CORONASTEP Report 13 October 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 **265 samples so far** (Table 3). For the wastewater treatment plants (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 and fluxes in influents of WWTPs has followed the dynamics of active COVID-19 cases observed at the national level (Figure 1). Similar patterns are observed for each WWTP individually, especially for the four most impacted WWTP (Beggen, Schifflange, Bettembourg and Pétange). For the other WWTP, the detected SARS CoV-2 RNA signal is generally lower, and the dynamics may sometimes appear fuzzier, due to the large number of samples close to the detection limit of the analytical method. However, increases in the number of cases in the population are also visible in the sewage from the contributing area.

According to the latest samples analysed (week 41), the national level of SARS-CoV-2 contamination of the country's wastewater treatment plants was comparable to that of the previous week (Table 1). The general situation is very similar to that described last week for each wastewater treatment plants (Table 2). In more details, the SARS-CoV-2 signal was present in eleven of the thirteen sewage treatment plants analysed and absent from both others (Echternach and Troisvierges). Among the eleven positive treatment plants, six (Mersch, Boevange-sur-Attert, Bleesbruck, Grevenmacher, Uebersyren and Pétange) presented a contamination level very close to the detection limit of the analytical test, while the others (Hespérange, Bettembourg and Wiltz) presented a level of contamination stable in comparison to previous week, with the highest values for Schifflange and Beggen.

It is interesting to note that after several weeks with a high normalised flux of SARS-CoV-2, the viral signal decreases significantly in the Pétange wastewater. This reduction can have two explanations, either a real decrease in the number of people infected in the contributory area, or a decrease in the signal due to a dilution phenomenon as a result of heavy rainfall. This second explanation remains plausible as the number of new infections in the area does not seem to be decreasing. On the contrary, an increase of the normalised flux is observed in Schifflange wastewater, that seems concordant with the number of new cases reported last week in the contributory area.

The detailed picture of each wastewater treatment plant situation is shown in Table 2 and Figures 2, 3, 4 and 5.

Table 1 – National level of SARS-CoV-2 contamination of wastewaters in Luxembourg.

Dark green: negative samples for SARS-CoV-2 gene E (-), Green to red: positive samples for SARS-CoV-2 gene E. The intensity of the color is related to the national SARS-CoV-2 flux (RNA copies / day / 100 000 equivalent inhabitants).

Year	2019				2020																														
Week	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	Week 36	Week 37	Week 38	Week 39	Week 40	Week 41
National Contamination Level	-	-	-	-	-	-													-																	

Figure 1 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Luxembourgish wastewater samples from March to October 2020. Grey squares: daily-confirmed cases for Luxembourgish residents (https://data.public.lu/fr/datasets/donnees-covid19/), dots: cumulative SARS-CoV-2 flux (RNA copies / day / 100 000 equivalent inhabitants).

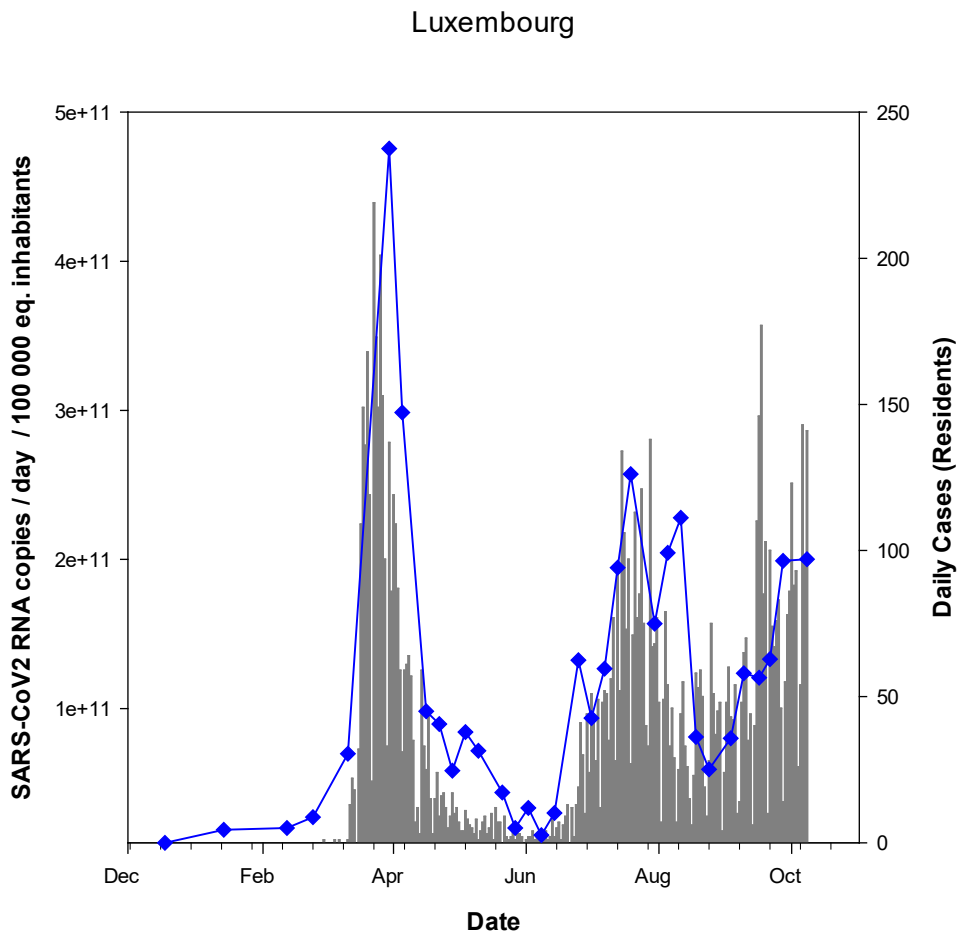

Table 2 - Level of SARS-CoV-2 contamination of each analyzed wastewater treatment plant in Luxembourg. BEG: Beggen, BET: Bettembourg, SCH: Schifflange, BLE: Bleesbruck, MER: Mersch, PET: Pétange, HES: Hespèrange, ECG: Echternach, UEB: Uebersyren, GRE: Grevenmacher, TRO: Troisvierges, BOE: Boevange sur Attert, WIL: Wiltz

Dark green: negative samples for SARS-CoV-2 gene E (-), Green to red: positive samples for SARS-CoV-2 gene E. The intensity of the color is related to the RT-qPCR signal (Ct values)

	2019						2020																													
	Before first case						1st wave														2nd wave																
WWTP	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	Week 36	Week 37	Week 38	Week 39	Week 40	Week 41
BEG																																				
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Figure 2 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in the four most impacted wastewater treatment plants from March to September 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

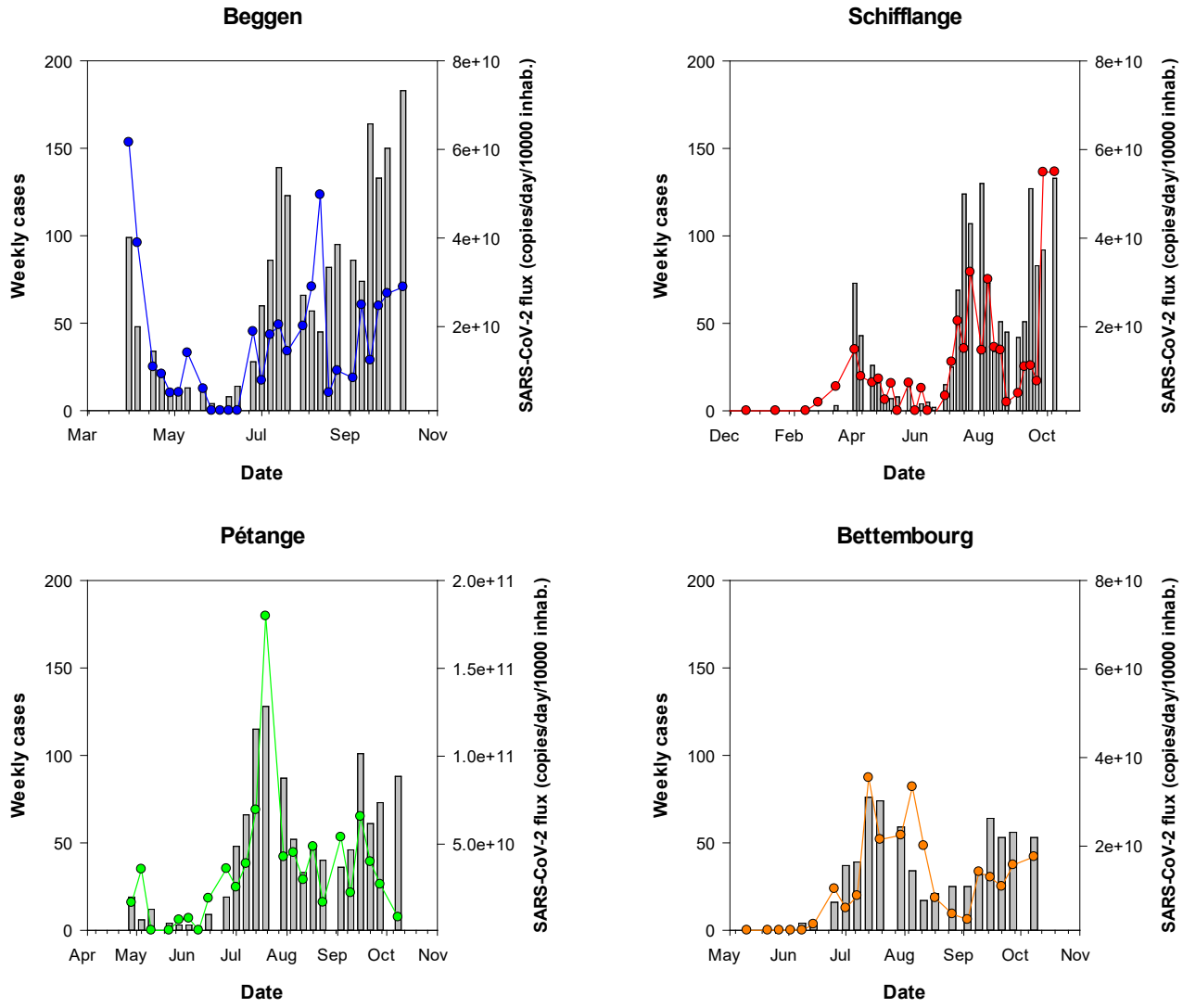

Figure 3 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Hespérange, Mersch and Boevange-sur-Attert wastewater treatment plants from March to September 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

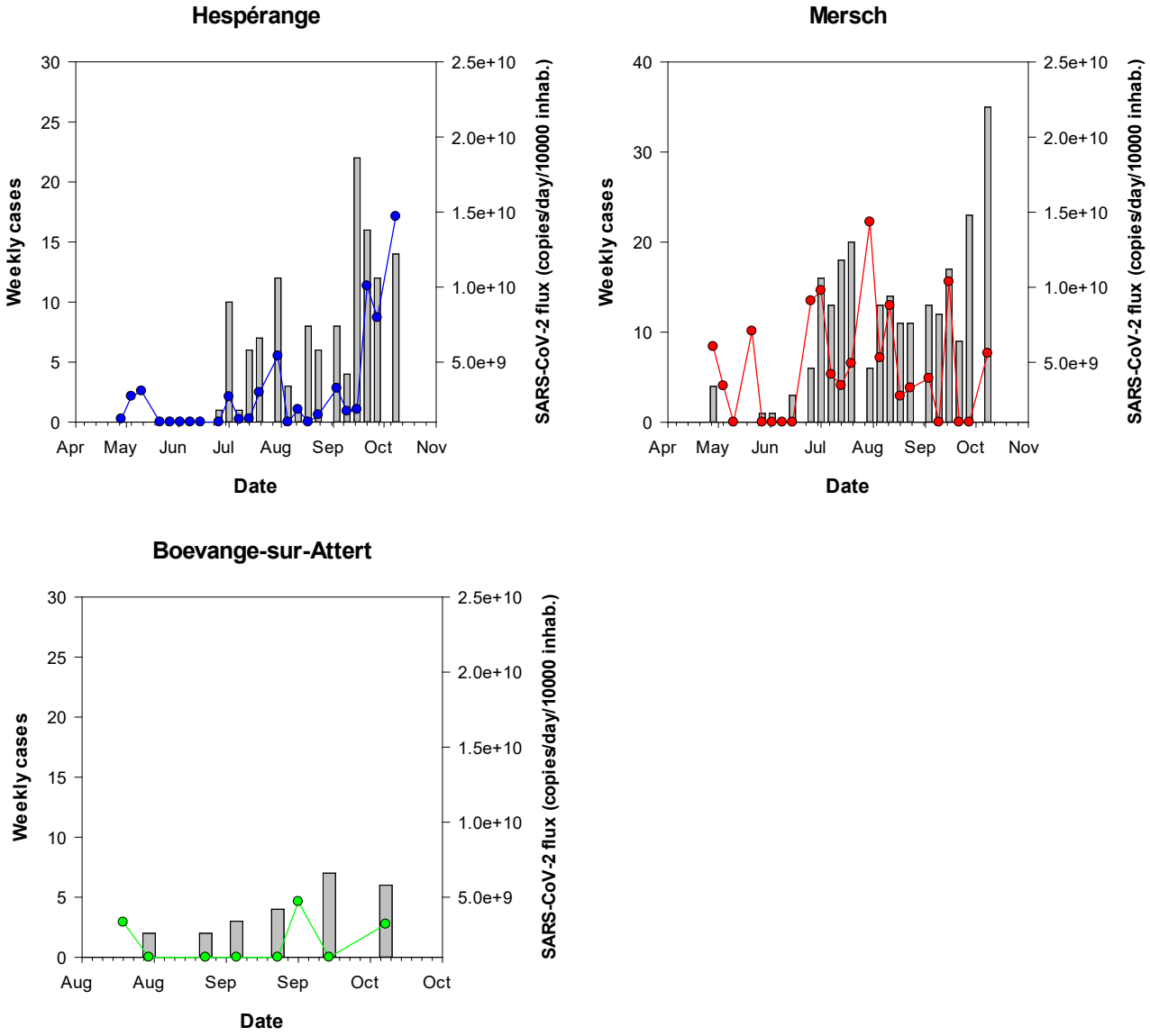

Figure 4 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from March to September 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent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

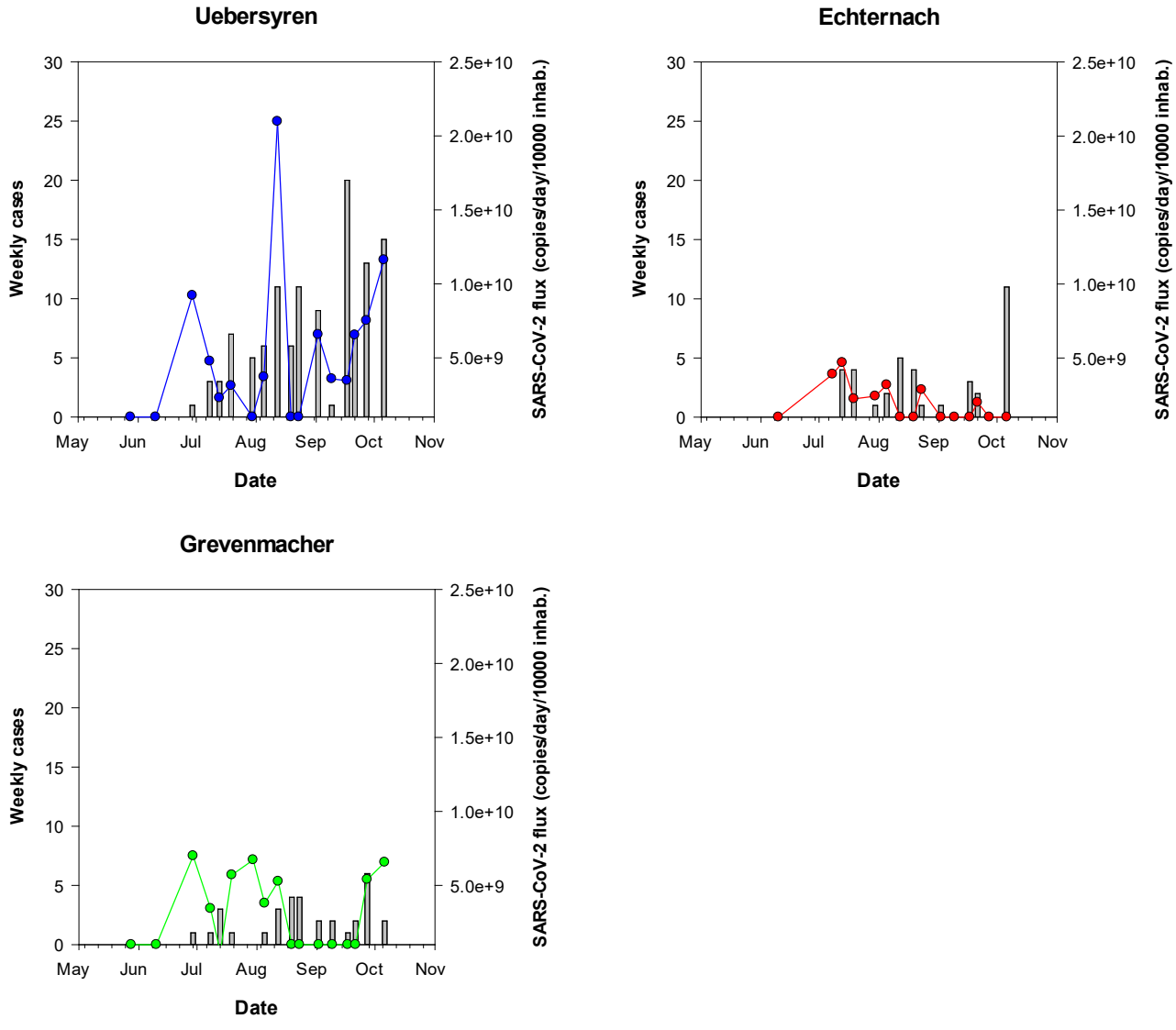

Figure 5 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from March to September 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

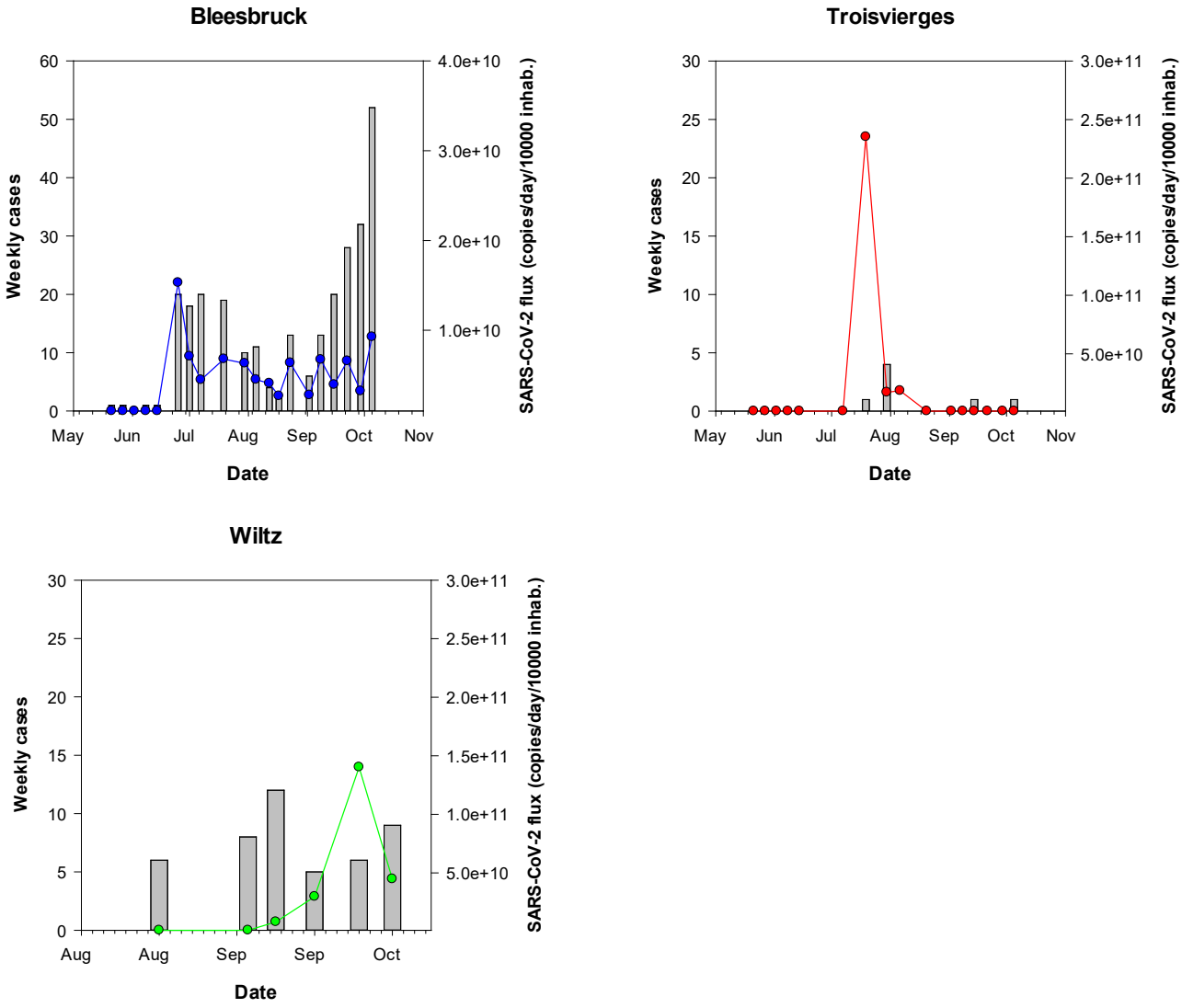

Table 3- Timing of sewage sampling

Wastewater Treatment Plant	Nominal capacity (eq. inhabitants)	Inhabitants connected	2019	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	Week 36	Week 37	Week 38	Week 39	Week 40	Week 41	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	x	x	x	x	x	x	**28**
Bettembourg	95000	53606												x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**22**
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	x	x	**36**
Bleesbrück	80000	30930													x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	**20**
Mersch	70000	30473										x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**24**
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	x	x	**32**
Hesperange	36000	15479										x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	**24**
Echternach	36000	7499																x				x	x	x	x	x	x	x	x	x	x	x	x	x	**15**
Uebersyren	35000	18600														x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	**17**
Grevenmacher	47000	9835														x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	**17**
Troisvierges	5000	3411													x	x	x	x	x			x		x	x	x		x		x	x	x	x	x	**16**
Boevange	15000	1170																										x	x	x	x	x	x	x	**8**
Wiltz	16500	6944																											x		x	x	x	x	**6**
Total	**785500**	**445302**	**8**	**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**	**12**	**13**	**13**	**13**	**13**	**13**	**265**

Materials and Methods

Sewage samples

From March to October 2020, up to thirteen WWTPs were sampled at the inlet of the plant according to the planning presented in Table 2. 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 3. The RT-qPCR protocols and reagents were all provided by the LIH.

Table 4 – 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'	

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.

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

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 6).

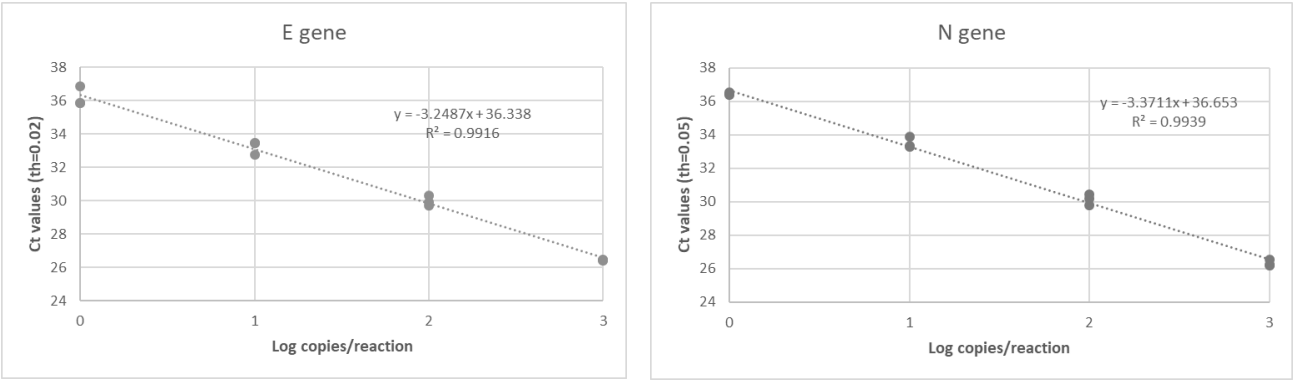

Figure 6 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

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.

As shown in Figure 7, 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, only the E gene results were presented in this report.

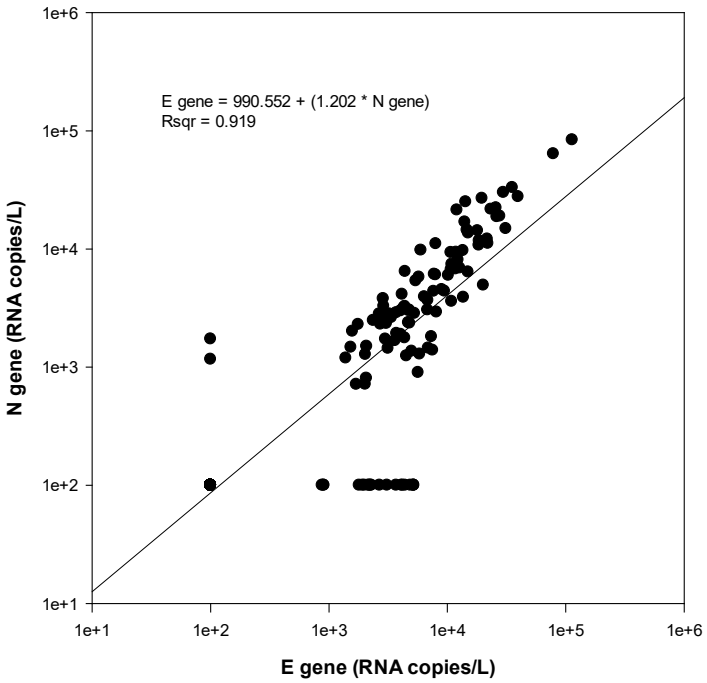

Figure 7 - Relationship between the SARS-CoV-2 RNA concentration (RNA copies / L of wastewater) estimated by the both distinct RT-qPCR systems targeting the E and N gene, respectively