CORONASTEP Report 20 October 2020
SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

In October, the dynamics of SARS-CoV-2 RNA copies and fluxes in influents of wastewater treatments plants (WWTPs) (Figure 1) continued to follow closely the dynamics of active COVID-19 cases observed at the national level (Figure 1). During week 42, both the total flux at the inlet of the wastewater and the number of active cases showed steep increases, reaching higher levels than in March 2020 during the maximum of the first wave. The National level of SARS-CoV-2 contamination of wastewaters in Luxembourg is the highest since the beginning of the study (Table 1).

Steep increase of the SARS-CoV-2 RNA copies fluxes are observed at the level of each WWTP individually (Figures 2, 3, 4 and 5), except at the WWTPs of Wiltz and Troisvierges (representing together 21,500 inhabitants-equivalents) which behave differently from the other WWTP since the beginning of the study.

It must be noted that the quantification of SARS-CoV-2 RNA at the inlet of the WWTPs is expressed as fluxes, (i.e. quantities of virus RNA flowing into the wastewater treatment plant per day), not concentration. The fluxes correspond to the concentrations of SARS-CoV-2 RNA in the wastewater multiplied by the daily water flow. During rain events, the concentration of viruses in the water certainly decreases, being diluted but the water flow is also higher. The resulting fluxes can be very high. The flux quantification is not affected per se by the dilution effect of the rain, except if the dilution is so high than the virus becomes undetectable. In this case, the fluxes cannot be calculated, although they can be significant. This has been not the case during last weeks, except probably at Troisvierges (5,000 inhabitants-equivalents).

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).

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	Week 42
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).

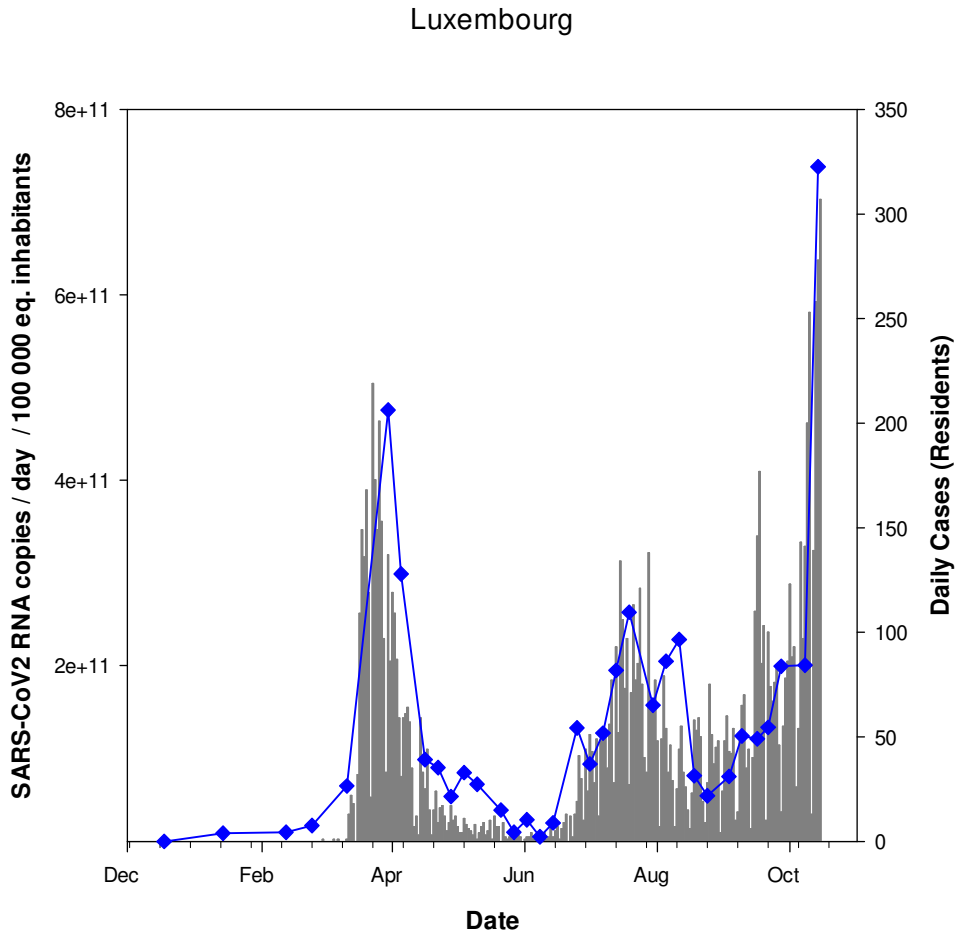

Table 2 - Level of SARS-CoV-2 contamination of each analyzed wastewater treatment plant in Luxembourg. BEG: Beggen, BET: Bettembourg, SCH: Schifflange, BLE: Bleesbruck, MER: Mersch, PET: Pétange, HES: Hespèrange, ECG: Echternach, UEB: Uebersyren, GRE: Grevenmacher, TRO: Troisvierges, BOE: Boevange sur 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	Week 42
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Figure 2 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in the three most impacted wastewater treatment plants from March to October 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant (only available until week 41 included), dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

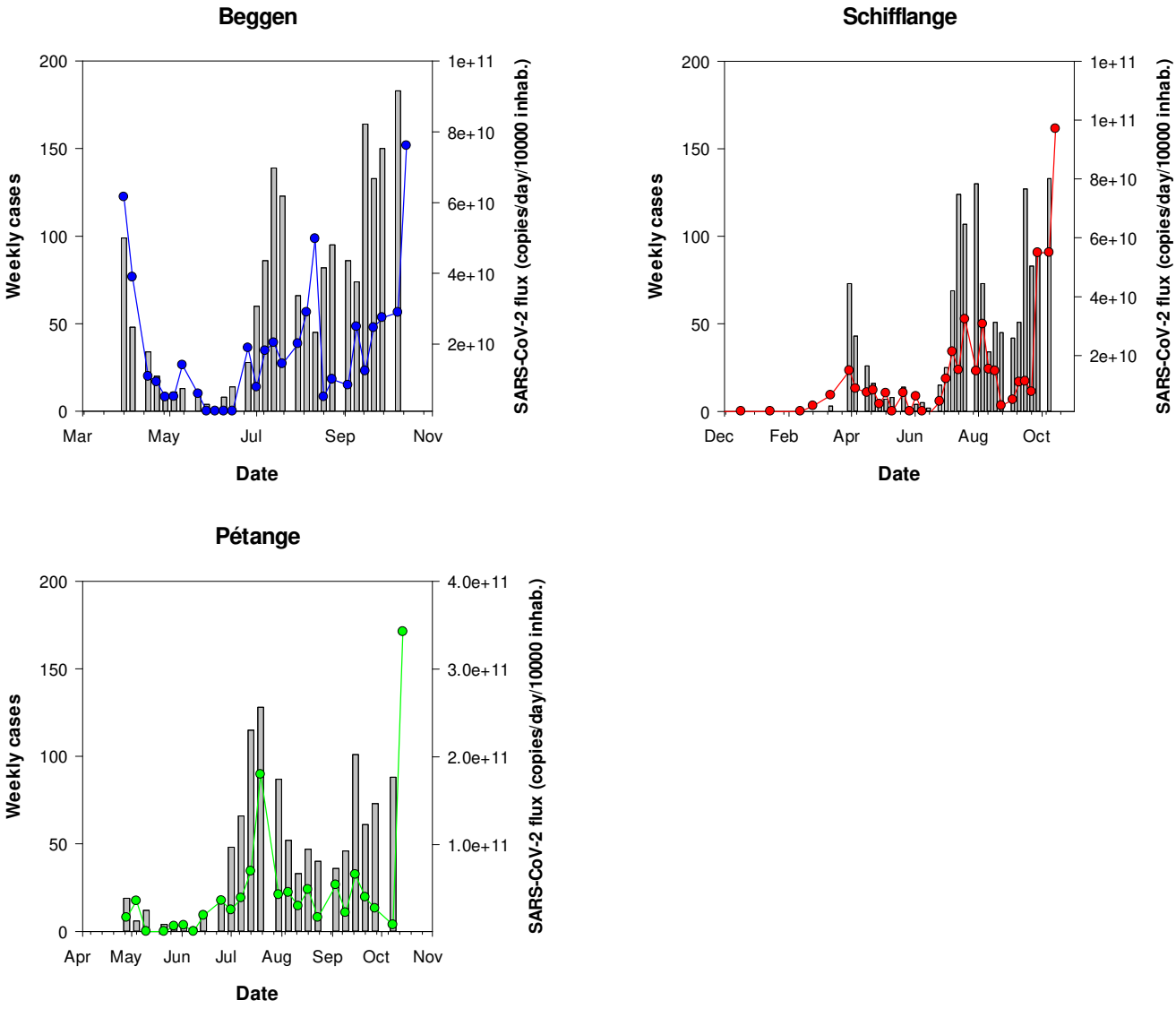

Figure 3 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Hespérange, Mersch and Boevange-sur-Attert wastewater treatment plants from March to October 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant (only available until week 41 included), dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

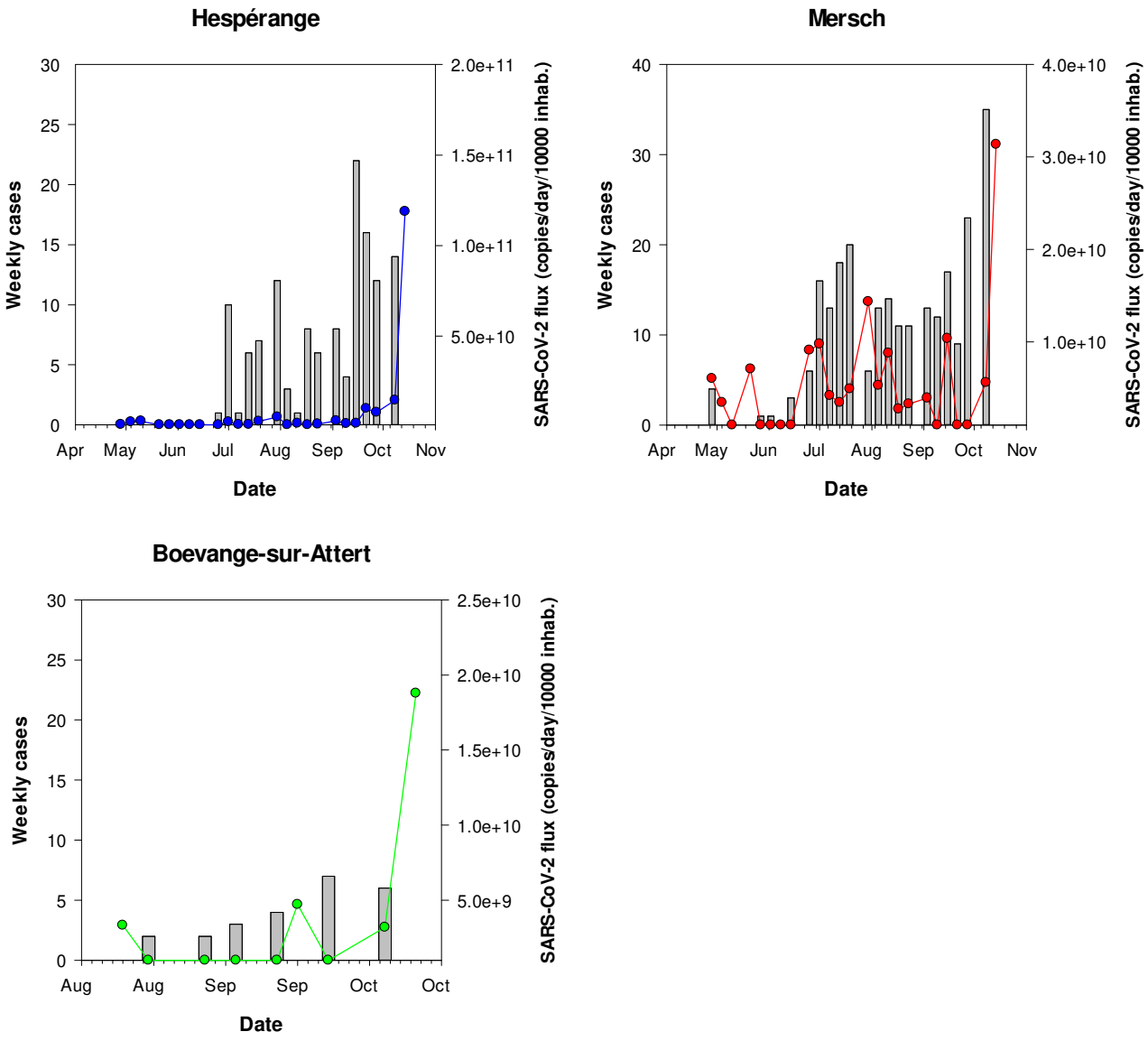

Figure 4 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from March to October 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant (only available until week 41 included), dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

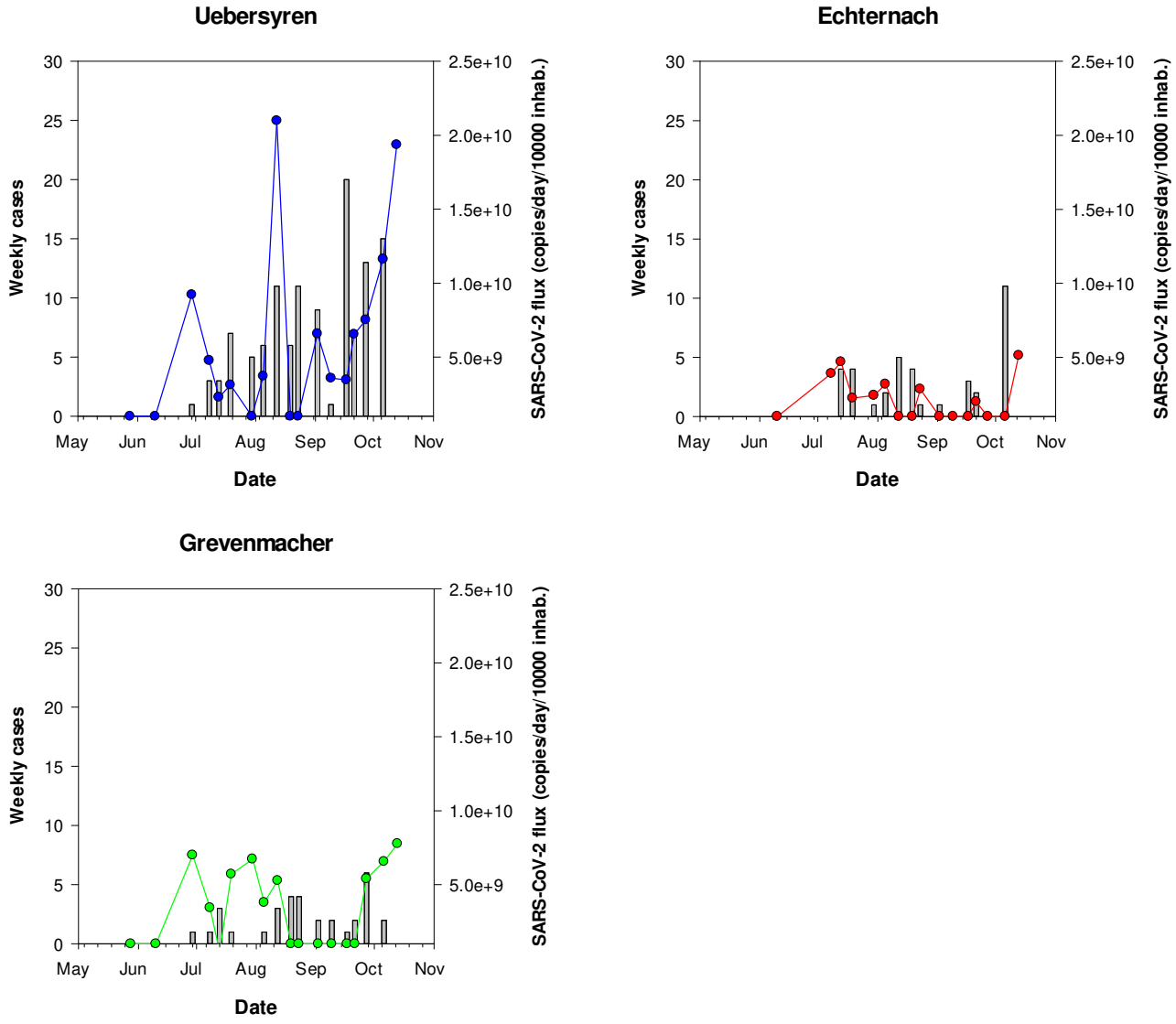

Figure 5 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from March to October 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant (only available until week 41 included), dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

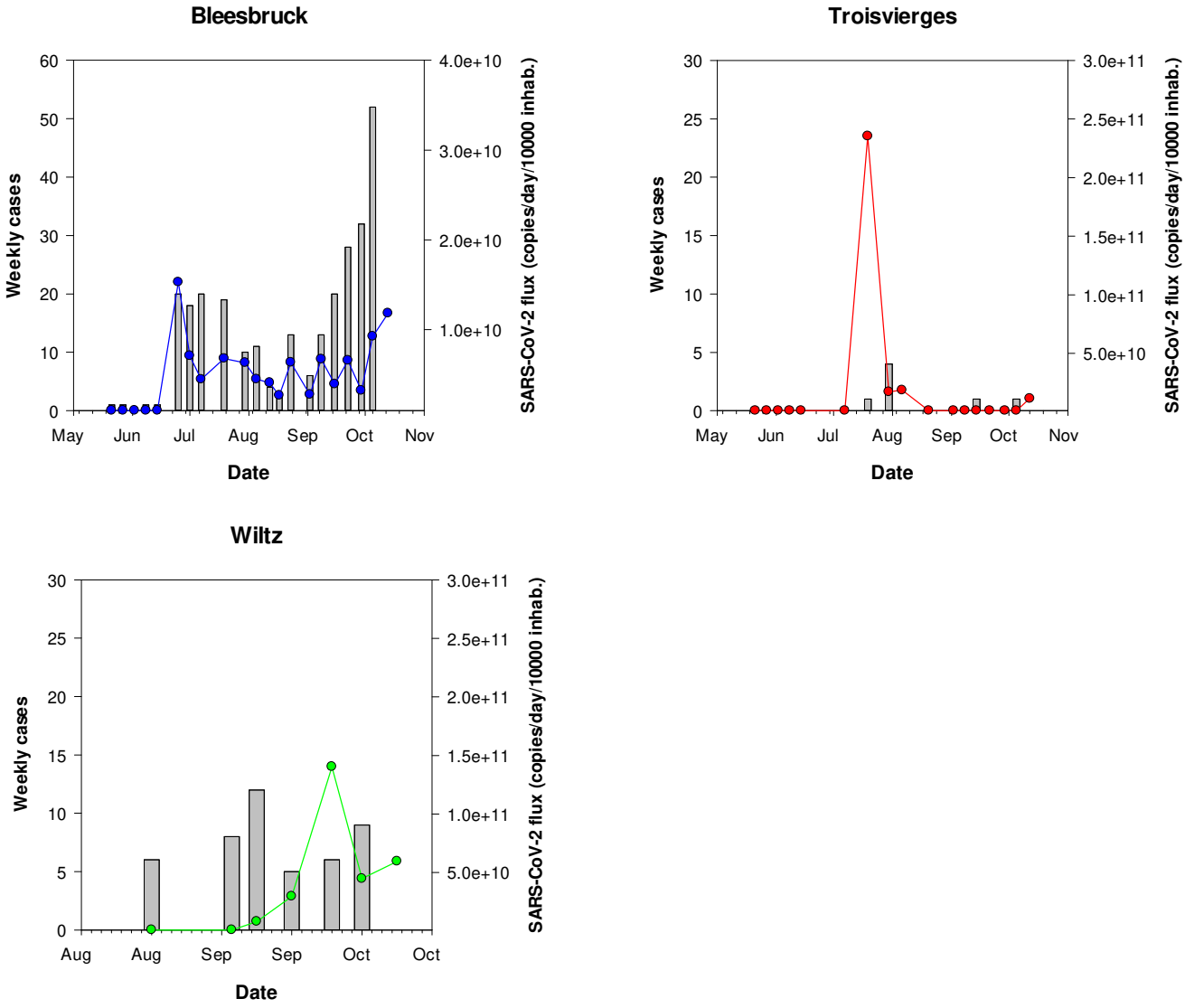

Table 3- Timing of sewage sampling

Wastewater Treatment Plant	Nominal capacity (eq. inhabitants)	Inhabitants connected	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	Week 42	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	x	**29**
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	x	**37**
Bleesbrück	80000	30930												x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	**21**
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	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	**33**
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	x	**25**
Echternach	36000	7499															x				x	x	x	x	x	x	x	x	x	x	x	x	x	x	**16**
Uebersyren	35000	18600													x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	**18**
Grevenmacher	47000	9835													x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	**18**
Troisvierges	5000	3411												x	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	x	x	**8**
Wiltz	16500	6944																										x		x	x	x	x	x	x	**6**
Total	**785500**	**445302**	**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**	**12**	***277***

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).

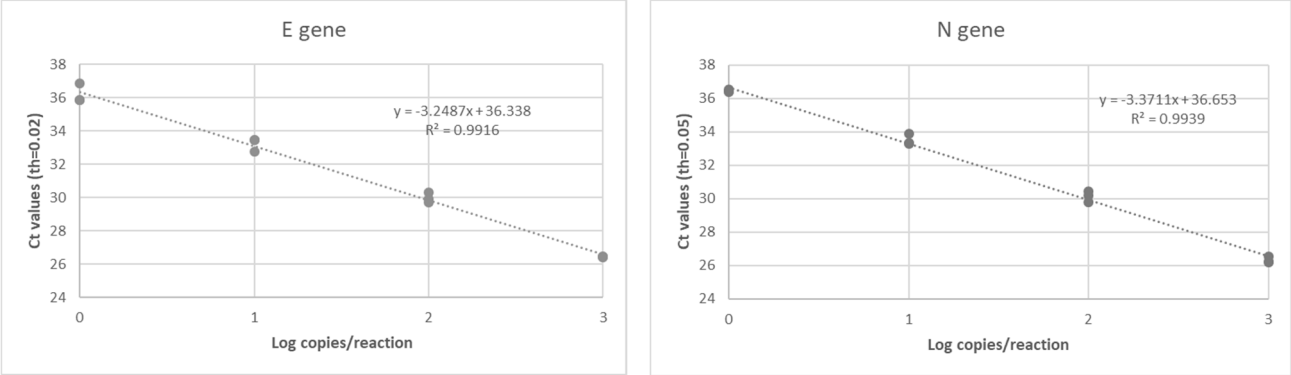

Figure 6 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

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.

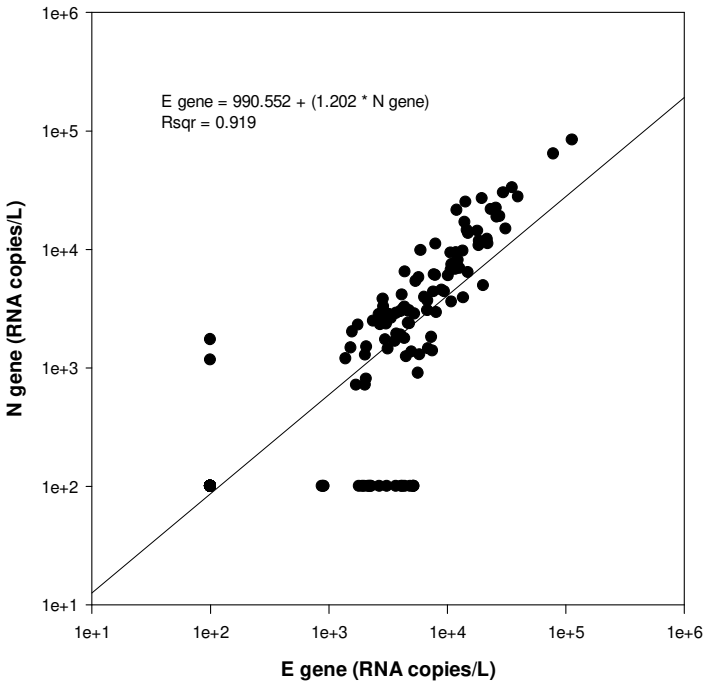

Figure 7 - Relationship between the SARS-CoV-2 RNA concentration (RNA copies / L of wastewater) estimated by the both distinct RT-qPCR systems targeting the E and N gene, respectively