

CORONASTEP Report 164 (2023 - Weeks 30 and 31) SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

This report 164 presents the results of SARS-CoV-2 contamination of wastewater at the entrance of the 13 wastewater treatment plants (WWTPs) analysed during the weeks 30 and 31 of 2023. All WWTPs were tested on a weekly basis during this period.

On the national and regional scale, the SARS-CoV-2 RNA flux measured in WWTPs during weeks 30 and 31 of 2023 confirms the low prevalence of the virus observed since the week 20, even if a small increase was noticed. The national SARS-CoV-2 flux ranged between 1×10^{11} and 3×10^{11} RNA copies per day per 100,000 population equivalents.

Remark: Following the recent adoption of the Act amending the Act of 17 July 2020 on measures to combat the COVID-19 pandemic and the decisions of the Government Council regarding the surveillance of the COVID-19 epidemic, the data on clinical surveillance will no longer be reported on the national and regional charts. Indeed, the number of tests performed has already started to decrease drastically and is therefore no longer as representative of the virus circulation in the population as before.

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	
National SARS-CoV-2 Level	Week
Green	Week 3
Green	Week 7
Green	Week 9
Green	Week 11
Yellow	Week 14
Yellow	Week 15
Yellow	Week 16
Yellow	Week 17
Yellow	Week 18
Yellow	Week 19
Yellow	Week 20
Yellow	Week 21
Yellow	Week 22
Yellow	Week 23
Yellow	Week 24
Yellow	Week 25
Yellow	Week 26
Yellow	Week 27
Yellow	Week 28
Yellow	Week 29
Yellow	Week 30
Yellow	Week 31
Yellow	Week 32
Yellow	Week 33
Yellow	Week 34
Yellow	Week 35
Yellow	Week 36
Yellow	Week 37
Yellow	Week 38
Yellow	Week 39
Yellow	Week 40
Yellow	Week 41
Yellow	Week 42
Yellow	Week 43
Orange	Week 44-1
Orange	Week 44-2
Orange	Week 45-1
Orange	Week 45-2
Orange	Week 45-3
Orange	Week 46-1
Orange	Week 46-2
Orange	Week 46-3
Orange	Week 47-1
Orange	Week 47-2
Orange	Week 48-1
Orange	Week 48-2
Orange	Week 48-3
Orange	Week 49-1
Orange	Week 49-2
Orange	Week 50-1
Orange	Week 50-2
Orange	Week 51-1
Orange	Week 51-2
Orange	Week 52
Orange	Week 53

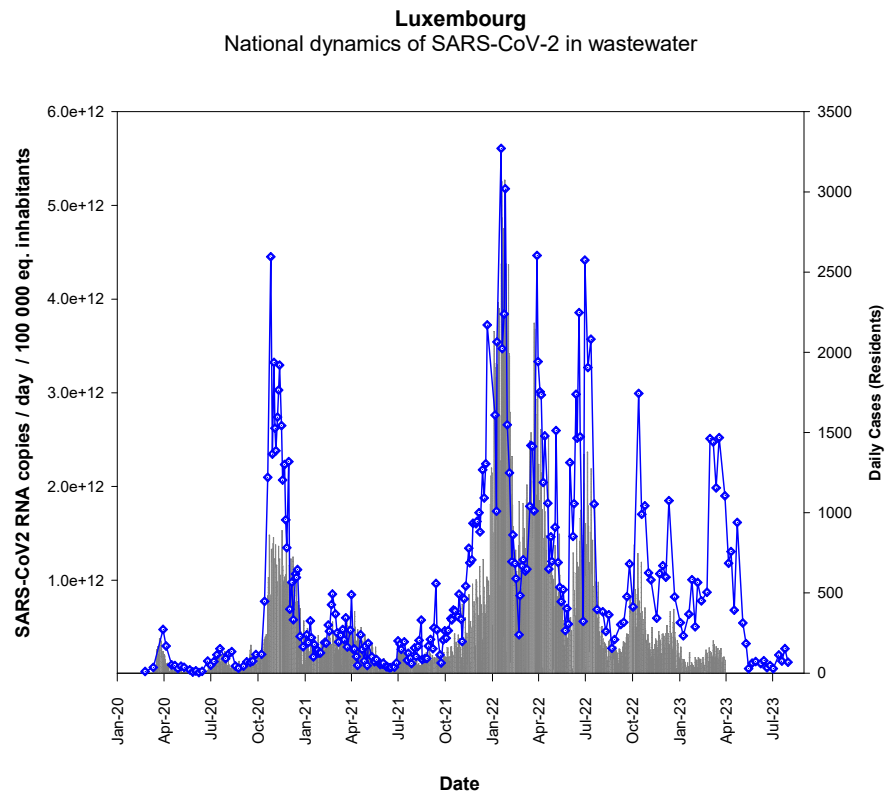
2021	
National SARS-CoV-2 Level	Week
Yellow	Week 01-1
Yellow	Week 01-2
Yellow	Week 02-1
Yellow	Week 02-2
Yellow	Week 03-1
Yellow	Week 03-2
Yellow	Week 04-1
Yellow	Week 04-2
Yellow	Week 05-1
Yellow	Week 06-1
Yellow	Week 06-2
Yellow	Week 07-1
Yellow	Week 07-2
Yellow	Week 08-1
Yellow	Week 08-2
Yellow	Week 09-1
Yellow	Week 09-2
Yellow	Week 10-1
Yellow	Week 10-2
Yellow	Week 11-1
Yellow	Week 11-2
Yellow	Week 12-1
Yellow	Week 12-2
Yellow	Week 13-1
Yellow	Week 13-2
Yellow	Week 14-1
Yellow	Week 14-2
Yellow	Week 15-1
Yellow	Week 15-2
Yellow	Week 16-1
Yellow	Week 16-2
Yellow	Week 17-1
Yellow	Week 17-2
Yellow	Week 18-1
Yellow	Week 18-2
Yellow	Week 19
Yellow	Week 20-1
Yellow	Week 20-2
Yellow	Week 21
Yellow	Week 22-1
Yellow	Week 22-2
Yellow	Week 23-1
Yellow	Week 23-2
Yellow	Week 24-1
Yellow	Week 24-2
Yellow	Week 25
Yellow	Week 26-1
Yellow	Week 26-2
Yellow	Week 27-1
Yellow	Week 27-2
Yellow	Week 28-1
Yellow	Week 28-2
Yellow	Week 29-1
Yellow	Week 29-2
Yellow	Week 30-1
Yellow	Week 30-2

2021		2022	
National SARS-CoV-2 Level	Week	National SARS-CoV-2 Level	Week
Yellow	Week 31-1	Yellow	Week 01
Yellow	Week 31-2	Yellow	Week 02-1
Yellow	Week 32-1	Yellow	Week 02-2
Yellow	Week 32-2	Yellow	Week 03-1
Yellow	Week 33-1	Yellow	Week 03-2
Yellow	Week 33-2	Yellow	Week 04-1
Yellow	Week 34-1	Yellow	Week 04-2
Yellow	Week 34-2	Yellow	Week 05-1
Yellow	Week 35-1	Yellow	Week 05-2
Yellow	Week 35-2	Yellow	Week 06-1
Yellow	Week 36-1	Yellow	Week 06-2
Yellow	Week 36-2	Yellow	Week 07-1
Yellow	Week 37-1	Yellow	Week 07-2
Yellow	Week 37-2	Yellow	Week 08-1
Yellow	Week 38-1	Yellow	Week 08-2
Yellow	Week 38-2	Yellow	Week 09-1
Yellow	Week 39-1	Yellow	Week 09-2
Yellow	Week 39-2	Yellow	Week 40-1
Yellow	Week 40-1	Yellow	Week 40-2
Yellow	Week 41-1	Yellow	Week 41-1
Yellow	Week 41-2	Yellow	Week 41-2
Yellow	Week 42-1	Yellow	Week 42-1
Yellow	Week 42-2	Yellow	Week 42-2
Yellow	Week 43-1	Yellow	Week 43-1
Yellow	Week 43-2	Yellow	Week 43-2
Yellow	Week 44-1	Yellow	Week 44-1
Yellow	Week 44-2	Yellow	Week 44-2
Yellow	Week 45-1	Yellow	Week 45-1
Yellow	Week 45-2	Yellow	Week 45-2
Yellow	Week 46-1	Yellow	Week 46-1
Yellow	Week 46-2	Yellow	Week 46-2
Yellow	Week 47-1	Yellow	Week 47-1
Yellow	Week 47-2	Yellow	Week 47-2
Yellow	Week 48-1	Yellow	Week 48-1
Yellow	Week 48-2	Yellow	Week 48-2
Yellow	Week 49-1	Yellow	Week 49-1
Yellow	Week 49-2	Yellow	Week 49-2
Yellow	Week 50-1	Yellow	Week 50-1
Yellow	Week 50-2	Yellow	Week 50-2
Yellow	Week 51-1	Yellow	Week 51-1
Yellow	Week 51-2	Yellow	Week 51-2
Yellow	Week 01	Yellow	Week 01
Yellow	Week 02-1	Yellow	Week 02-1
Yellow	Week 02-2	Yellow	Week 02-2
Yellow	Week 03-1	Yellow	Week 03-1
Yellow	Week 03-2	Yellow	Week 03-2
Yellow	Week 04-1	Yellow	Week 04-1
Yellow	Week 04-2	Yellow	Week 04-2
Yellow	Week 05-1	Yellow	Week 05-1
Yellow	Week 05-2	Yellow	Week 05-2
Yellow	Week 06-1	Yellow	Week 06-1
Yellow	Week 06-2	Yellow	Week 06-2
Yellow	Week 07-1	Yellow	Week 07-1
Yellow	Week 07-2	Yellow	Week 07-2

2022	
National SARS-CoV-2 Level	Week
Yellow	Week 08-1
Yellow	Week 08-2
Yellow	Week 09-1
Yellow	Week 09-2
Yellow	Week 10-1
Yellow	Week 10-2
Yellow	Week 11-1
Yellow	Week 11-2
Yellow	Week 12-1
Yellow	Week 12-2
Yellow	Week 13-1
Yellow	Week 13-2
Yellow	Week 14-1
Yellow	Week 14-2
Yellow	Week 15-1
Yellow	Week 15-2
Yellow	Week 16-1
Yellow	Week 16-2
Yellow	Week 17-1
Yellow	Week 17-2
Yellow	Week 18-1
Yellow	Week 18-2
Yellow	Week 19-1
Yellow	Week 19-2
Yellow	Week 20-1
Yellow	Week 20-2
Yellow	Week 21-1
Yellow	Week 21-2
Yellow	Week 22-1
Yellow	Week 22-2
Yellow	Week 23-1
Yellow	Week 23-2
Yellow	Week 24-1
Yellow	Week 24-2
Yellow	Week 25-1
Yellow	Week 25-2
Yellow	Week 26-1
Yellow	Week 26-2
Yellow	Week 27
Yellow	Week 28
Yellow	Week 29
Yellow	Week 30
Yellow	Week 31
Yellow	Week 32
Yellow	Week 33
Yellow	Week 34
Yellow	Week 35
Yellow	Week 36
Yellow	Week 37
Yellow	Week 38
Yellow	Week 39
Yellow	Week 40
Yellow	Week 41
Yellow	Week 42
Yellow	Week 43

Figure 1 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (*E* gene) in Luxembourgish wastewater samples from December 2019 to August 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) Log_{10} scale

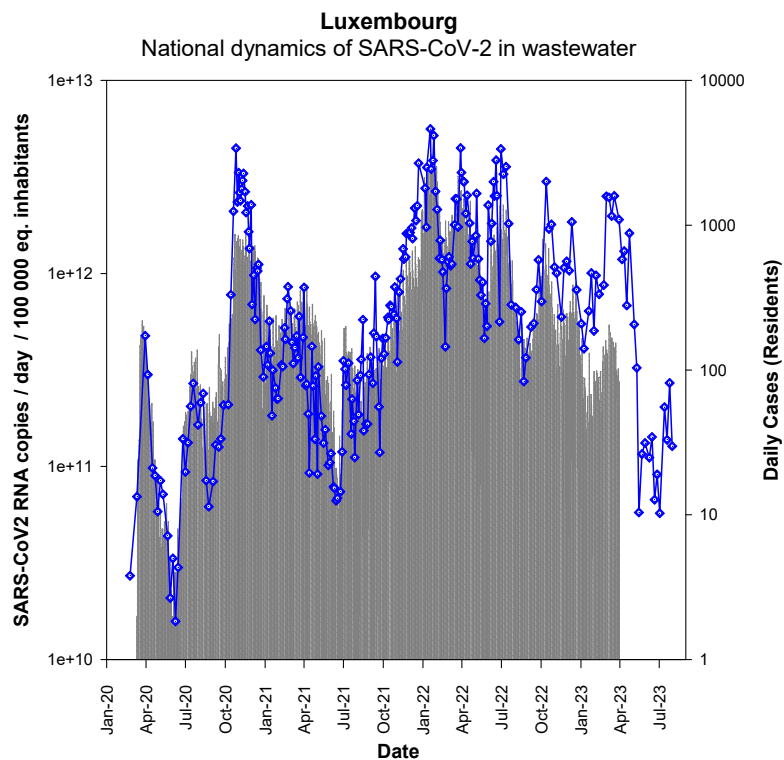


Figure 2a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in the four most impacted wastewater treatment plants from March 2020 to August 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies /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

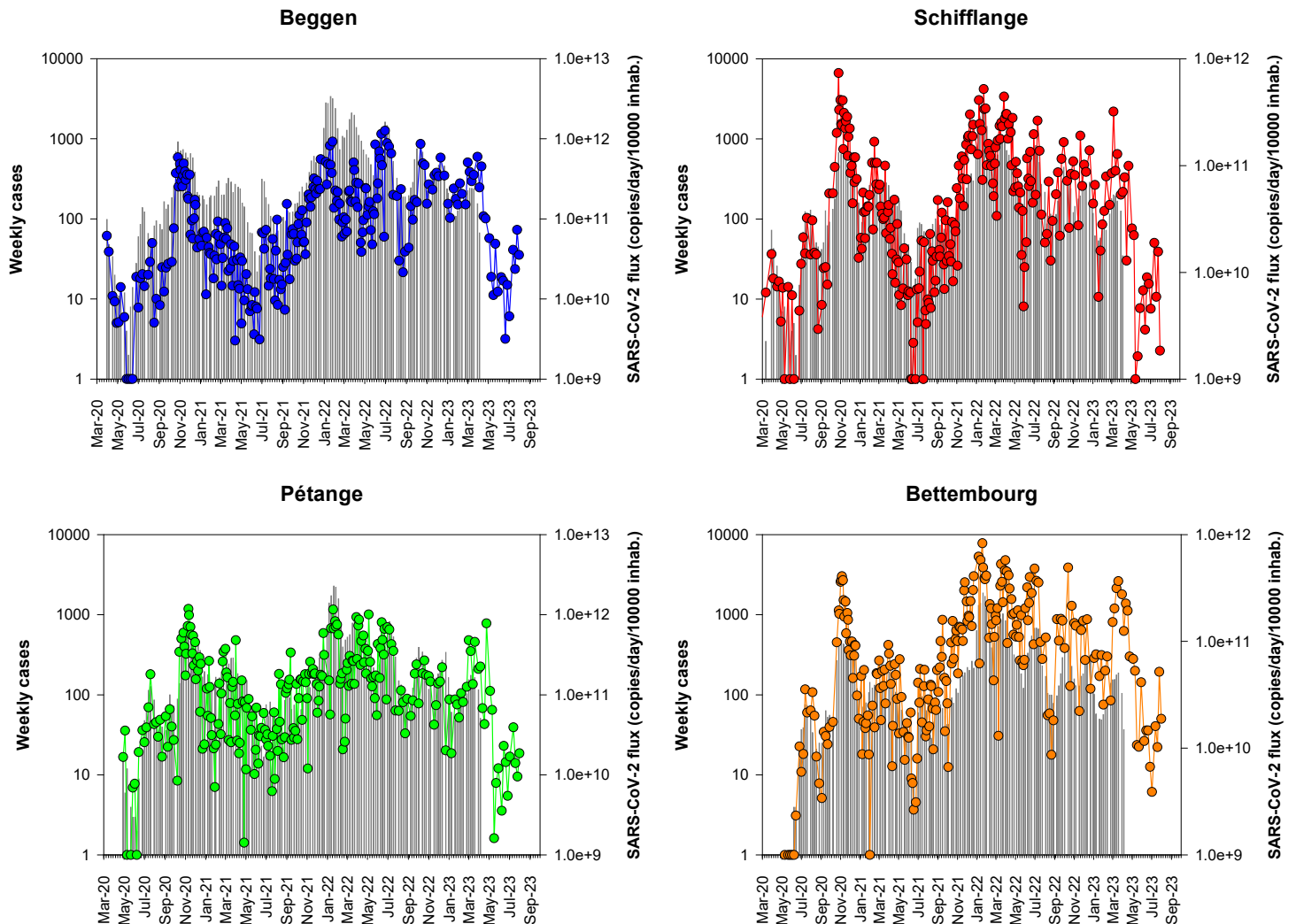


Figure 2b – Close-up of Figure 2a showing results from January 1st, 2022 on.

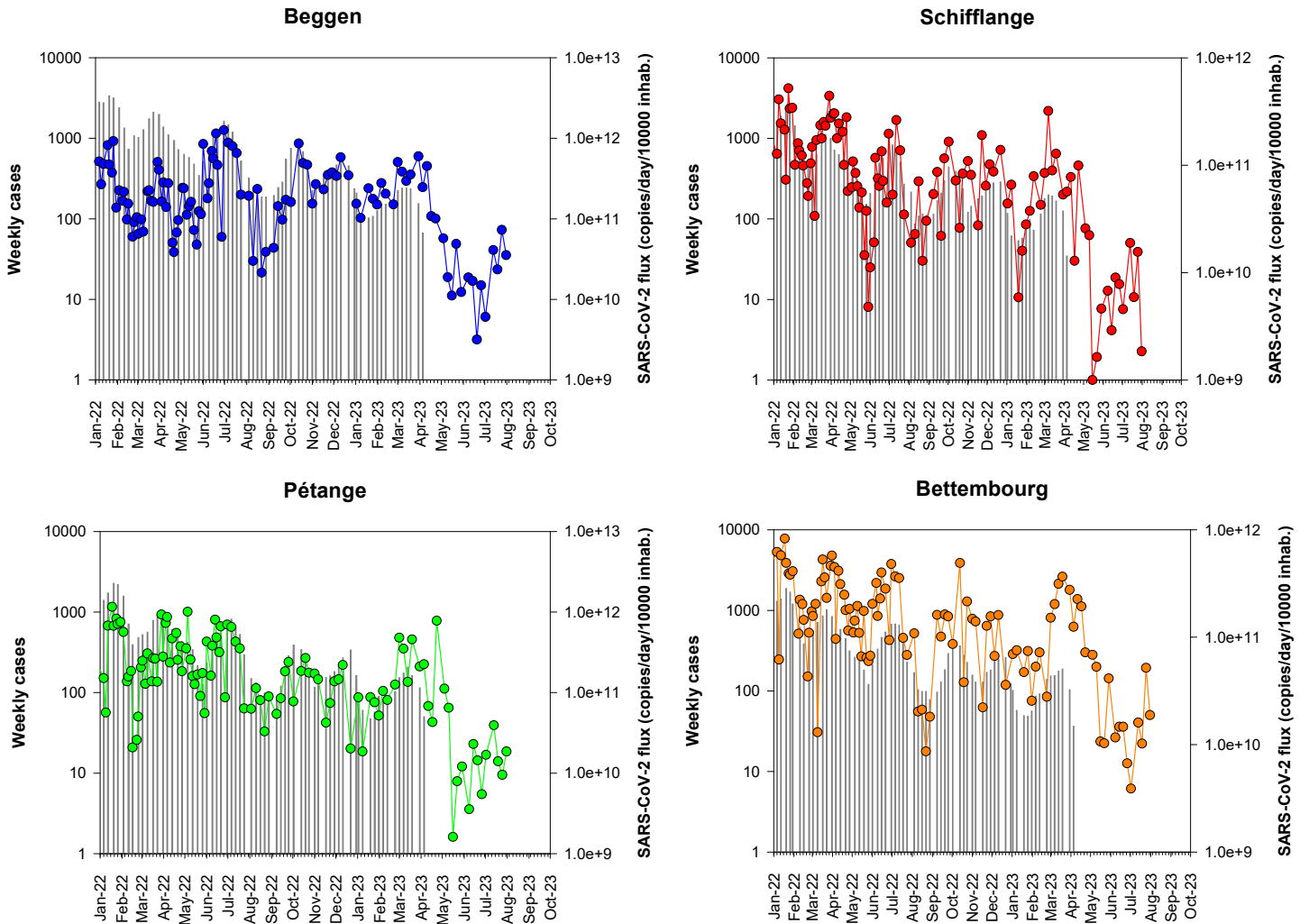


Figure 3a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (*E* gene) in Hesperange, Mersch and Boevange-sur-Attert wastewater treatment plants from May 2020 to August 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2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

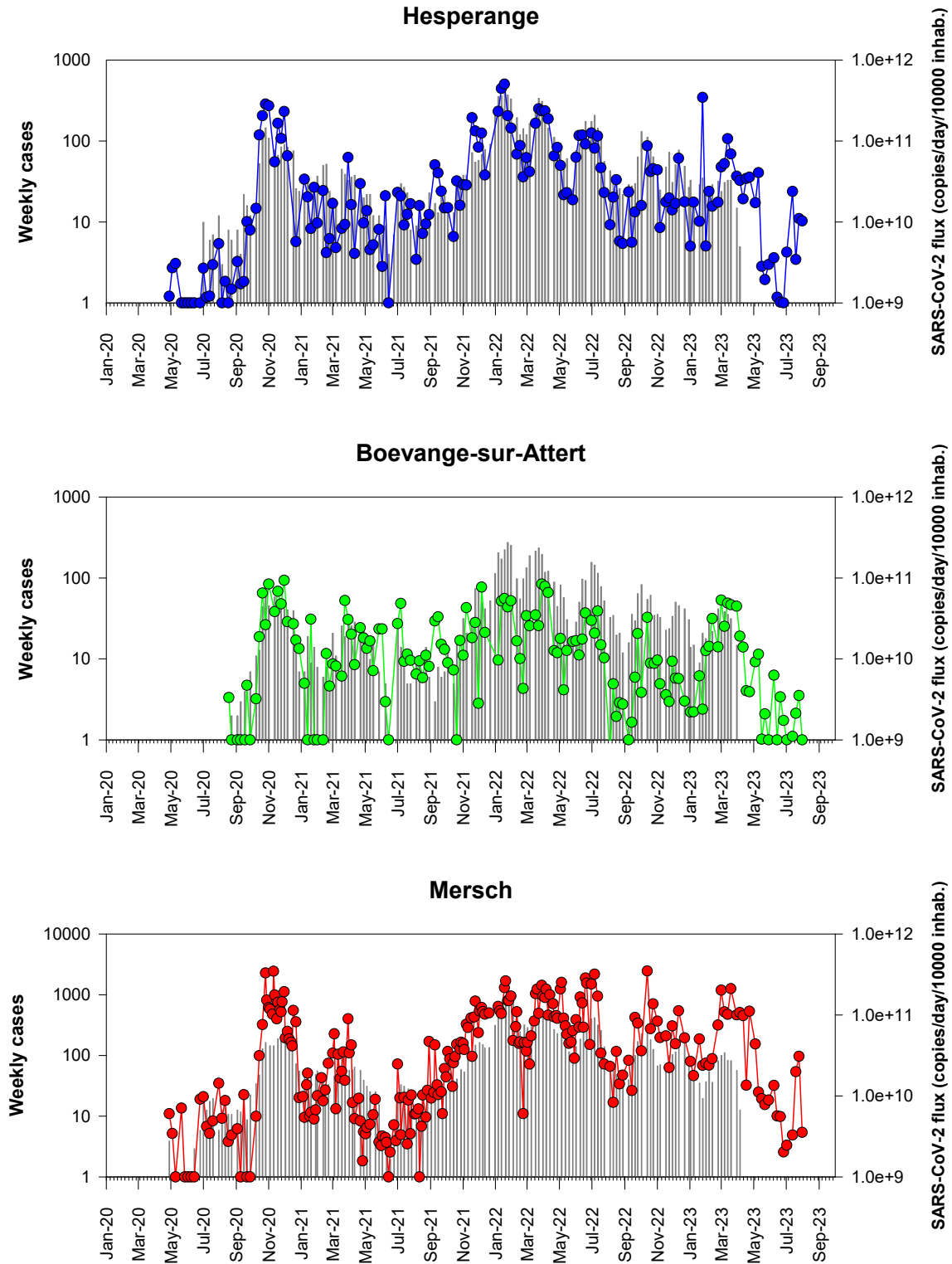


Figure 3b – Close-up of Figure 3a showing results from January 1st, 2022 on.

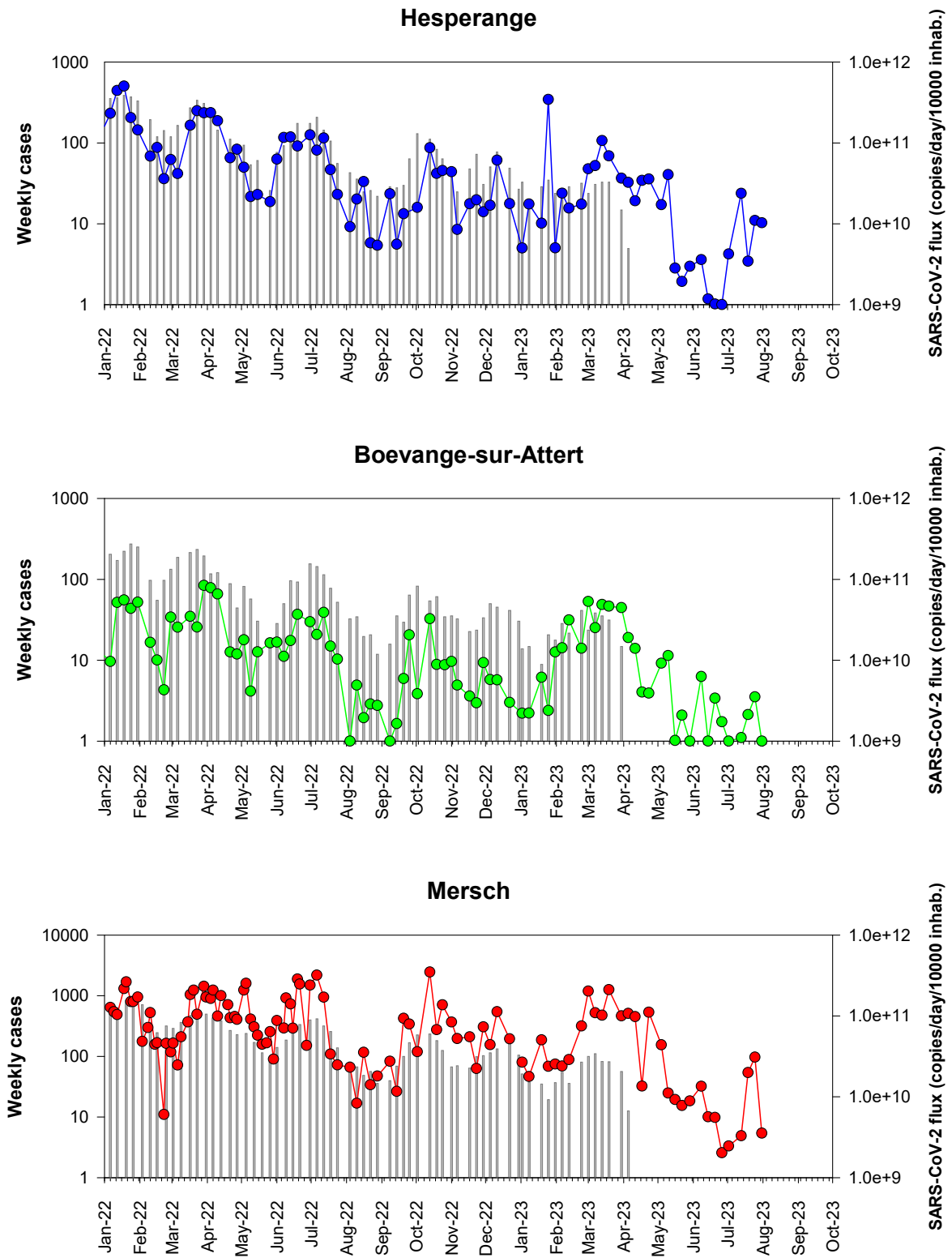


Figure 4a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from May 2020 to August 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent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

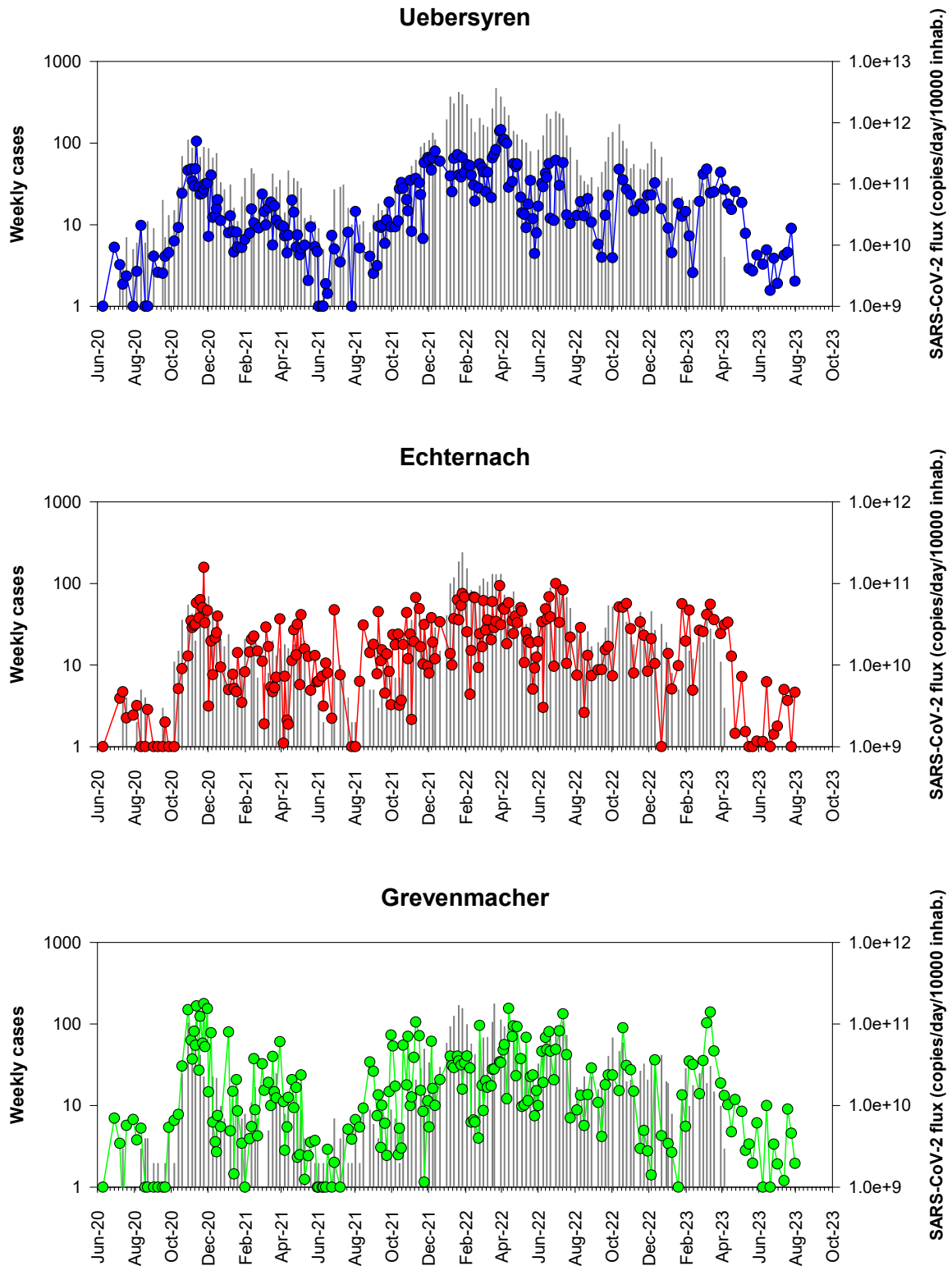


Figure 4b – Close-up of Figure 4a showing results from January 1st, 2022 on.

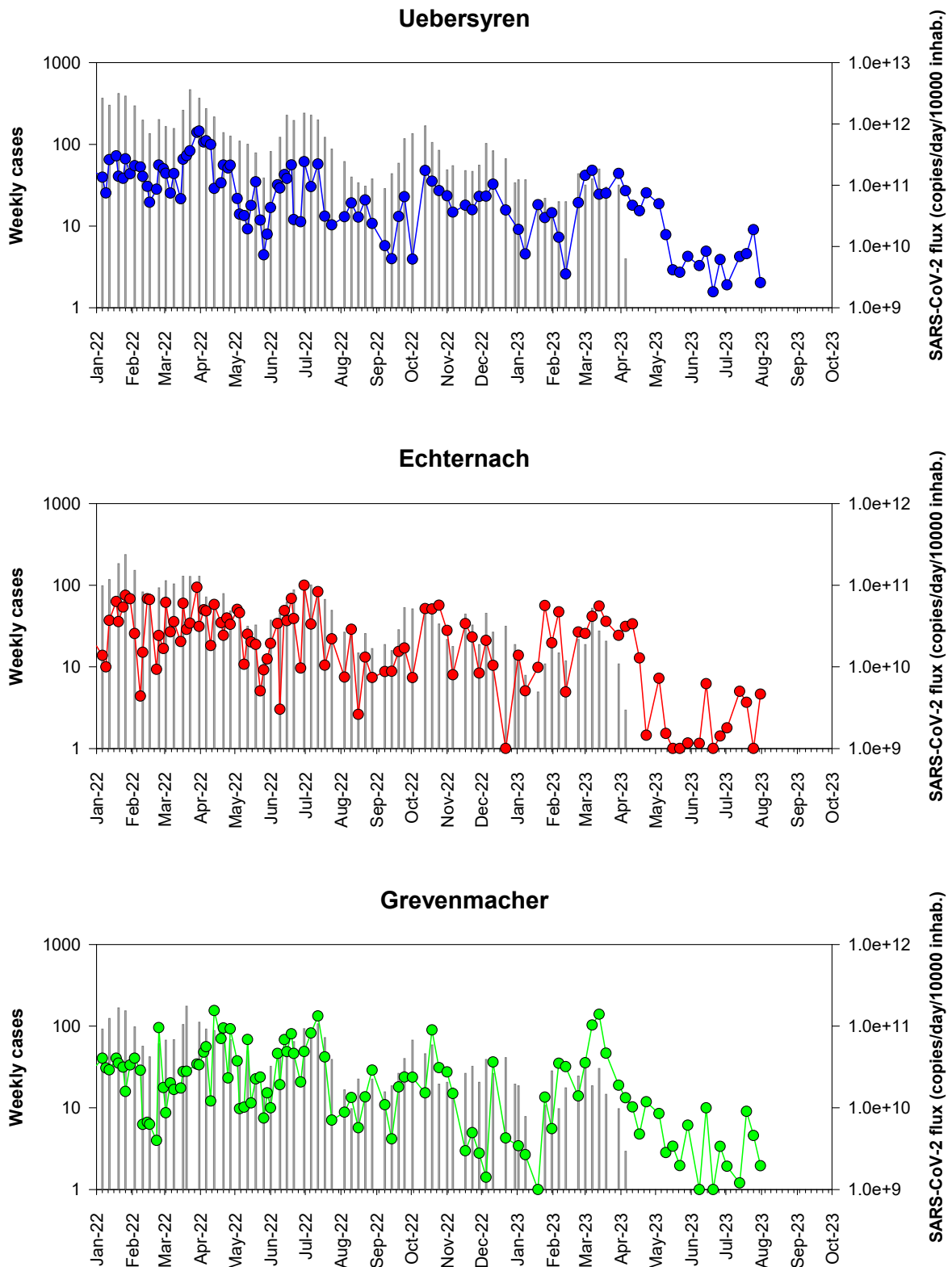


Figure 5a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from May 2020 to August 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent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

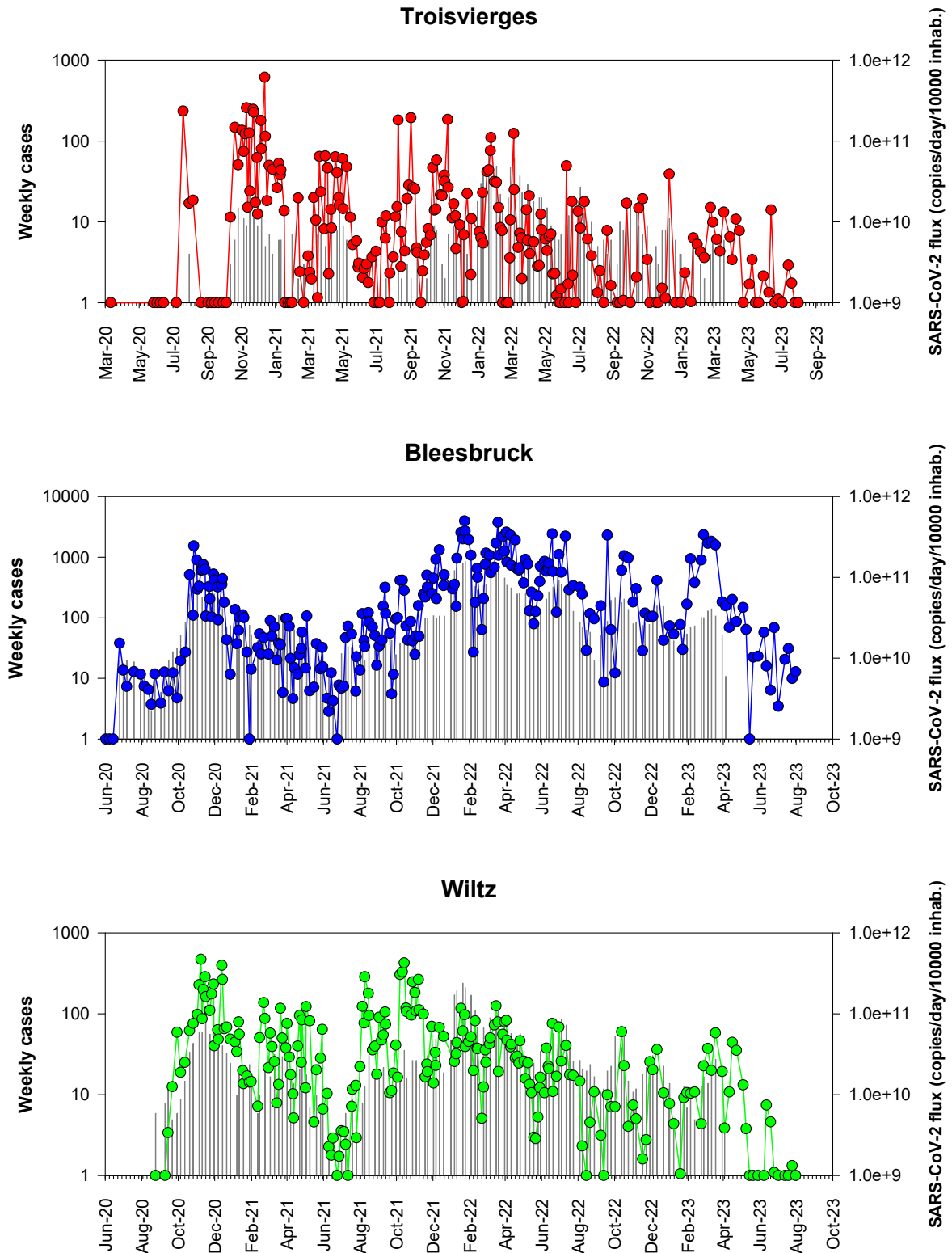


Figure 5b – Close-up of Figure 5a showing results from January 1st, 2022 on.

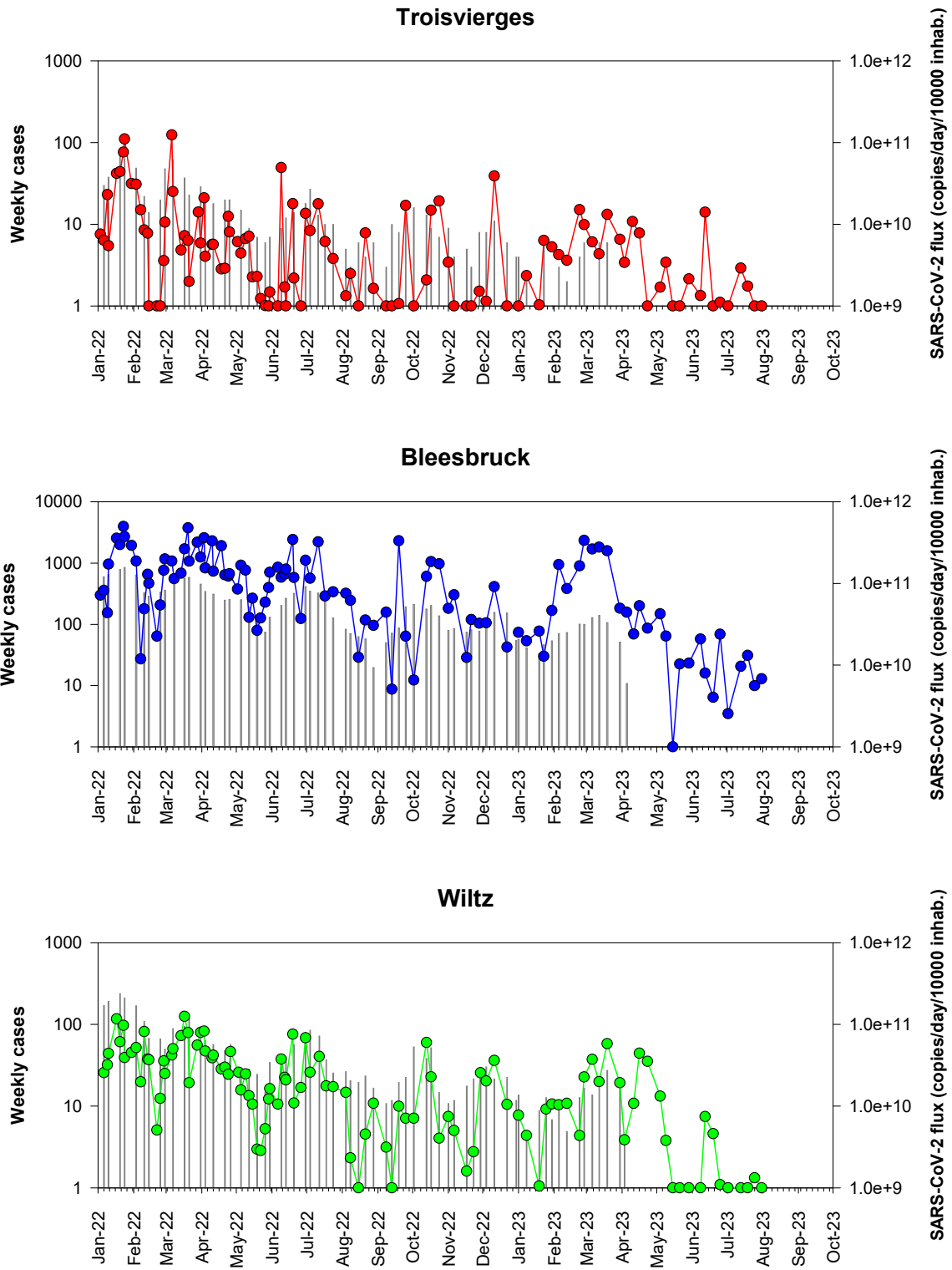


Table 3- Sewage sampling since the beginning of the CORONASTEP study

WWTP	2019	2020	2021	2022	2023	Total
Beggen		52	92	77	30	251
Bettembourg		45	91	75	29	240
Schifflange	4	56	96	76	30	262
Bleesbrück		44	92	77	30	243
Mersch		47	91	77	30	245
Pétange	4	51	96	77	30	258
Hesperange		35	48	50	30	163
Echternach		36	79	77	30	222
Uebersyren		38	78	77	30	223
Grevenmacher		38	81	76	30	225
Troisvierges		38	92	77	30	237
Boevange sur Attert		20	48	51	30	149
Wiltz		28	92	77	30	227
Total	8	528	1076	944	389	2945

Materials and Methods

Sewage samples

From March 2020 to August 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 protocol¹. 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).

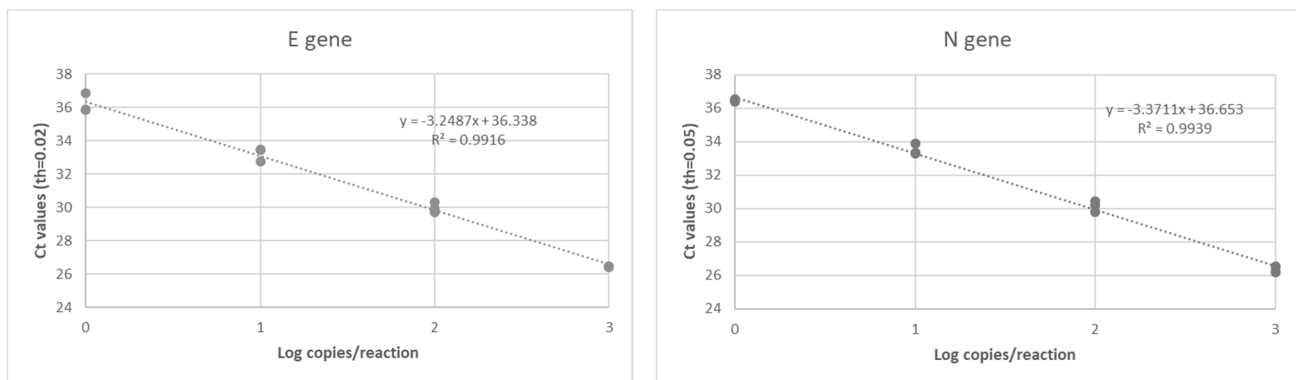


Figure 6 – RT-qPCR standard curves established for both target genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

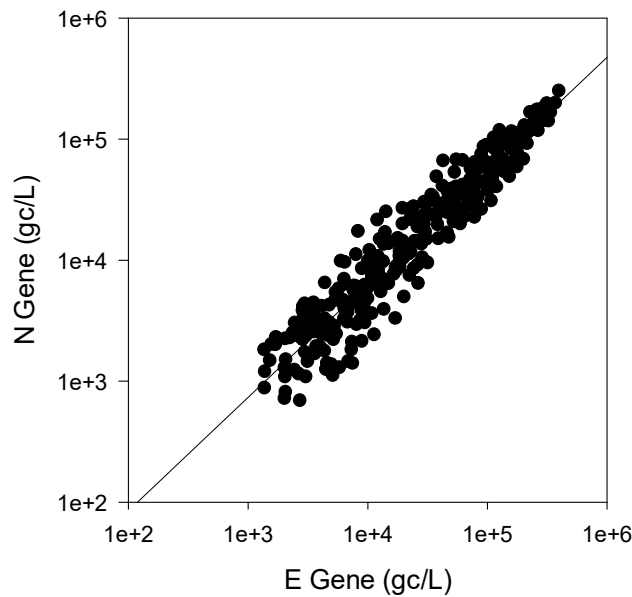
Data interpretation

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, $R^2=0.964$, $p = 5.979 \cdot 10^{-24}$) 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

This work is supported by the Fond National de la Recherche (FNR) under project 14806023 - CORONASTEP+ and is conducted in collaboration with the Luxembourg Institute of Health (LIH), the “Laboratoire National de Santé” (LNS) and the University of Luxembourg (LCSB).

In addition, the authors of this report would like to thank all the wastewater syndicates (SIACH, SIVÉC, STEP, SIDERO, SIDEN and SIDEST), the “Ville du Luxembourg”, the Hesperange city as well as the “Administration de la Gestion de l’Eau” (AGE) for their kind and valuable assistance in the sample collection, the acquisition of wastewater parameters and the collection of demographic data. The authors would also like to thank the Ministry of Health and the Inspection Sanitaire for their valuable contribution in providing the COVID-19 data at the national and regional scale.