CORONASTEP Report 150 (2023 - Weeks 02 and 03) SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

This report 150 presents the results of SARS-CoV-2 contamination of wastewater at the entrance of the 13 wastewater treatment plants (WWTPs) analysed during the weeks 02 and 03 of 2023. All WWTPs were tested on a weekly basis during this period.

The SARS-CoV-2 RNA flux measured in WWTPs during weeks 02 and 03 of 2023 shows a still high national prevalence of the virus, with a SARS-CoV-2 flux of between and 4 and 6 x 10^{11} RNA copies per day per 100,000 population equivalents. The current trend is towards a decrease of the SARS-CoV-2 RNA fluxes circulating in wastewater in comparison to the two previous weeks, both at national and regional level.

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).

	2020																																																					
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	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 52	Week 53
National SARS-CoV-2 Level																																																							

	2021																																																					
Week	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	Week 07-2	Week 08-1	Week 08-2	Week 09-1	Week 09-2	Week 10-1	Week 10-2	Week 11-1	Week 11-2	Week 12-1	Week 12-2	Week 13-1	Week 13-2	Week 14-1	Week 14-2	Week 15-1	Week 15-2	Week 16-1	Week 16-2	Week 17-1	Week 17-2	Week 18-1	Week 18-2	Week 19	Week 20-1	Week 20-2	Week 21	Week 22-1	Week 22-2	Week 23-1	Week 23-2	Week 24-1	Week 24-2	Week 25	Week 26-1	Week 26-2	Week 27-1	Week 27-2	Week 28-1	Week 29-1	Week 29-2	Week 30-1	Week 30-2
National SARS-CoV-2 Level																																																							

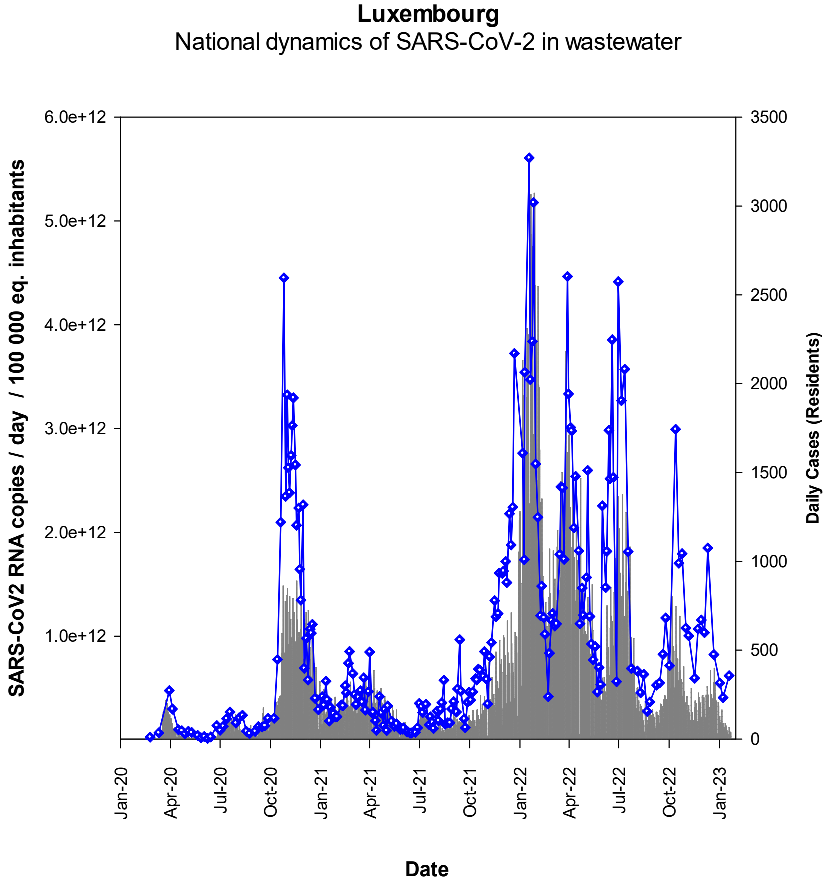
	2021																																										2022												
Week	Week 31-1	Week 31-2	Week 32-1	Week 32-2	Week 33-1	Week 33-2	Week 34-1	Week 34-2	Week 35-1	Week 35-2	Week 36-1	Week 36-2	Week 37-1	Week 37-2	Week 38-1	Week 38-2	Week 39-1	Week 39-2	Week 40-1	Week 40-2	Week 41-1	Week 41-2	Week 42-1	Week 42-2	Week 43-1	Week 43-2	Week 44-1	Week 44-2	Week 45-1	Week 45-2	Week 46-1	Week 46-2	Week 47-1	Week 47-2	Week 48-1	Week 48-2	Week 49-1	Week 49-2	Week 50-1	Week 50-2	Week 51-1	Week 51-2	Week 01	Week 02-1	Week 02-2	Week 03-1	Week 03-2	Week 04-1	Week 04-2	Week 05-1	Week 05-2	Week 06-1	Week 06-2	Week 07-1	Week 07-2
National SARS-CoV-2 Level																																																							

	2022																																																					
Week	Week 08-1	Week 08-2	Week 09-1	Week 09-2	Week 10-1	Week 10-2	Week 11-1	Week 11-2	Week 12-1	Week 12-2	Week 13-1	Week 13-2	Week 14-1	Week 14-2	Week 15-1	Week 15-2	Week 16-1	Week 16-2	Week 17-1	Week 17-2	Week 18-1	Week 18-2	Week 19-1	Week 19-2	Week 20-1	Week 20-2	Week 21-1	Week 21-2	Week 22-1	Week 22-2	Week 23-1	Week 23-2	Week 24-1	Week 24-2	Week 25-1	Week 25-2	Week 26-1	Week 26-2	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	Week 43
National SARS-CoV-2 Level																																																							

	2022								2023																																														
Week	Week 44	Week 45	Week 46	Week 47	Week 48	Week 49	Week 50	Week 51	Week 01	Week 02	Week 03																																												
National SARS-CoV-2 Level																																																								

Figure 1 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Luxembourgish wastewater samples from December 2019 to January 2023. 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).

a) Linear scale



b) Log10 scale

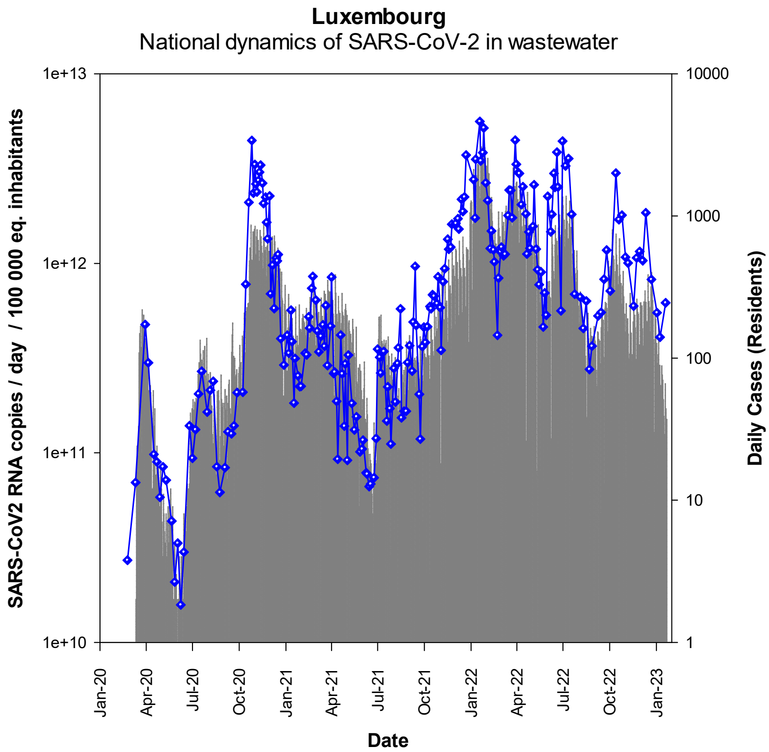


Table 2 - Level of SARS-CoV-2 contamination of each analyzed wastewater treatment plant in Luxembourg in 2022 and 2023. BEG: Beggen, BET: Bettembourg, SCH: Schifflange, BLE: Bleesbruck, MER: Mersch, PET: Pétange, HES: Hesperange, ECG: Echternach, UEB: Uebersyren, GRE: Grevenmacher, TRO: Troisvierges, 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

	2022																																																									2023			
WWTP	Week 10-1	Week 10-2	Week 11-1	Week 11-2	Week 12-1	Week 12-2	Week 13-1	Week 13-2	Week 14-1	Week 14-2	Week 15-1	Week 15-2	Week 16-1	Week 16-2	Week 17-1	Week 17-2	Week 18-1	Week 18-2	Week 19-1	Week 19-2	Week 20-1	Week 20-2	Week 21-1	Week 21-2	Week 22-1	Week 22-2	Week 23-1	Week 23-2	Week 24-1	Week 24-2	Week 25-1	Week 25-2	Week 26-1	Week 26-2	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	Week 43	Week 44	Week 45	Week 46	Week 47	Week 48	Week 49	Week 50	Week 51	Week 01	Week 02	Week 03
BEG																																																														
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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 January 2023. 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).

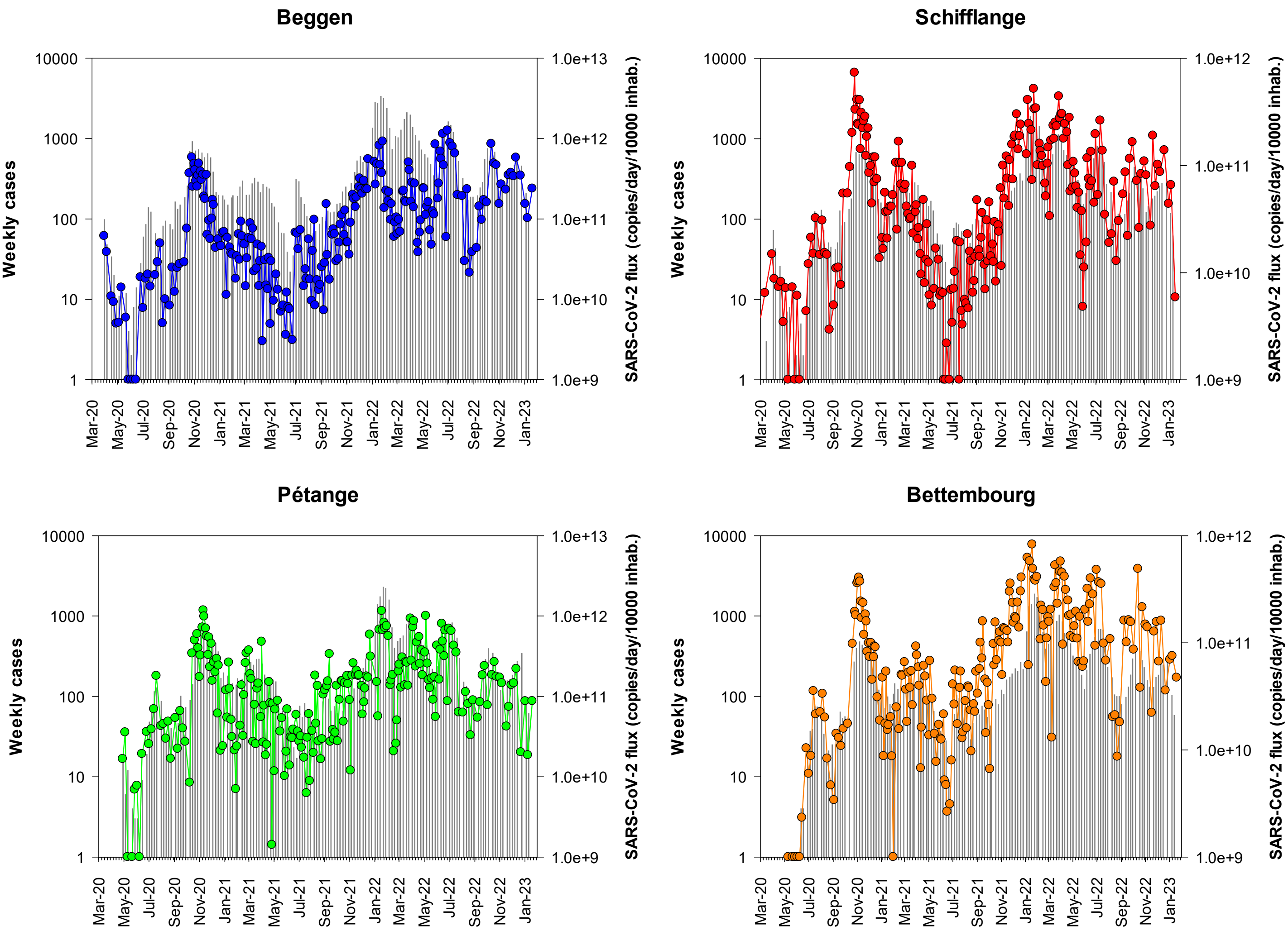


Figure 2b – Close-up of Figure 2a showing results from June 1st, 2021 on

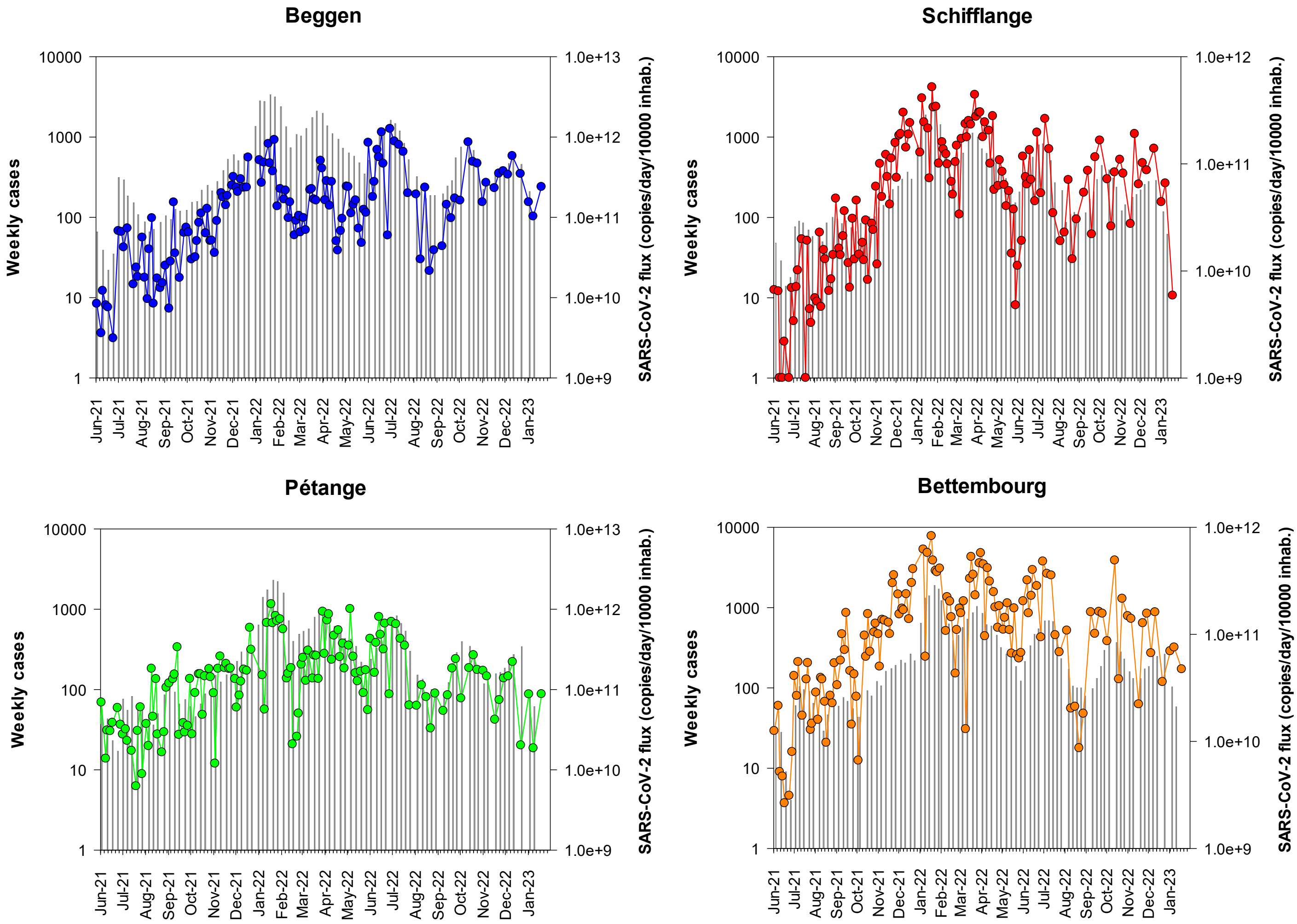
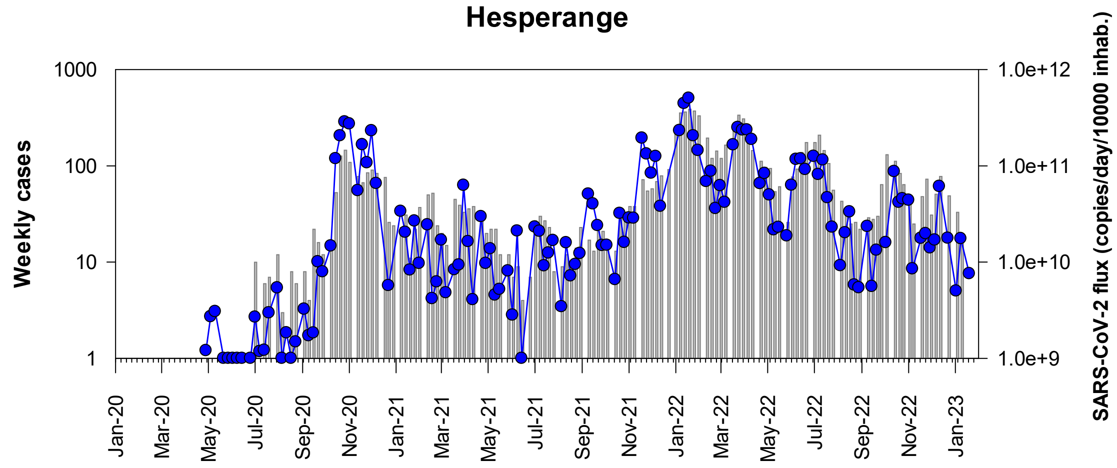
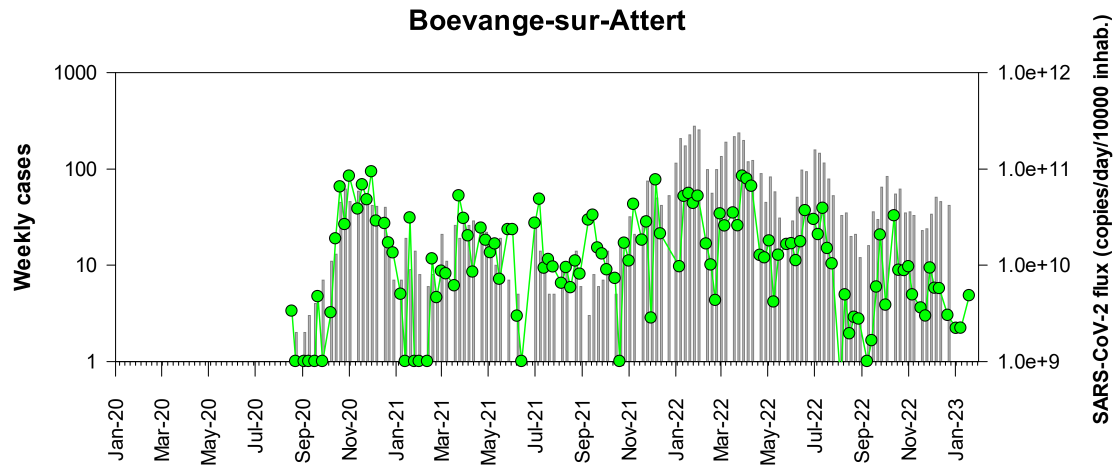


Figure 3a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Hesperange, Mersch and Boevange-sur-Attert wastewater treatment plants from May 2020 to January 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).





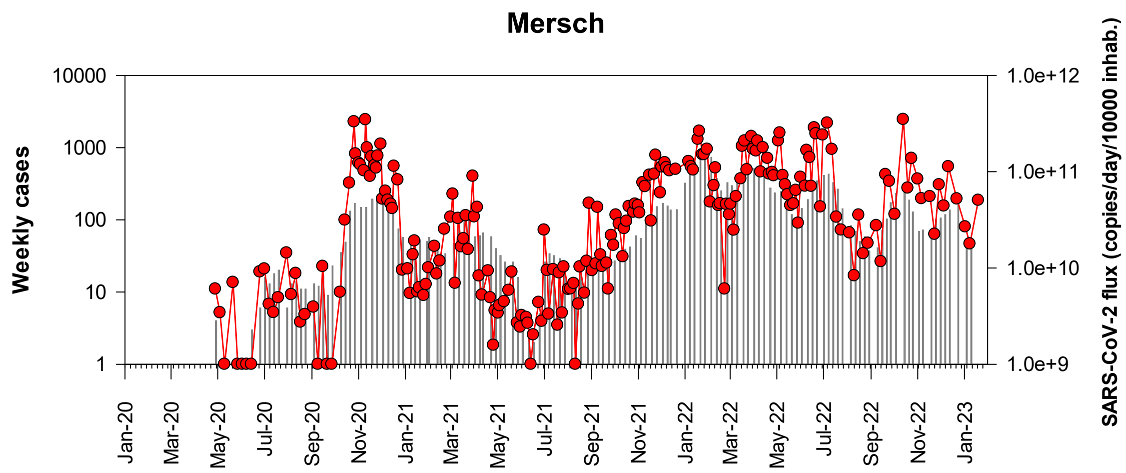


Figure 3b – Close-up of Figure 3a showing results from June 1st, 2021 on.

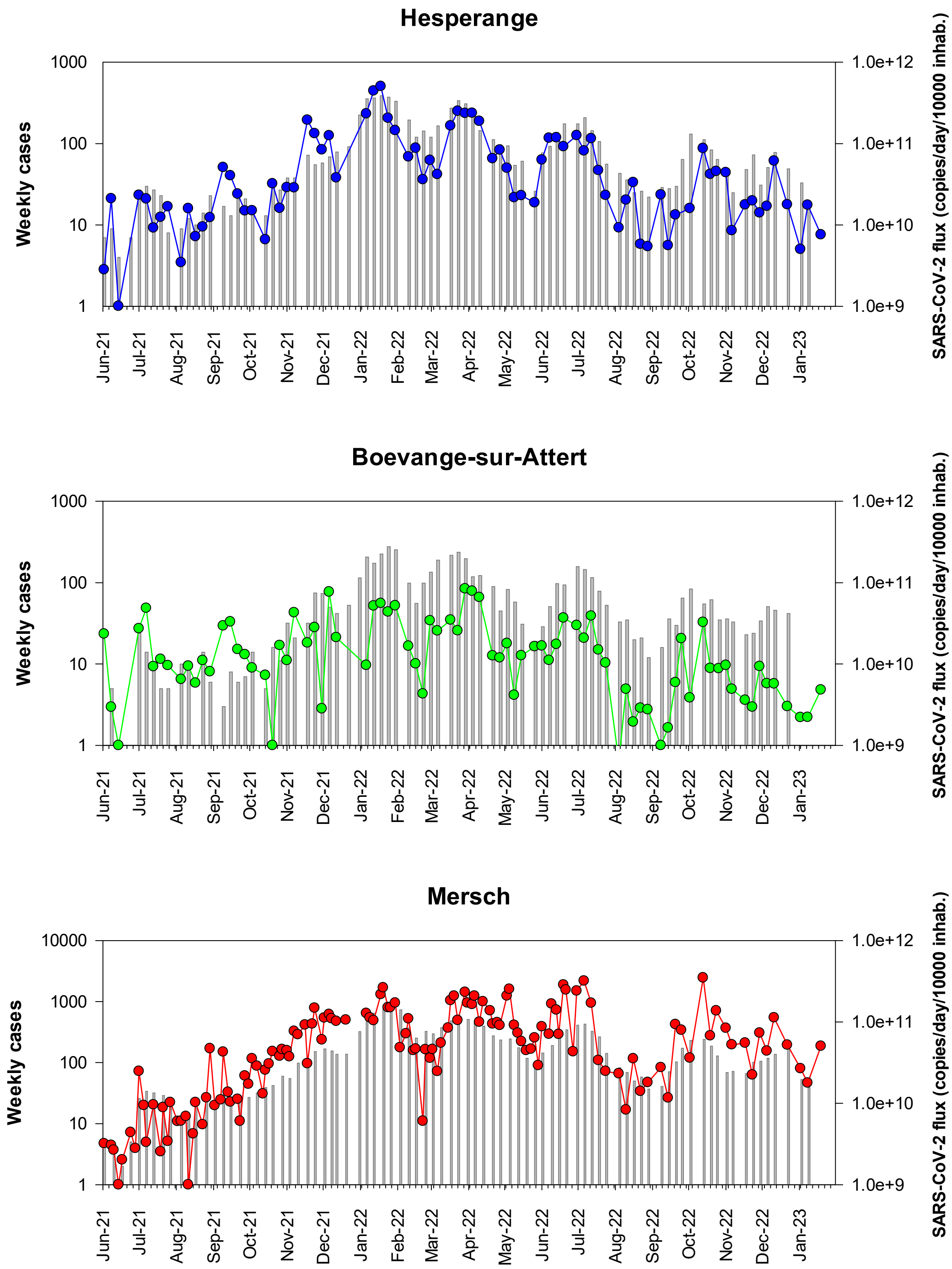
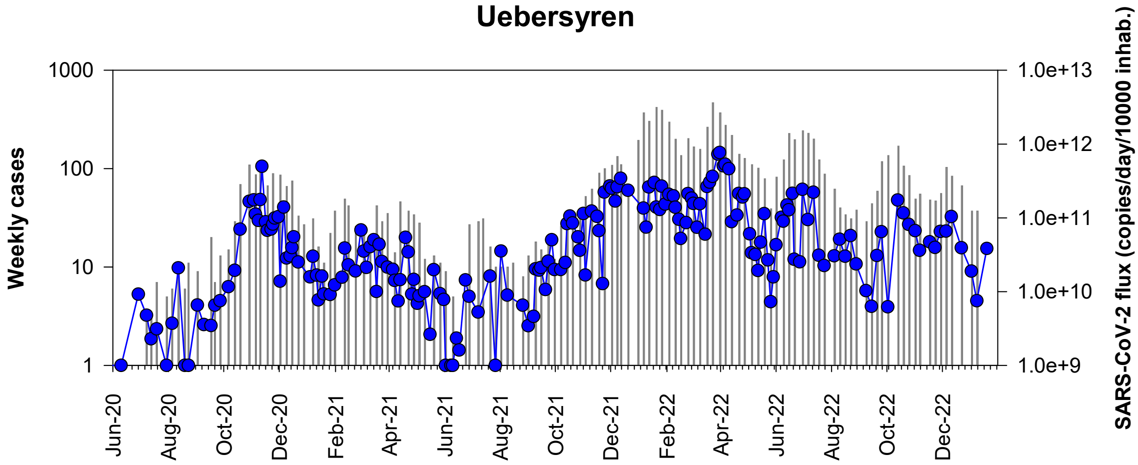
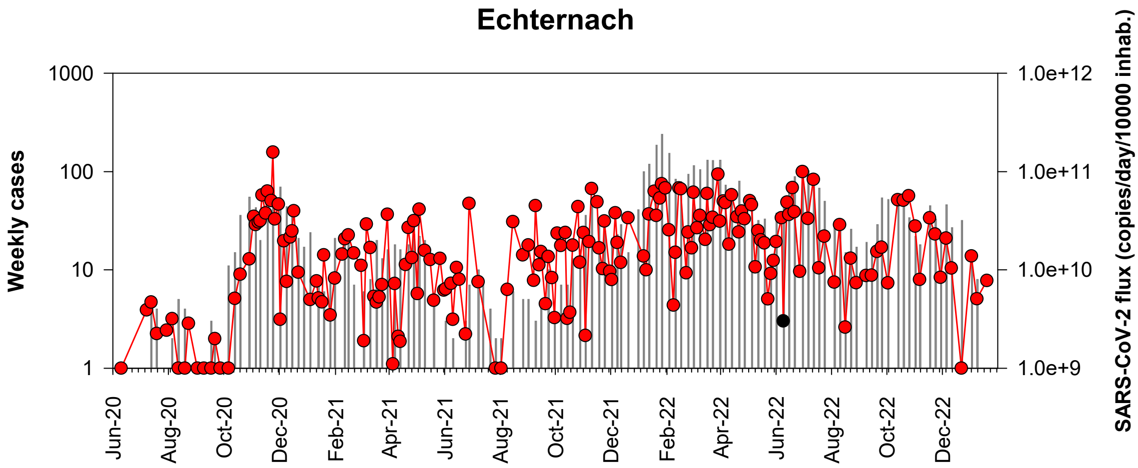


Figure 4a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from May 2020 to January 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).





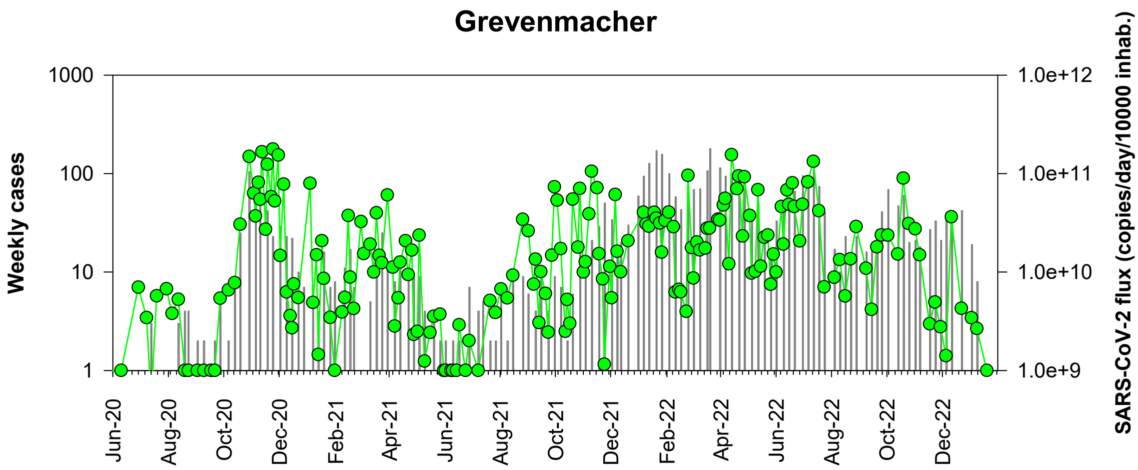


Figure 4b – Close-up of Figure 4a showing results from June 1st, 2021 on

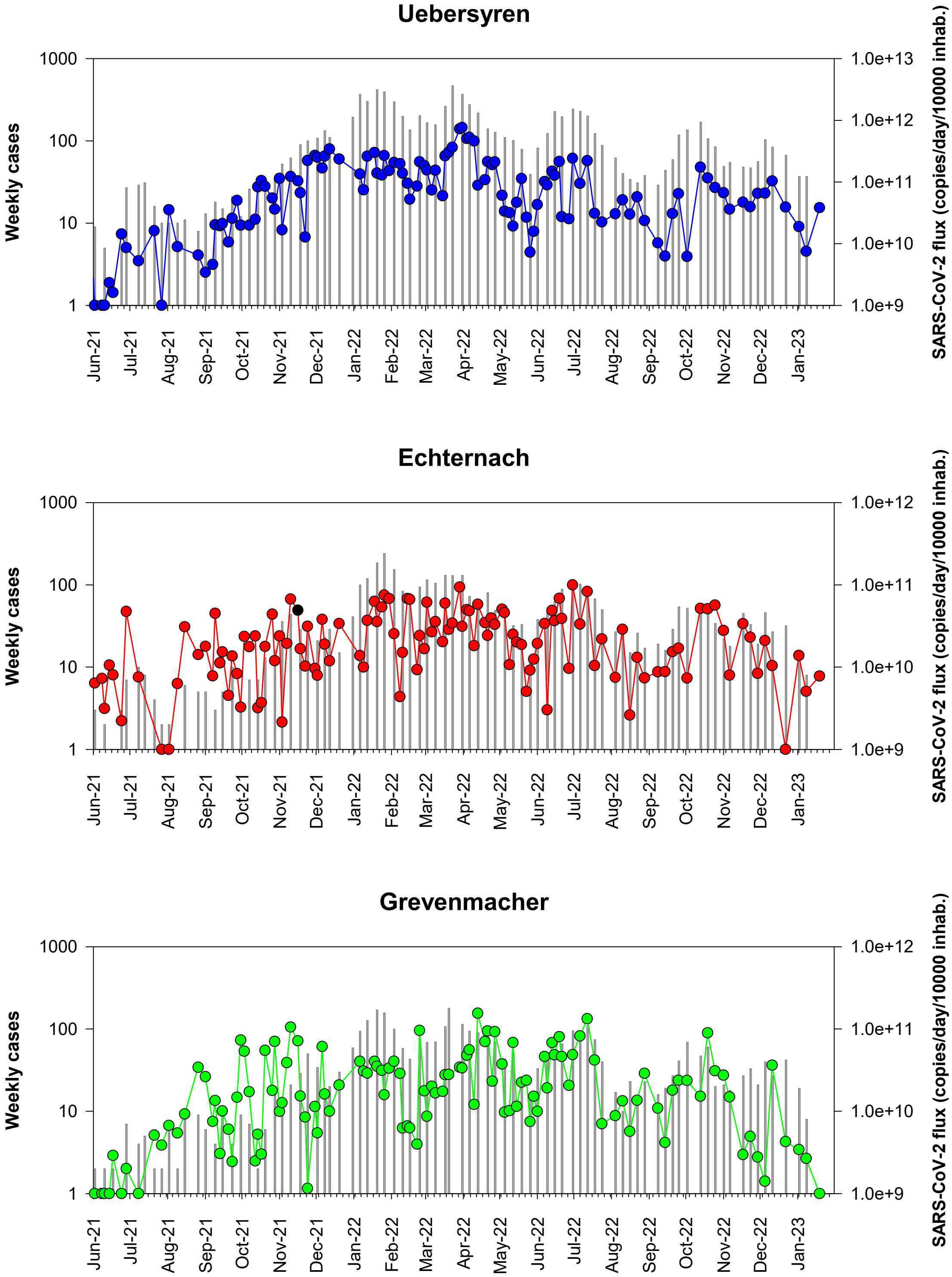


Figure 5a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from May 2020 to January 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants)

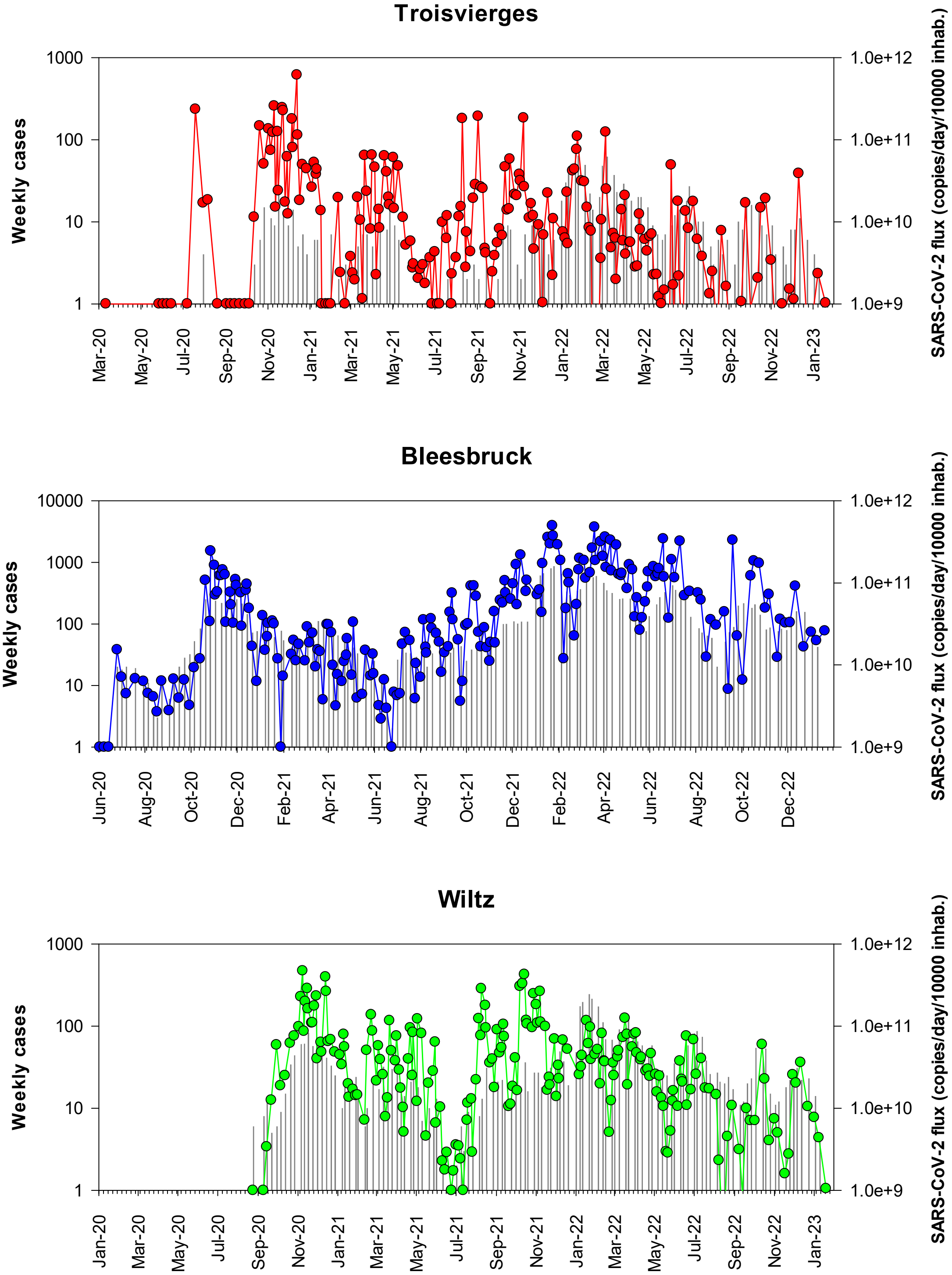


Figure 5b – Close-up of Figure 5a showing results from September 1st, 2020 on.

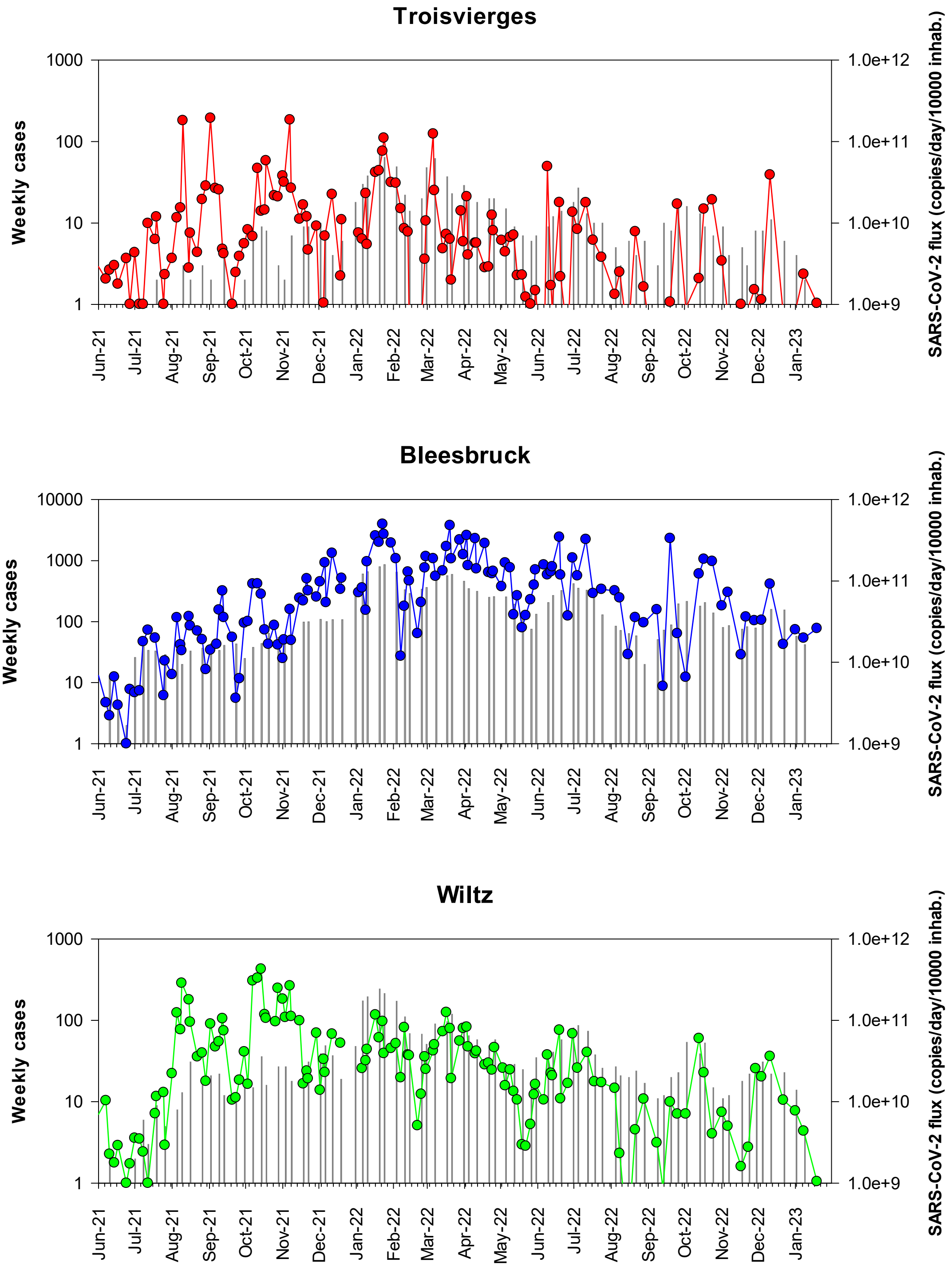


Table 3- Sewage sampling since the beginning of the CORONASTEP study

WWTP	2019	2020	2021	2022	2023	Total
Beggen		52	92	77	3	**224**
Bettembourg		45	91	75	3	**214**
Schifflange	4	56	96	76	3	**235**
Bleesbrück		44	92	77	3	**216**
Mersch		47	91	77	3	**218**
Pétange	4	51	96	77	3	**231**
Hesperange		35	48	50	3	**136**
Echternach		36	79	77	3	**195**
Uebersyren		38	78	77	3	**196**
Grevenmacher		38	81	76	3	**198**
Troisvierges		38	92	77	3	**210**
Boevange sur Attert		20	48	51	3	**122**
Wiltz		28	92	77	3	**200**
Total	**8**	**528**	**1076**	**944**	**39**	**2595**

Materials and Methods

Sewage samples

From March 2020 to January 2023, up to thirteen wastewater treatment plants (WWTPs) were sampled at their inlet according to the planning presented in Table 3. The operators of the WWTPs collected a 24-h composite sample according to their routine 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 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 were screened for the presence of *Sarbecovirus* (*Coronaviridae, Betacoronaviruses*) and/or SARS-CoV-2 virus RNA by two distinct real-time one-step RT-PCR assays, targeting the E gene (Envelope small membrane protein) and 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
	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).

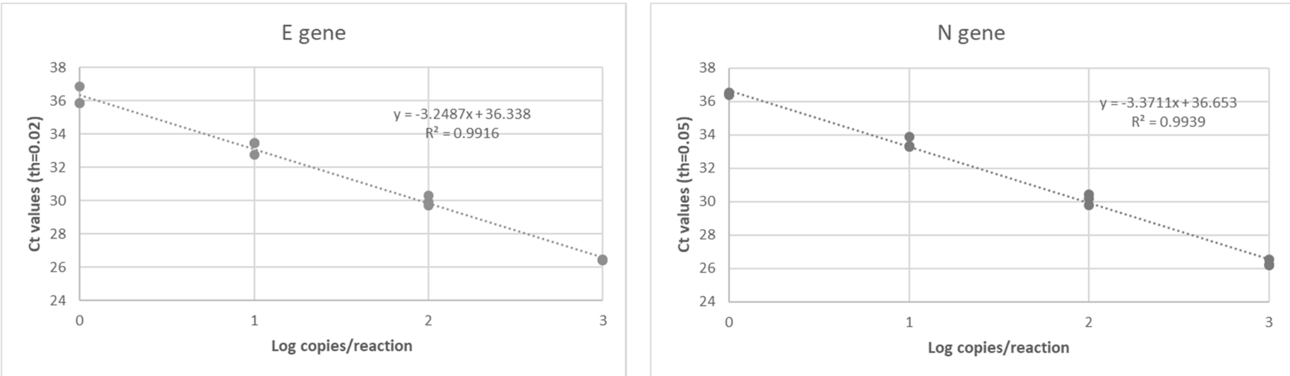


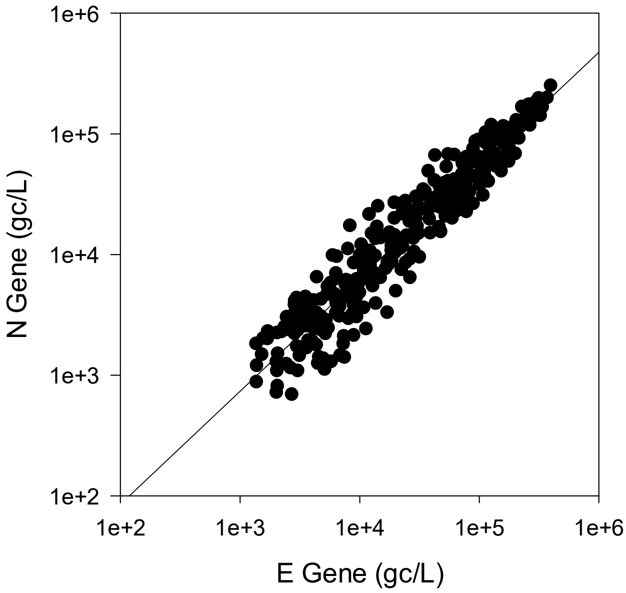
Figure 6 – RT-qPCR standard curves established for both target genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

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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