CORONASTEP Report 49
(Week 07- Partial)
SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

This report 49 presents the results of SARS-CoV-2 contamination of wastewater at the entrance of 11 wastewater treatment plants at the beginning of the week 07 of 2021. Hespérange and Boevange-sur-Attert treatment plants were not analysed.

The SARS-CoV-2 RNA fluxes present in wastewater treatment plants at the beginning of the week 06 indicate a high prevalence of the virus in wastewater at national. A slight upward trend was perceptible again at the beginning of the week 07. However, we must remain cautious in our interpretation and wait for the results of the next analyses to be able to identify a real trend.

At the level of individual wastewater treatment plants, certain geographical disparities appear. An upward dynamic is notably observed at the Schifflange, Beggen and Grevenmacher wastewater treatment plants, while the RT-qPCR signal is constant for other treatment plants.

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 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																																	

Week	Week 43	Week 44-1	Week 44-2	Week 45-1	Week 45-2	Week 45-3	Week 46-1	Week 46-2	Week 46-3	Week 47-1	Week 47-2	Week 48-1	Week 48-2	Week 48-3	Week 49-1	Week 49-2	Week 50-1	Week 50-2	Week 51-1	Week 51-2	Week 51-2	Week 52	Week 53	Week 01-1	Week 01-2	Week 02-1	Week 02-2	Week 03-1	Week 03-2	Week 04-1	Week 04-2	Week 05-1	Week 06-1	Week 06-2	Week 07-1
National Contamination Level																																			

Figure 1a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Luxembourgish wastewater samples from December 2019 to February 2021. Grey squares: daily-confirmed cases for Luxembourgish residents (https://data.public.lu/fr/datasets/donnees-covid19/), Blue dots: cumulative SARS-CoV-2 flux (RNA copies / day / 100 000 equivalent inhabitants).

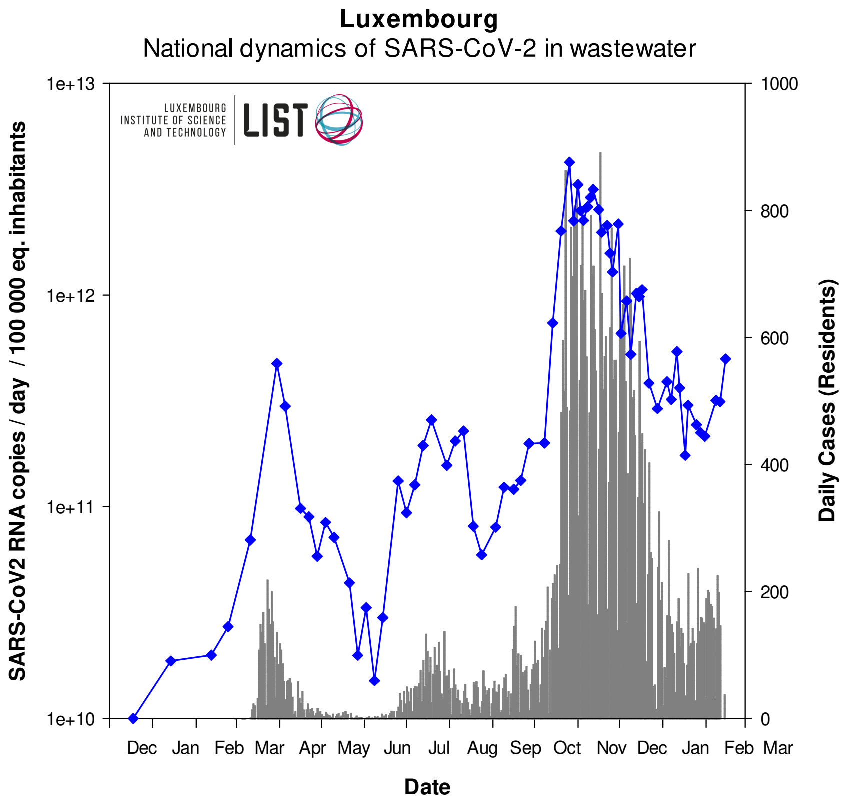


Figure 1b – Close-up of Figure 1a showing results from September 1st on.

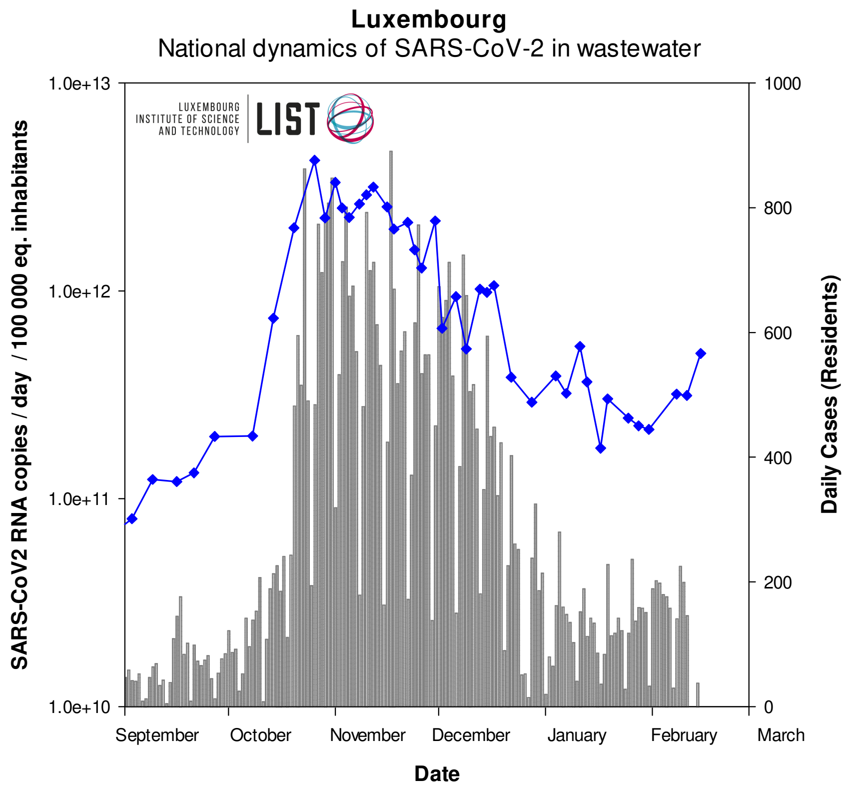


Table 2 - Level of SARS-CoV-2 contamination of each analyzed wastewater treatment plant in Luxembourg during the second wave. BEG: Beggen, BET: Bettembourg, SCH: Schifflange, BLE: Bleesbruck, MER: Mersch, PET: Pétange, HES: Hespérange, ECG: Echternach, UEB: Uebersyren, GRE: Grevenmacher, TRO: Troisvierges, 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) Grey boxes: no data

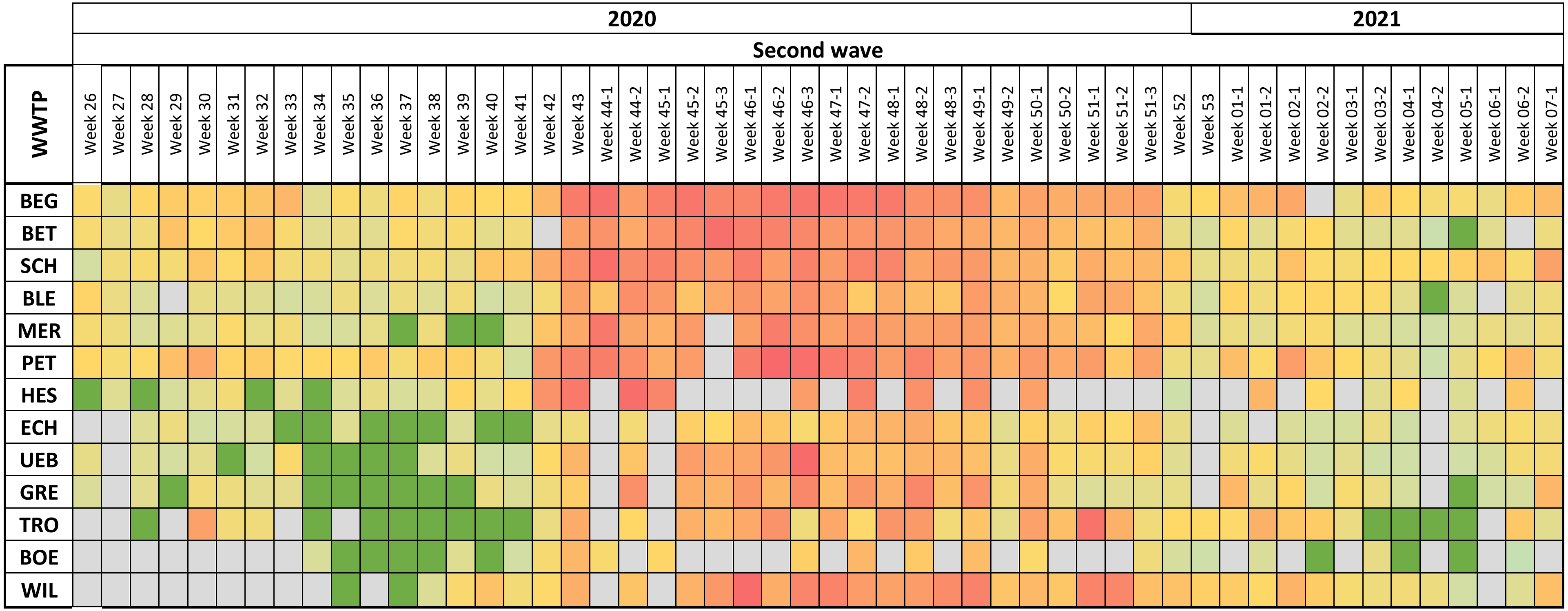


Figure 2a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in the four most impacted wastewater treatment plants from March 2020 to February 2021. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

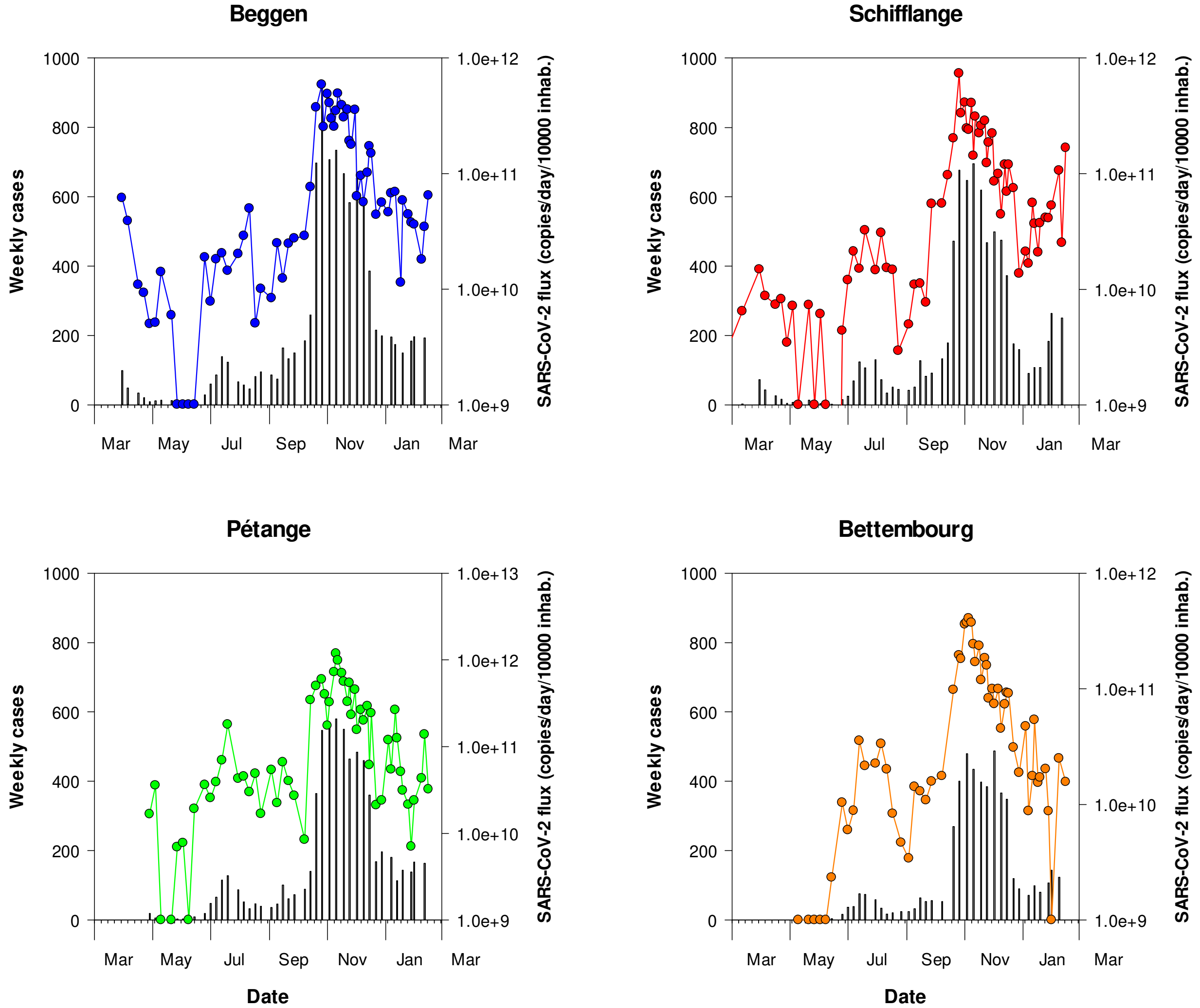


Figure 2b – Close-up of Figure 2a showing results from September 1st on.

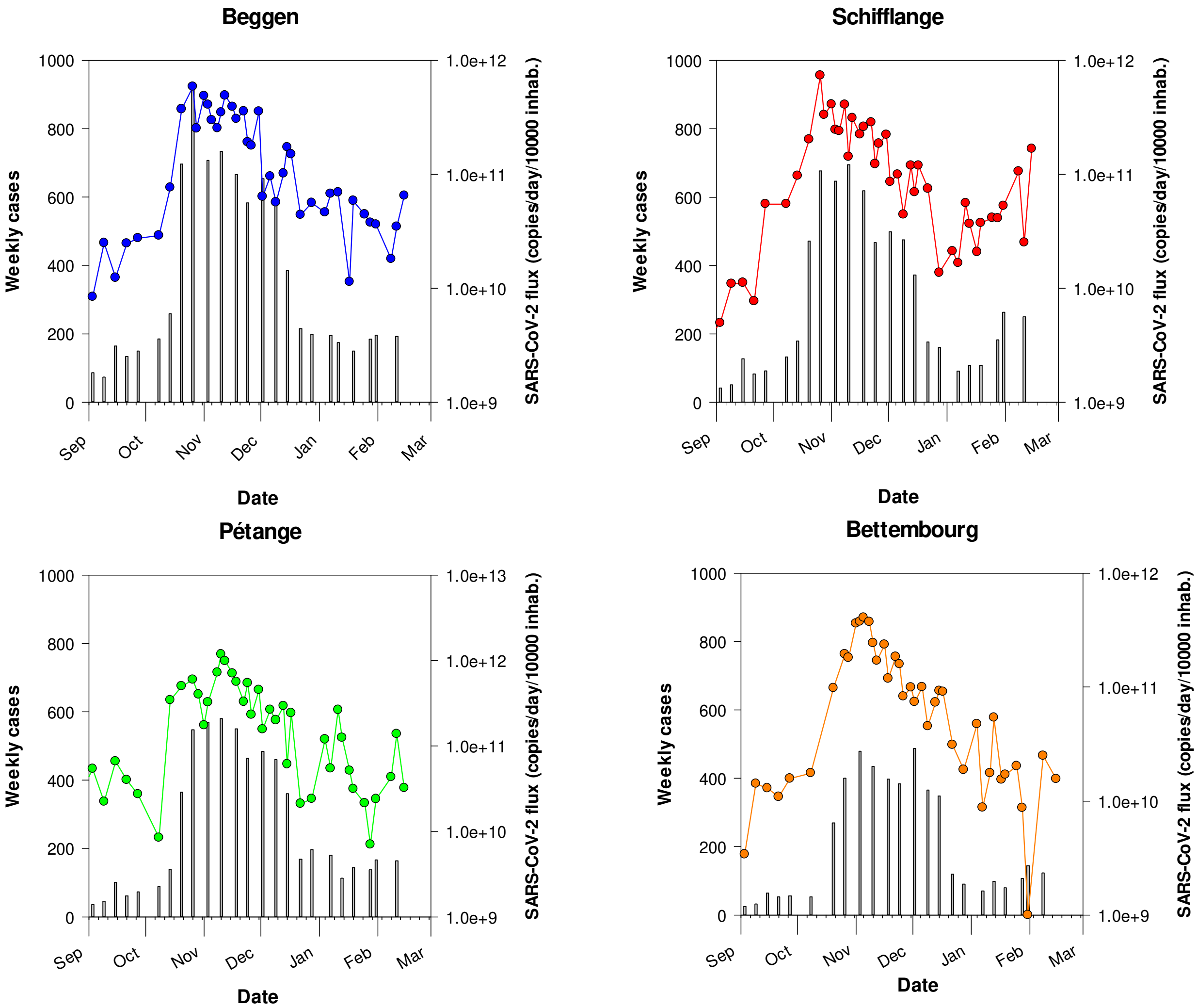
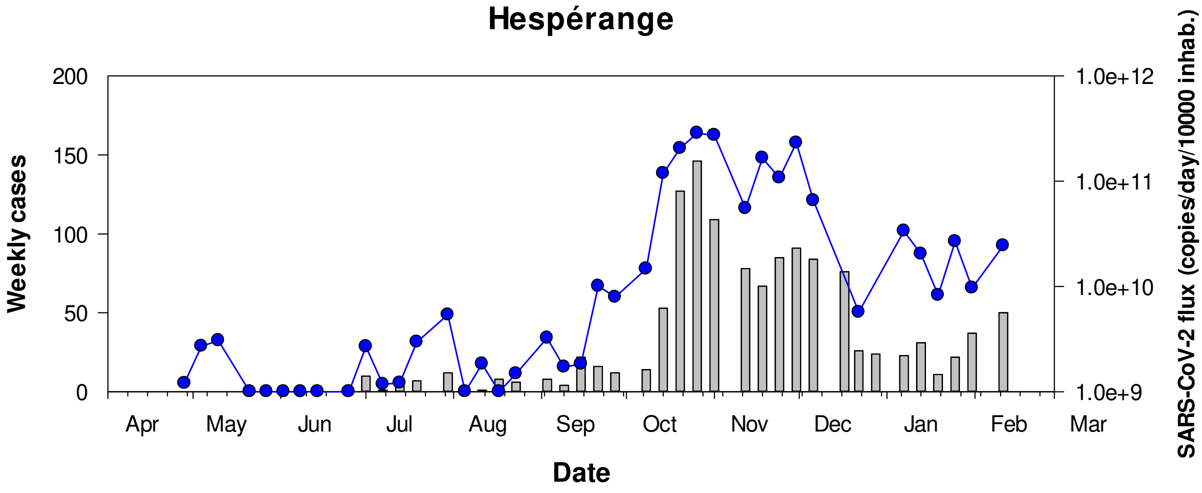
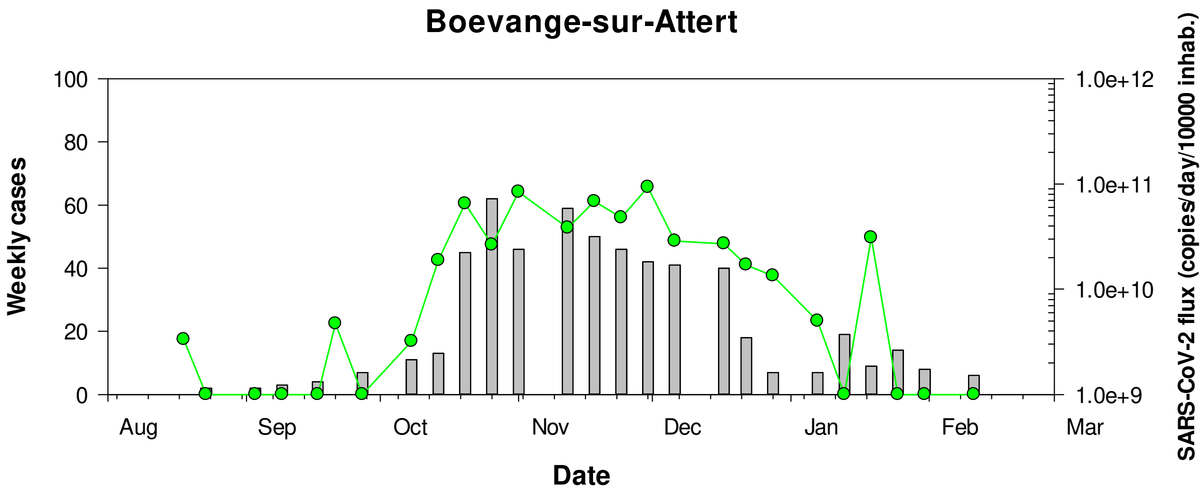


Figure 3a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Hespérange, Mersch and Boevange-sur-Attert wastewater treatment plants from March 2020 to February 2021. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).





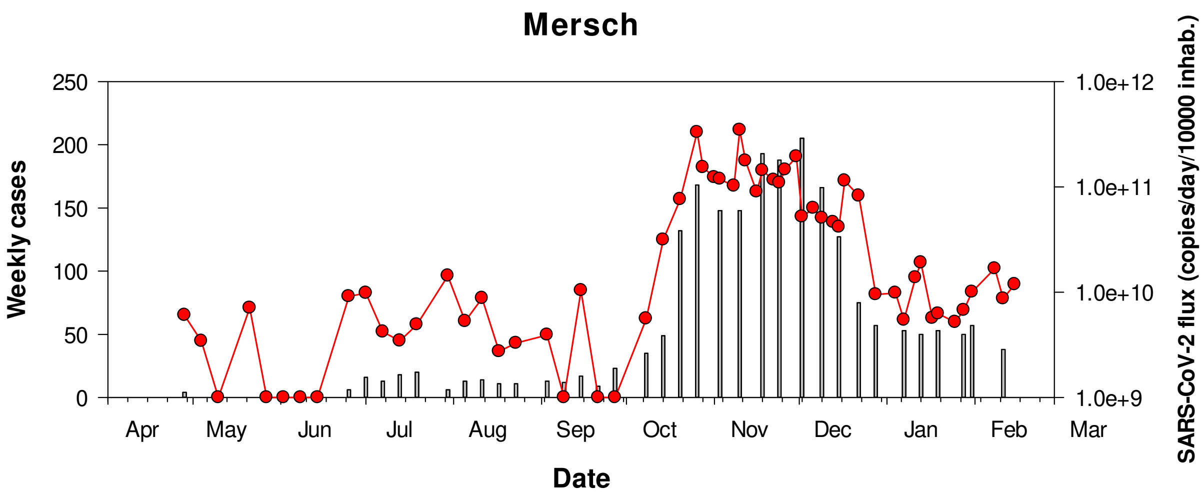
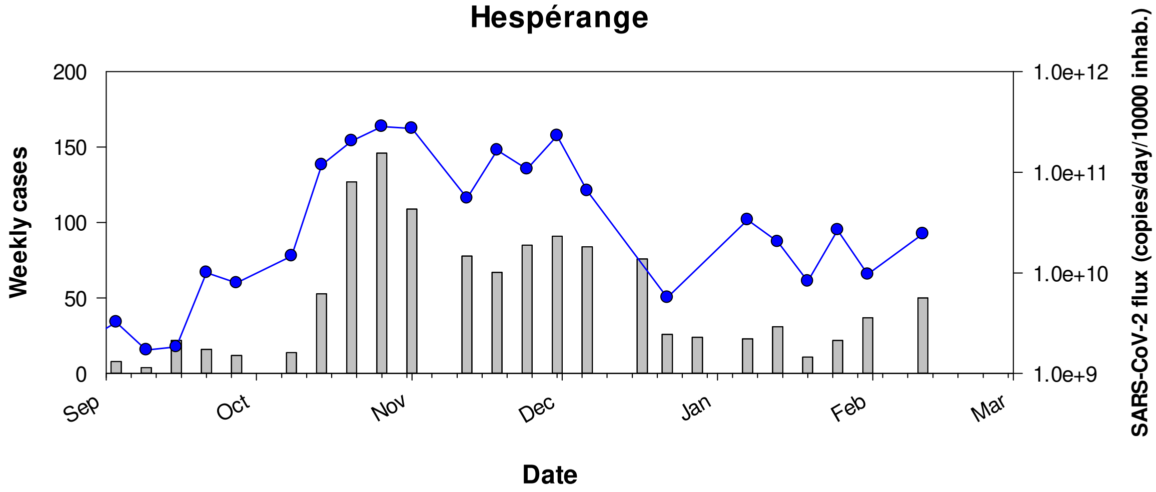
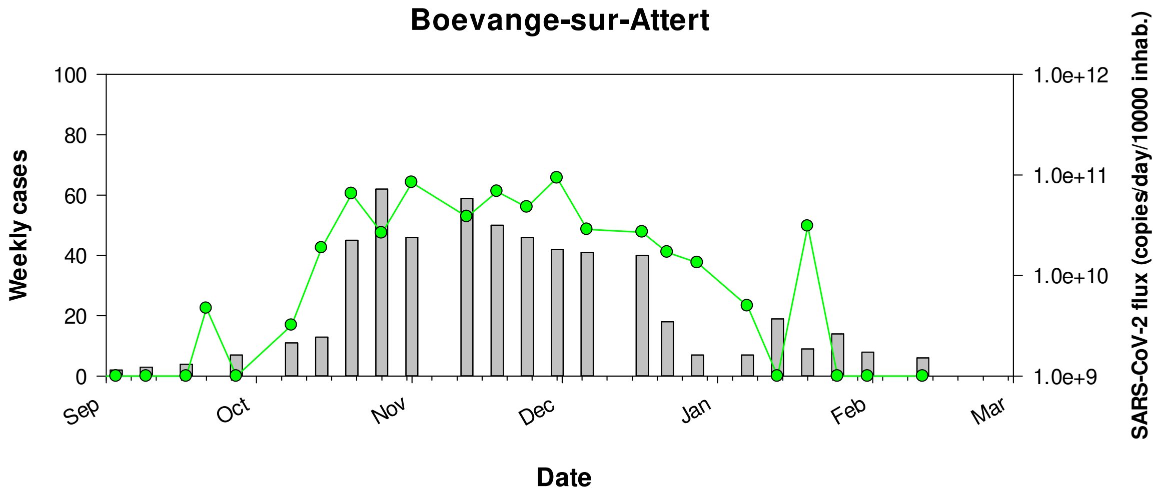


Figure 3b – Close-up of Figure 3a showing results from September 1st on.





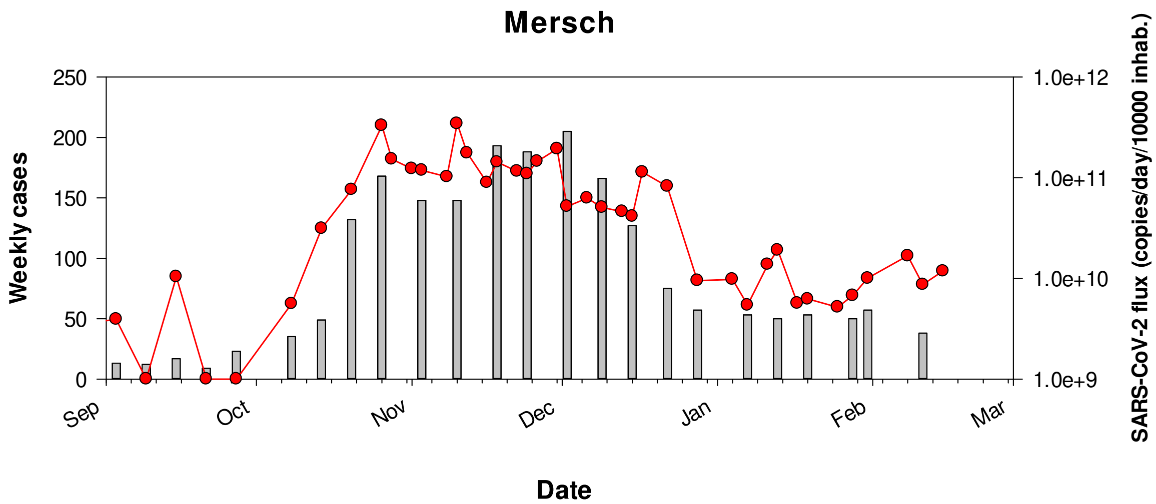


Figure 4a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from March 2020 to February 2021. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

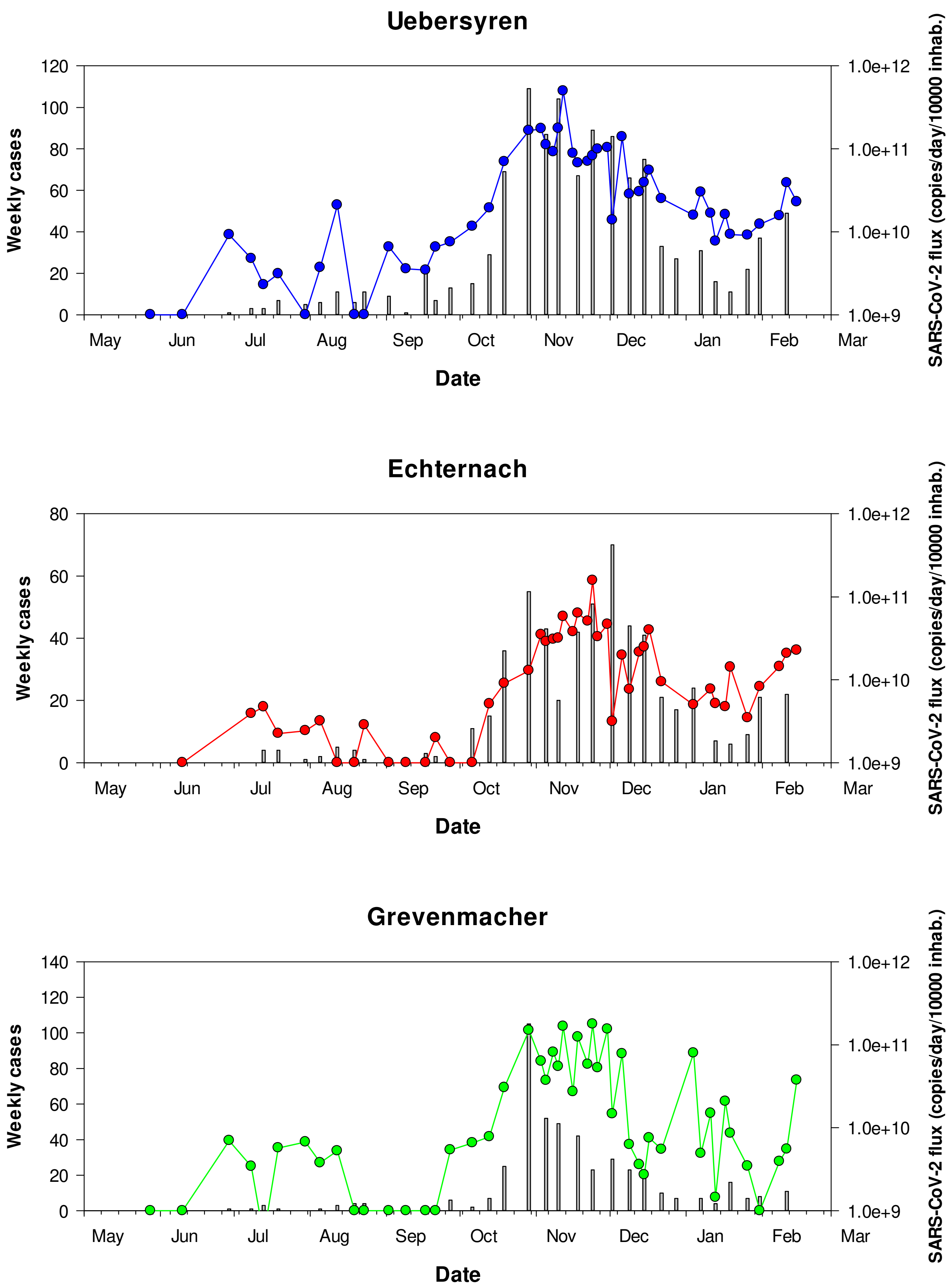
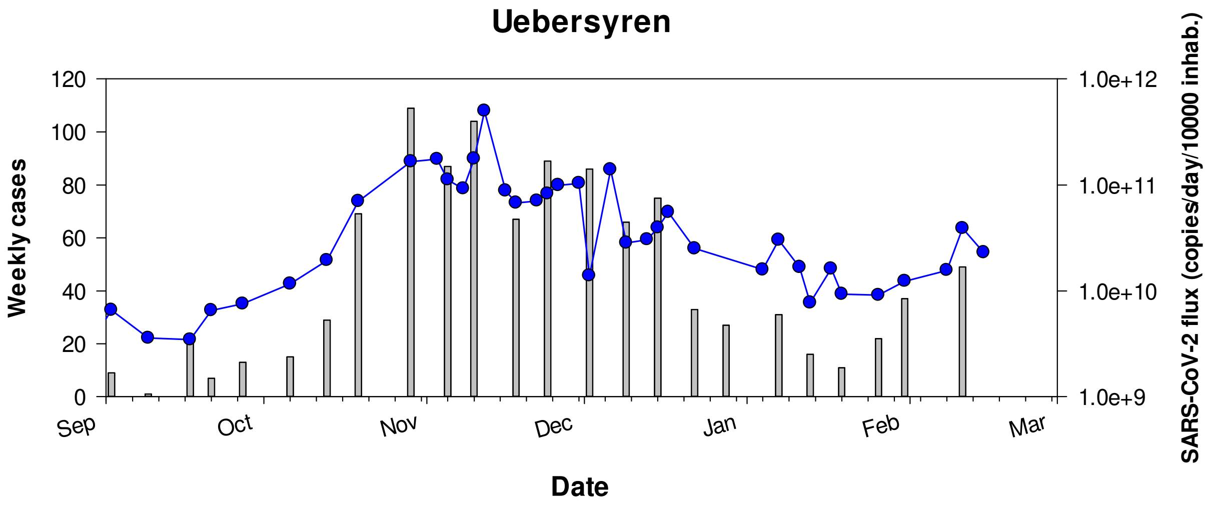
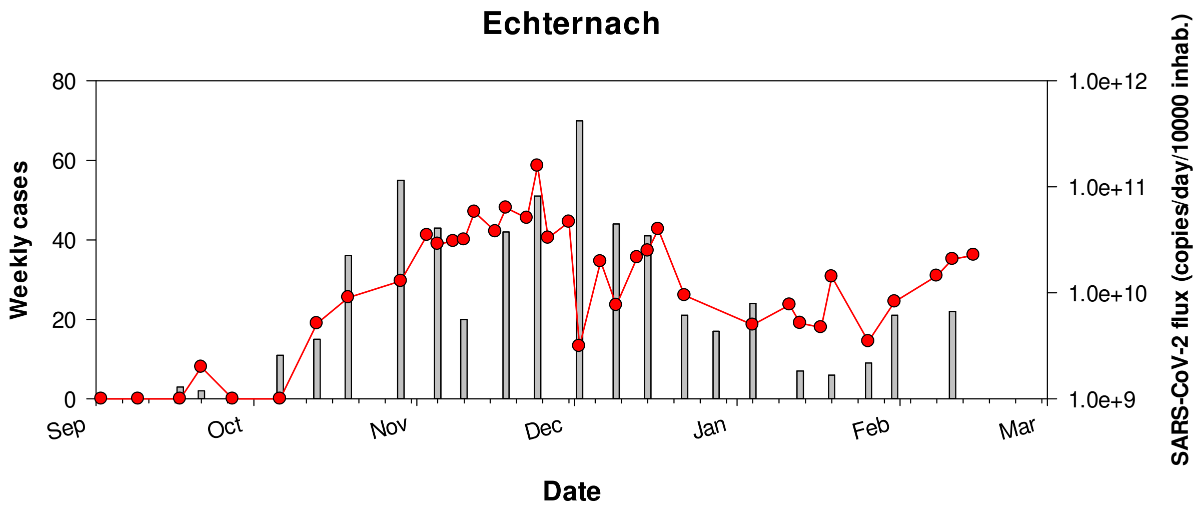


Figure 4b – Close-up of Figure 4a showing results from September 1st on.





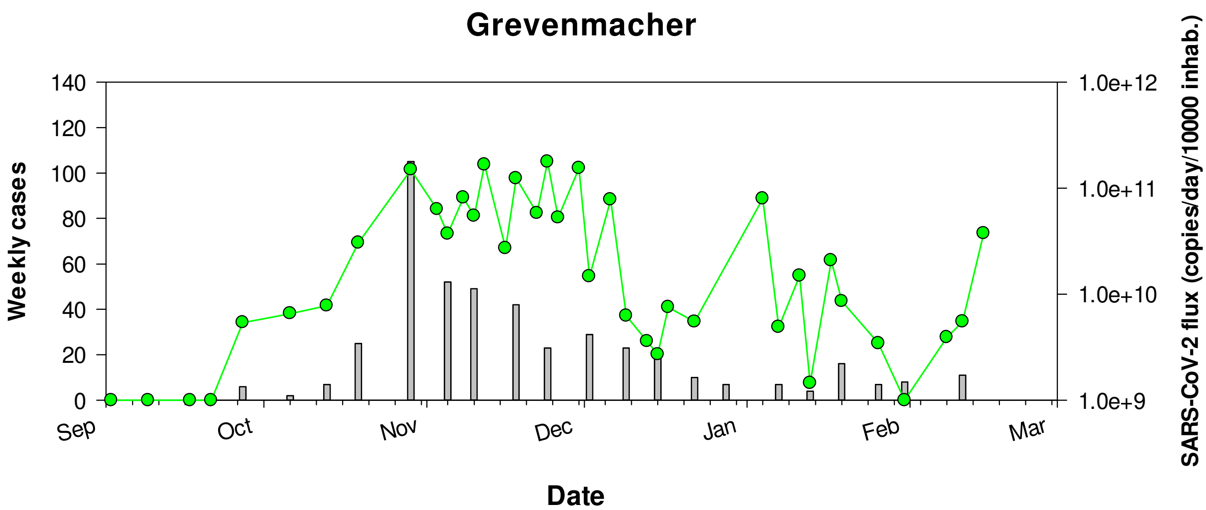


Figure 5a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from March 2020 to February 2021. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

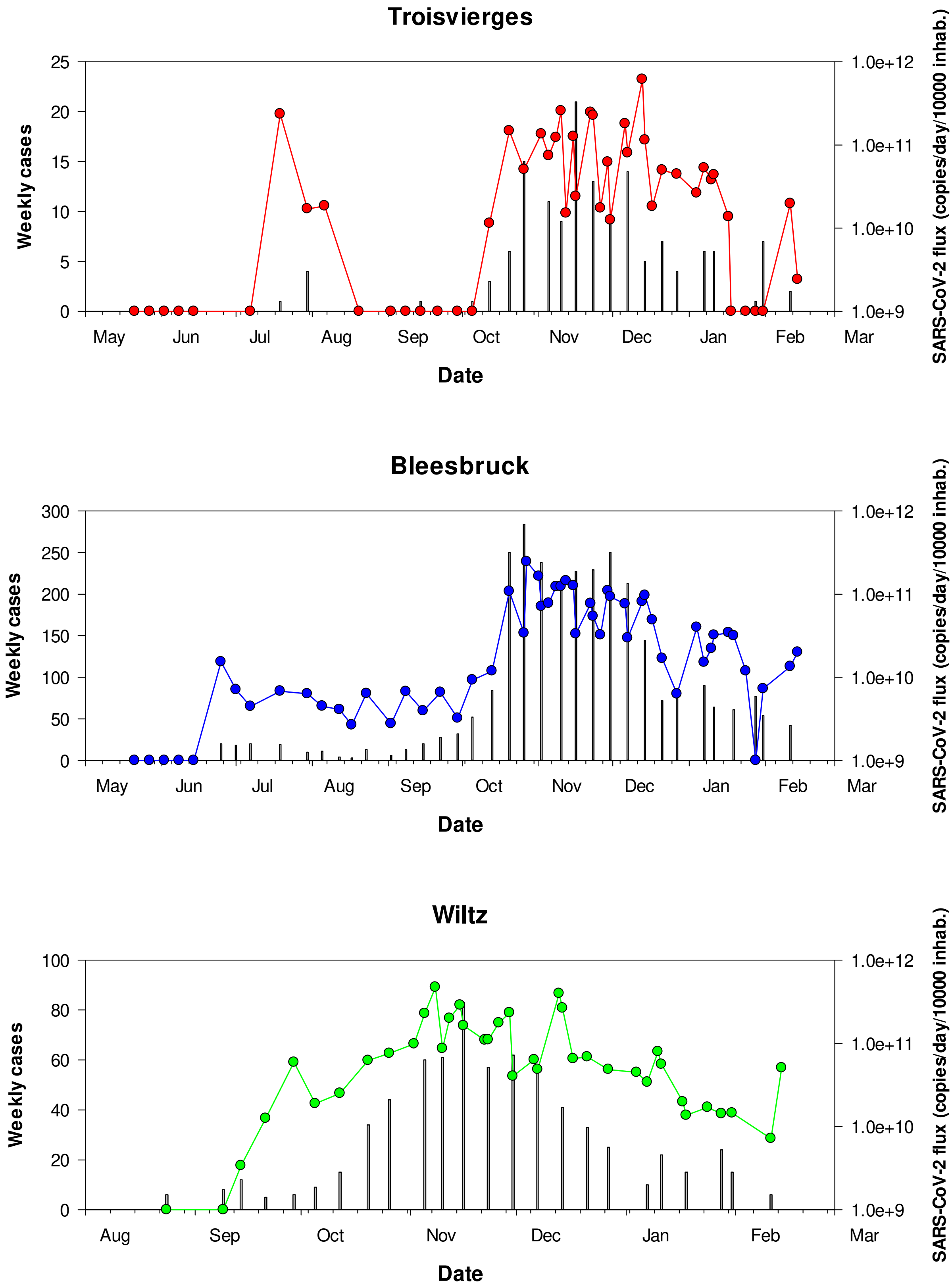
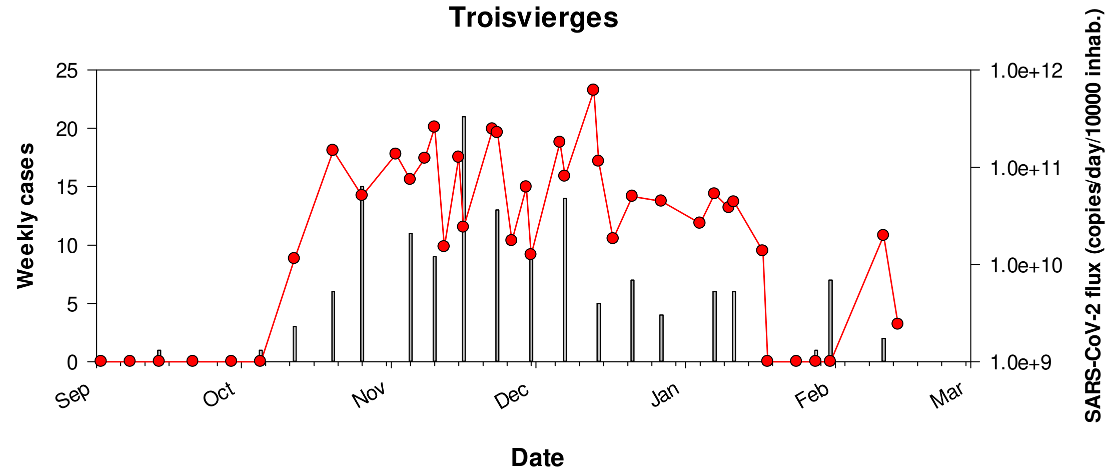
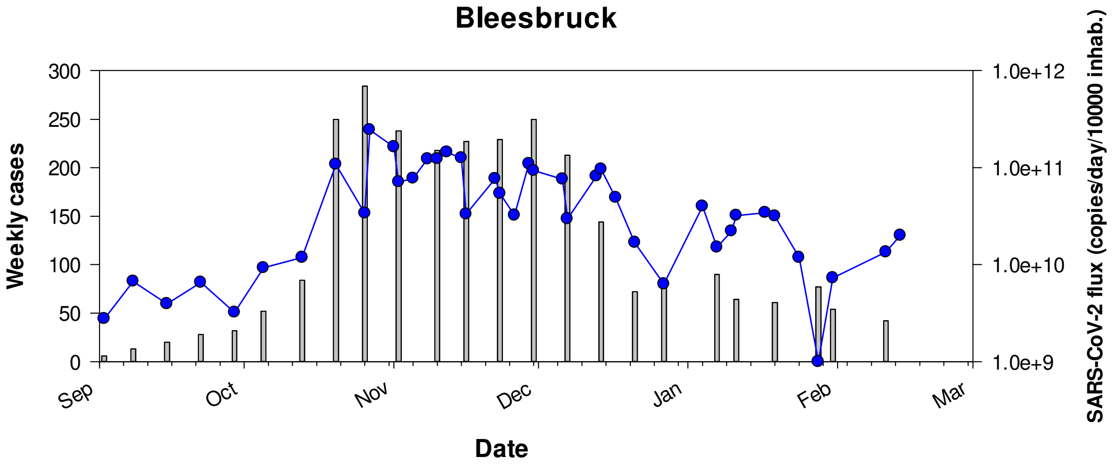


Figure 5b – Close-up of Figure 5a showing results from September 1st on.





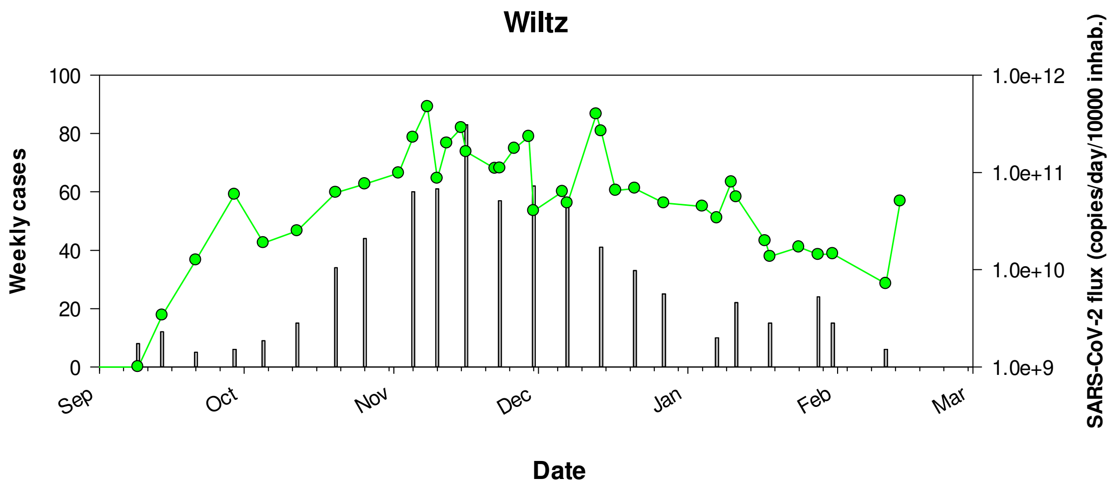


Table 3- Timing of sewage sampling since the beginning of the CORONASTEP study

			2019				2020																		
WWTP	**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
Beggen	210000	139731									1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bettembourg	95000	53606															1	1	1	1	1	1	1	1	1
Schifflange	90000	68143	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bleesbrück	80000	30930																1	1	1	1	1	1	1	1
Mersch	70000	30473													1	1	1	1	1	1	1	1	1	1	1
Pétange	50000	59481	1	1	1	1	1	1	1	1					1	1	1	1	1	1	1	1	1	1	1
Hespérange	36000	15479													1	1	1	1	1	1	1	1	1	1	1
Echternach	36000	7499																			1				1
Uebersyren	35000	18600																	1		1		1		1
Grevenmacher	47000	9835																	1		1		1		1
Troisvierges	5000	3411																1	1	1	1	1			1
Boevange sur Attert	15000	1170																							
Wiltz	16500	6944																							
Total	**785500**	**445302**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**5**	**5**	**6**	**8**	**10**	**8**	**11**	**8**	**9**	**7**	**11**
Pop Lux (2019)		**613901**																							
		72.54%																							

	2020																									2021							
WWTP	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	Week 43	Week 44	Week 45	Week 46	Week 47	Week 48	Week 49	Week 50	Week 51	Week 52	Week 53	Week 01	Week 02	Week 03	Week 04	Week 05	Week 06	Week 07	**Total samples**
Beggen	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	3	2	3	2	2	3	1	1	2	1	2	2	2	2	1	**64**
Bettembourg	1	1	1	1	1	1	1	1	1	1	1	1	1		1	2	3	3	2	3	2	2	3	1	1	2	2	2	2	2	1	1	**57**
Schifflange	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	3	2	3	2	2	3	1	1	2	2	2	2	2	2	1	**73**
Bleesbrück		1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	3	2	3	2	2	3	1	1	2	2	2	2	2	1	1	**56**
Mersch	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	3	2	3	2	2	3	1	1	2	2	2	2	2	2	1	**60**
Pétange	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	3	2	3	2	2	3	1	1	2	2	2	2	2	2	1	**68**
Hespérange	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	0	1	1	1	1	1	1	0	**42**
Echternach	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	2	3	2	2	3	1	0	1	2	2	1	2	2	1	**47**
Uebersyren	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	2	3	2	2	3	1	0	2	2	2	1	2	2	1	**50**
Grevenmacher	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	3	2	3	2	2	3	1	0	2	2	2	1	2	2	1	**50**
Troisvierges		1	1	1		1		1	1	1	1	1	1	1	1	1	2	3	2	3	2	2	3	1	1	2	2	2	2	2	1	1	**50**
Boevange sur Attert						1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	0	**27**
Wiltz							1		1	1	1	1	1	1	1	1	2	3	2	3	2	2	3	1	1	2	2	2	2	2	1	1	**40**
Total	**9**	**11**	**11**	**11**	**10**	**12**	**12**	**12**	**13**	**13**	**13**	**13**	**13**	**12**	**13**	**19**	**28**	**35**	**24**	**37**	**24**	**24**	**35**	**13**	**9**	**23**	**23**	**24**	**21**	**24**	**24**	**11**	**###**
Pop Lux (2019)																																	

Materials and Methods

Sewage samples

From March 2020 to February 2021, up to thirteen WWTPs were sampled at the inlet of the plant according to the planning presented in Table 3. 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 55°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).

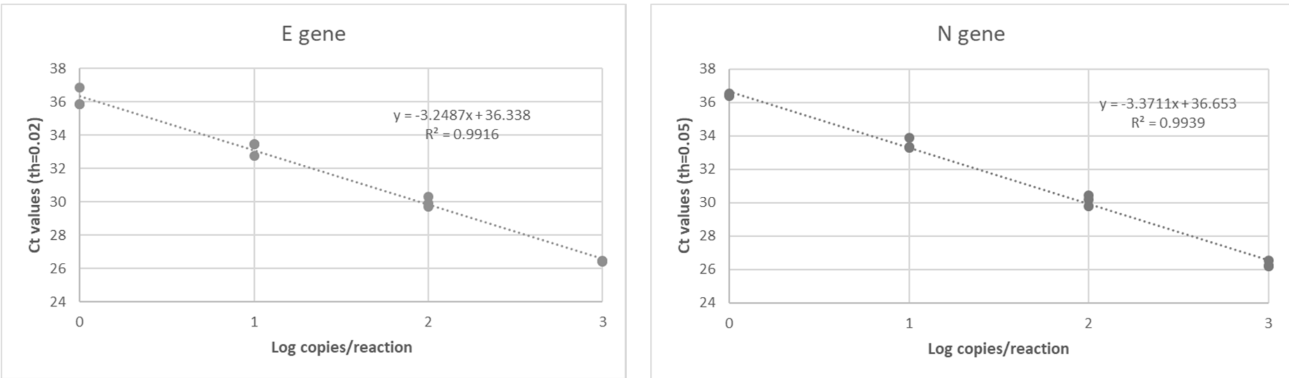


Figure 6 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

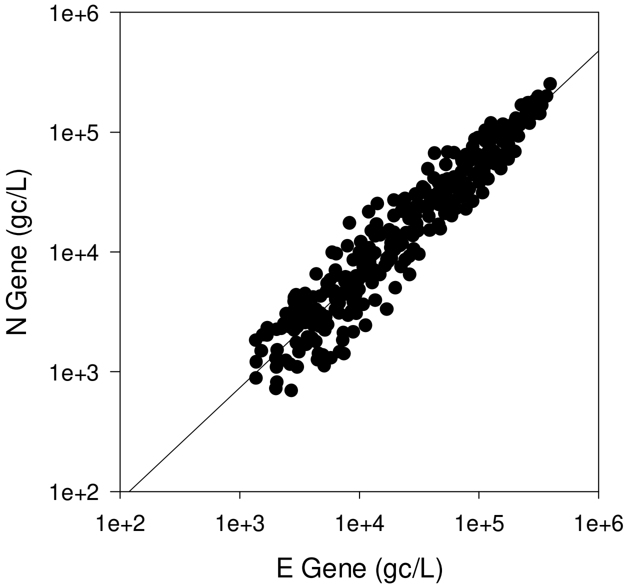
Data interpretation

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 highly significant correlation (Pearson Correlation, R^2=0.964, p = $5.979.10^{-24}$) 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 (n=415),



Acknowledgments

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