
Beggen	210000	139731									x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**22**
Bettembourg	95000	53606															x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**16**
Schifflange	90000	68143	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**30**
Bleesbrück	80000	30930																x	x	x	x	x	x	x	x		x	x	x	x	x	x	**14**
Mersch	70000	30473													x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**18**
Pétange	50000	59481	x	x	x	x	x	x	x	x					x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**26**
Hesperange	36000	15479													x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**18**
Echternach	36000	7499																			x				x	x	x	x	x	x	x	x	**9**
Uebersyren	35000	18600																	x		x		x		x	x	x	x	x	x	x	x	**11**
Grevenmacher	47000	9835																	x		x		x		x	x	x	x	x	x	x	x	**11**
Troisvierges	5000	3411																x	x	x	x	x			x		x	x	x		x		**10**
Boevange / Attert	15000	1170																													x	x	**2**
Wiltz		6944																														x	**1**
Total	**769000**	**445302**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**5**	**5**	**6**	**8**	**10**	**8**	**11**	**8**	**9**	**7**	**11**	**9**	**11**	**11**	**11**	**10**	**12**	**12**	**188**
Pop Lux (2019)		**613901**																															
		72.54%																															

Table 2 - Summary of the screening of SARS-CoV-2 gene E in 24-h composite samples of incoming wastewater at different WWTP in Luxembourg. White: not tested sample, Green: negative samples for SARS-CoV-2 gene E, Yellow to red: positive samples for SARS-CoV-2 gene E, the intensity of the color is depending to the Ct values (number in the cases).

		2019						2020																							
		Before 1st case						1st wave												2nd wave											
WWTP	**Inhabitants connected**	Week 41	Week 43	Week 46	Week 51	Week 3	Week 7	Week 9	Week 11	Week 14	Week 15	Week 16	Week 17	Week 18	Week 19	Week 20	Week 21	Week 22	Week 23	Week 24	Week 25	Week 26	Week 27	Week 28	Week 29	Week 30	Week 31	Week 32	Week 33	Week 34	Week 35
Beggen	139731									31.89	33.67	35.02	34.98	36.20	35.95	35.59	35.55	-	-	-	-	34.19	35.36	33.76	33.37	33.56	33.34	33.06	32.49	35.79	34.25
Bettembourg	53606															-	-	-	-	-	-	34.45	35.26	34.83	33.04	33.99	33.29	32.75	34.40	35.80	35.23
Schifflange	68143	-	-	-	-	-	-	37.04	36.04	34.04	35.70	35.87	35.16	36.61	35.77	-	35.20	-	36.60	-	-	36.65	34.90	34.36	34.59	33.17	34.12	33.18	34.66	34.80	35.68
Bleesbrück	30930																-	-	-	-	-	33.68	35.26	36.11		35.41	35.68	35.93	36.54	36.37	35.11
Mersch	30473													35.86	36.21	-	35.28	-	-	-	-	34.58	35.05	36.26	36.02	35.61	34.18	35.45	34.74	36.55	36.39
Pétange	59481	-	-	-	-	-	-	-	36.13					35.22	35.03	-	-	36.50	36.18	-	35.89	33.83	34.50	34.10	32.86	31.94	33.67	33.38	34.08	33.84	34.00
Hesperange	15479													36.99	36.13	36.75	-	-	-	-	-	-	36.00	-	36.41	35.57	34.72	-	35.81	-	36.14
Echternach	7499																			-				36.05	35.10	36.69	36.42	36.27	-	-	35.95
Uebersyren	18600																	-		-		35.50		35.86	36.59	35.68	-	36.68	34.29	-	-
Grevenmacher	9835																	-		-		36.26		35.82	-	34.81	35.13	35.81	35.65	-	-
Troisvierges	3411																-	-	-	-	-			-		31.61	34.75	34.92		-	
Boevange sur Attert	1170																													36.30	-
Wiltz	6944																														-
Total	**445302**																														

Figure 1 – Relationship between the SARS-CoV-2 RNA concentration (RNA copies / L of wastewater) estimated by the both distinct RT-qPCR systems targeting the E and N gene, respectively.



As shown in Figure 1, a good linear relationship (R^2: 0.92) was obtained between the SARS-CoV-2 RNA concentrations estimated using the E gene and the N gene, respectively. Therefore, in the remainder of this report, only the E gene results will be presented.

Figure 2 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in wastewater samples from the four most impacted wastewater treatment plants (Beggen, Schifflange, Pétange and Bettembourg) from March to August 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 normalized flux (RNA copies / day / 10 000 equivalent inhabitants).

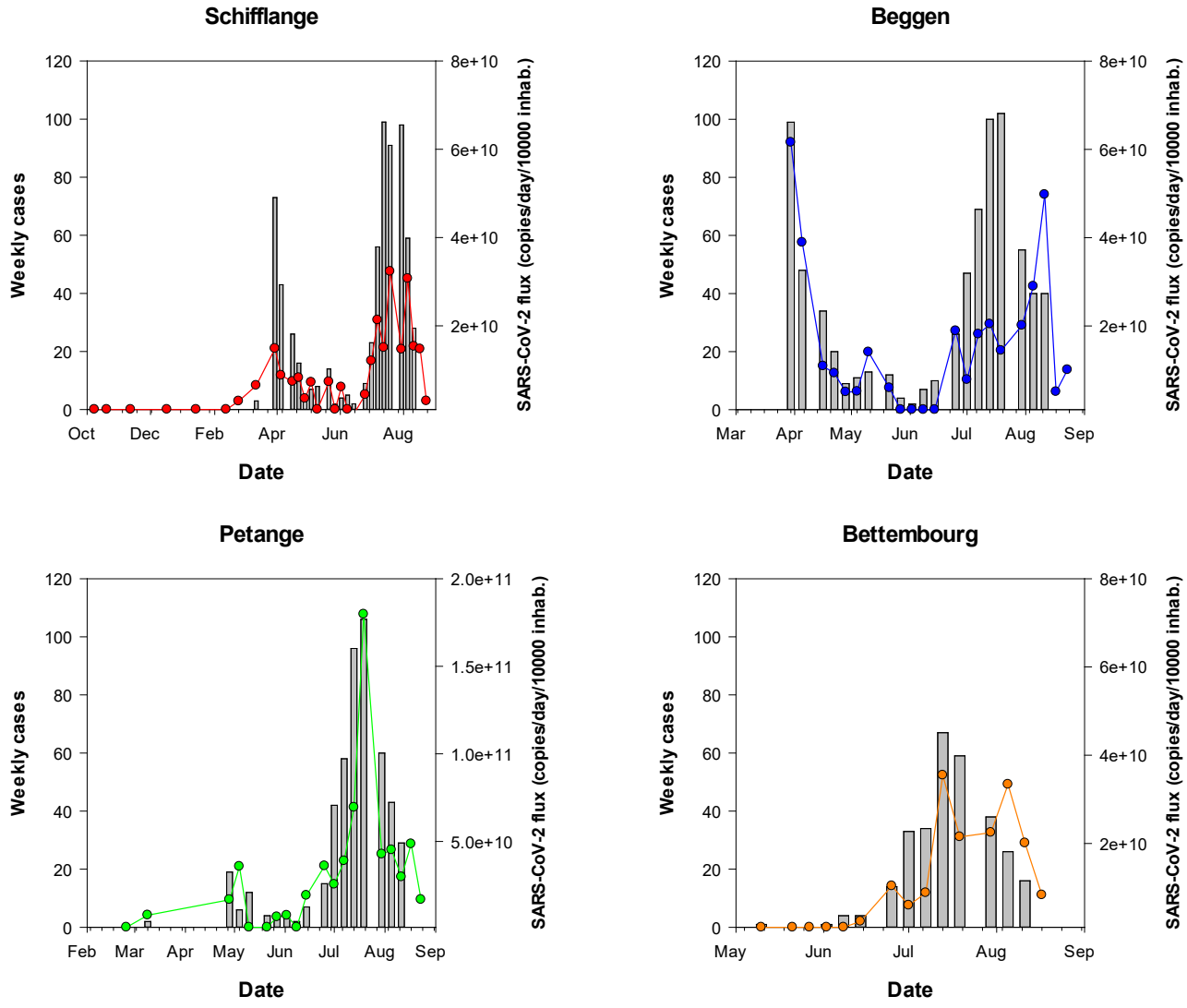


Figure 3 - RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in wastewater samples from the less impacted wastewater treatment plants (Mersch,, Hespérange Grevenmacker, Uebersyren, Echternach and Bleesbruck) from March to August 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 normalized flux (RNA copies / day / 10 000 equivalent inhabitants).

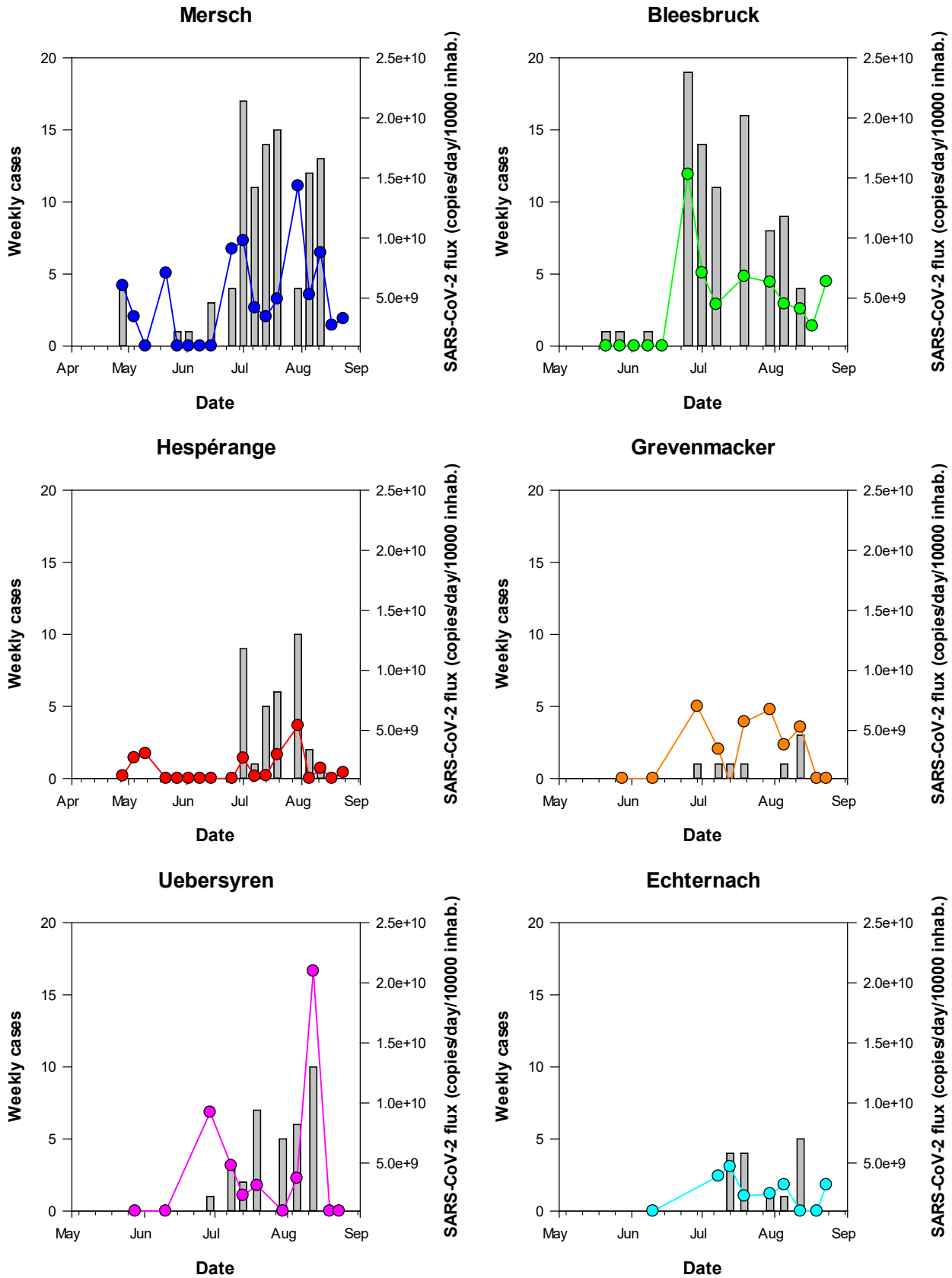
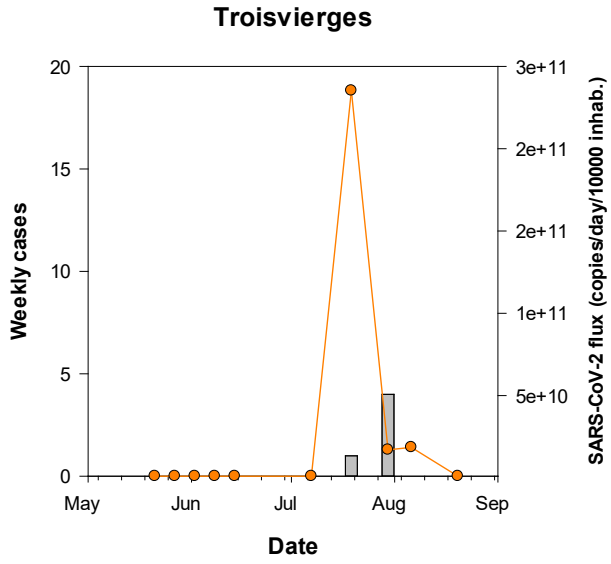


Figure 4 - RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in wastewater samples from the Troisvierges Wastewater treatment plant from March to August 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 normalized flux (RNA copies / day / 10 000 equivalent inhabitants).



Materials and Methods

Sewage samples

From March 31st to August 13th, 2020, up to eleven WWTPs were sampled at the inlet of the plant according to the planning presented in Table 1. The operators of the WWTPs sampled a 24-h composite sample of 96 samples according to your own sampling procedure. Composite sample was stored at 4°C until sample processing.

Sample processing

The samples were transported to the laboratory at 4°C and viral RNA was isolated on the day of sampling. Larger particles (debris, bacteria) were removed from the samples by pelleting using centrifugation at 2,400 x g for 20 min at 4°C. A volume of 120 mL of supernatant was filtered through Amicon® Plus-15 centrifugal ultrafilter with a cut-off of 10 kDa (Millipore) by centrifugation at 3,220 x g for 25 min at 4°C. The resulting concentrate was collected and 140 µL of each concentrate was then processed to extract viral RNA using the QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's protocol. Elution of RNA was done in 60 µL of elution buffer.

Real-time One-Step RT-PCR

Samples are screened for the presence of *Sarbecovirus* (*Coronaviridae, Betacoronaviruses*) and/or SARS-CoV-2 virus RNA by two distinct real-time one-step RT-PCR, one on the E gene (Envelope small membrane protein) and the second on the N gene (nucleoprotein). The E gene real-time RT-PCR can detect *Sarbecoviruses*, i.e. SARS-CoV, SARS-CoV-2 and closely related bat viruses. In the context of the COVID19 pandemic, it can be assumed that only SARS-CoV-2 strains will be detected by this assay given that SARS-CoV virus has been eradicated and other bat viruses do not commonly circulate in the human population. The E gene assay is adapted from Corman et al. [17]. The N gene real-time RT-PCR assay (N1 assay) specifically detects SARS-CoV-2 virus. It is adapted from the CDC protocol[1]. The two primers/probe sets are presented in Table 2. The RT-qPCR protocols and reagents were all provided by the LIH.

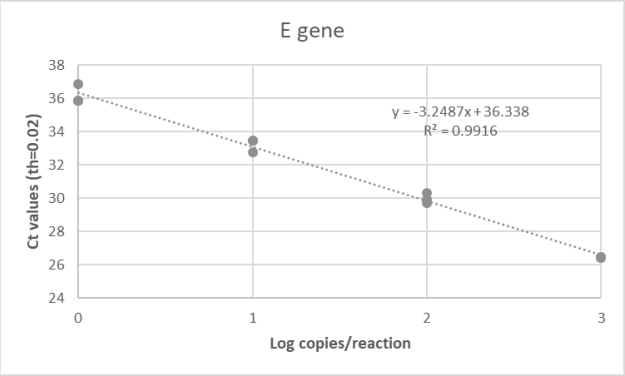
Each reaction contained 5 µL of RNA template, 5 µL of TaqPath 1-step RT-qPCR MasterMix (A15299, Life Technologies), 0.5 µL of each primer (20 µM) and probe (5 µM) and the reaction volume was adjusted to a final volume of 20 µL with molecular biology grade water. Thermal cycling reactions were carried out at 50 °C for 15 min, followed by 95 °C for 2 min and 45 cycles of 95 °C for 3 sec and 58°C (E gene) or 53°C (N gene) for 30 sec using a Viia7 Real-Time PCR Detection System (Life Technologies). Reactions were considered positive (limit of detection – LOD) if the cycle threshold (Ct value) was below 40 cycles.

Controls

A non-target RNA fragment commercially available (VetMAX™ Xeno™ IPC and VetMAX™ Xeno™ IPC Assay, ThermoFischer Scientific) was added to the viral RNA extract from sewage concentrates as an internal positive control (IPC). This IPC-RNA is used to control the performance of the RT-qPCR (E gene) and to detect the presence of RT-qPCR inhibitors.

Viral RNA copies quantification of both targeting genes in wastewater samples was performed using RT-qPCR standard curves generated using EDX SARS-CoV-2 Standard (Biorad). This standard is manufactured with synthetic RNA transcripts containing 5 targets (E, N, S, ORF1a, and RdRP genes of SARS-CoV-2, 200,000 copies/mL each).Using such a standard, the limits of quantification (LOQ) of both RT-qPCR assays were estimated to 1 RNA copy per reaction (Figure 1).

[1] https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf



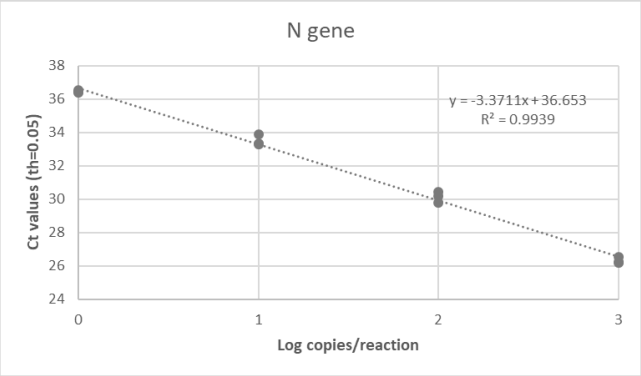


Figure 5 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

Table 3 – RT-qPCR primer-probe sets

Target	Primer name	Primer sequence (5' to 3')	References
E gene	E_Sarbeco_F1	5-ACAGGTACGTTAATAGTTAATAGCGT-3	Corman et al., 2020
	E_Sarbeco_R2	5-ATATTGCAGCAGTACGCACACA-3	
	E_Sarbeco_P1	5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	
N gene	2019-nCoV_N1_Fw	5'-GAC CCC AAA ATC AGC GAA AT-3'	CDC
	2019-nCoV_N1_Rv	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	
	2019-nCoV_N1 Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'	

Data interpretation

A sample is declared positive for the presence of SARS-CoV-2 if both targets (E and N gene) are detected with Ct values less than or equal to the LOQ. If only one target is detected or if target genes are detected with Ct values between the LOD and the LOQ, samples are reported as presumptive positive (+/-). A sample is declared negative when no target genes are detected (Ct values superior to the LOD).
In case of presumptive positive, sample is tested again using another RT-qPCR detection assay (Allplex 2019-nCoV Assay, Seegene). This commercially available detection kit is a multiplex real-time RT-PCR assay for simultaneous detection of three target genes of SARS-CoV-2 in a single tube. The assay is designed to detect RdRP and N genes specific for SARS-CoV-2, and E gene specific for all *Sarbecovirus* including SARS-CoV-2.