

CORONASTEP Report 79 (Week 24-Partial) SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

This report 79 presents the results of SARS-CoV-2 contamination of wastewater at the entrance of the 13 wastewater treatment plants analysed at the beginning of the week 24 of 2021.

The SARS-CoV-2 RNA flux measured in wastewater treatment plants shows a moderate national prevalence of the virus. The flux has around 6 to 7 x 10¹⁰ RNA copies per day per 100,000 equivalent-inhabitants, with a continuing downward trend for several weeks.

At the individual wastewater treatment plant scale, the trend is similar with most of the plants having a SARS-CoV-2 flux near the detection limit of the detection assay, or even below. SARS-CoV-2 genome is no longer detectable in 5 out of 13 stations and present in trace amounts for others.

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).

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

| National Contamination Level | Week |
|------------------------------|-----------|
| Light Green | Week 50-2 |
| Light Green | Week 51-1 |
| Light Green | Week 51-2 |
| Light Green | Week 51-2 |
| Light Green | Week 52 |
| Light Green | Week 53 |
| Light Green | Week 01-1 |
| Light Green | Week 01-2 |
| Light Green | Week 02-1 |
| Light Green | Week 02-2 |
| Light Green | Week 03-1 |
| Light Green | Week 03-2 |
| Light Green | Week 04-1 |
| Light Green | Week 04-2 |
| Light Green | Week 05-1 |
| Light Green | Week 06-1 |
| Light Green | Week 06-2 |
| Light Green | Week 07-1 |
| Light Green | Week 07-2 |
| Light Green | Week 08-1 |
| Light Green | Week 08-2 |
| Light Green | Week 09-1 |
| Light Green | Week 09-2 |
| Light Green | Week 10-1 |
| Light Green | Week 10-2 |
| Light Green | Week 11-1 |
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| Light Green | Week 12-1 |
| Light Green | Week 12-2 |
| Light Green | Week 13-1 |
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| Light Green | Week 16-1 |
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| Light Green | Week 18-2 |
| Light Green | Week 19 |
| Light Green | Week 20-1 |
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| Light Green | Week 21 |
| Light Green | Week 22-1 |
| Light Green | Week 22-2 |
| Light Green | Week 23-1 |
| Light Green | Week 23-2 |
| Light Green | Week 24-1 |

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

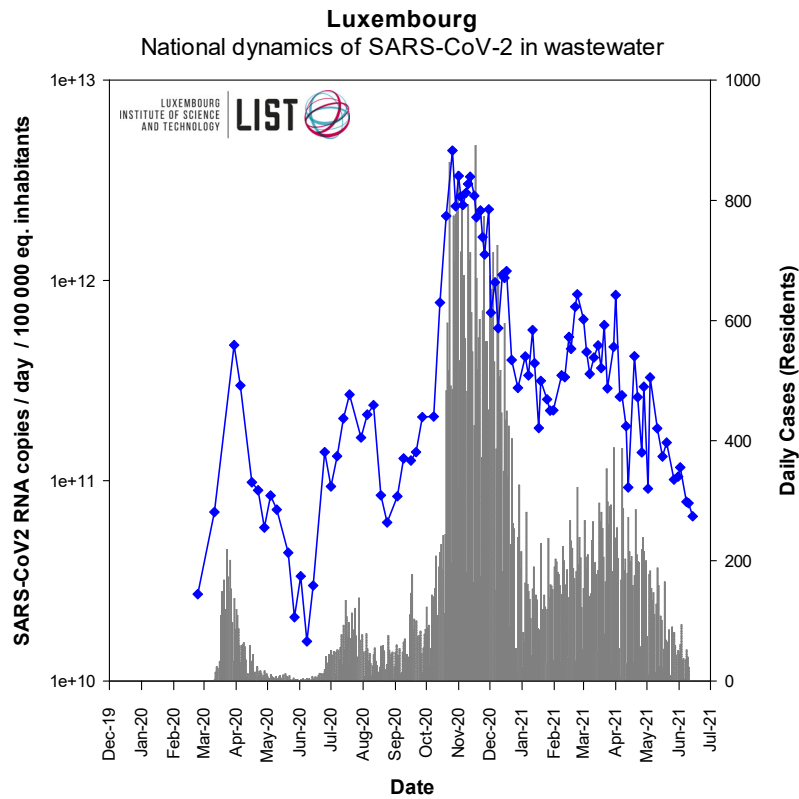


Figure 1b – Close-up of Figure 1a showing results from September 1st on.

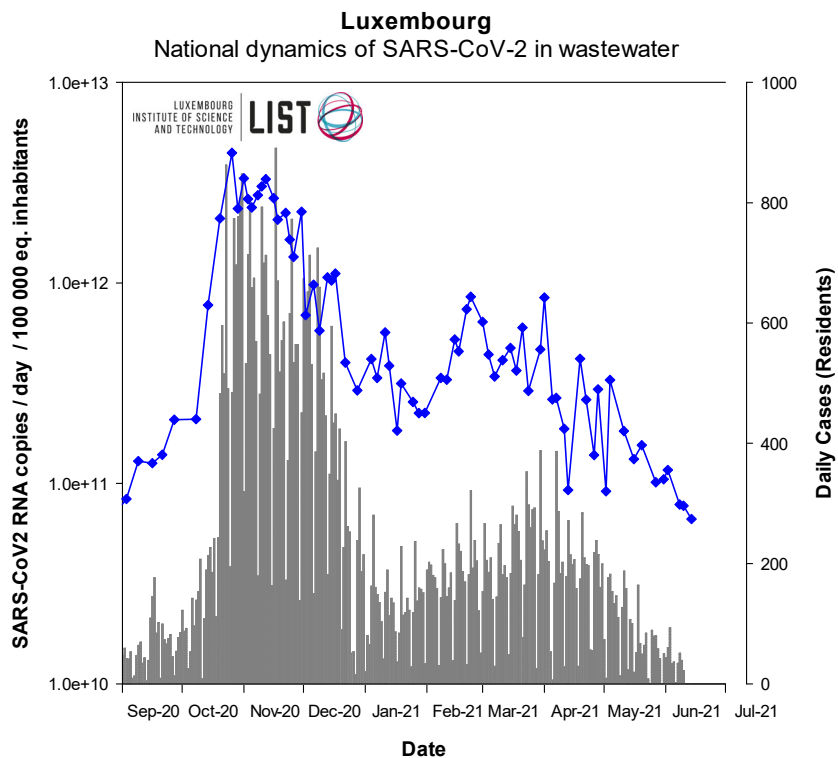


Table 2 - Level of SARS-CoV-2 contamination of each analyzed wastewater treatment plant in Luxembourg during the second wave. BEG: Beggen, BET: Bettembourg, SCH: Schifflange, BLE: Blesbruck, MER: Mersch, PET: Pétange, HES: Hespérange, ECG: Echternach, UEB: Uebersyren, GRE: Grevenmacher, TRO: Troisvierges, 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

| WWTP | 2021 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|---------|-----------|-----------|---------|-----------|-----------|-----------|-----------|-----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| | 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 | | | | | | | | | |
| BEG | Orange | Orange | Orange | Grey | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | | | | |
| BET | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Green | Green | Yellow | Grey | Yellow | Grey | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | | | |
| SCH | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | | |
| BLE | Yellow | Yellow | Yellow | Yellow | Yellow | Green | Green | Yellow | Grey | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | | | |
| MER | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | | |
| PET | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | |
| HES | Grey | Orange | Grey | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | | |
| ECH | Yellow | Grey | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | | |
| UEB | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | | |
| GRE | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange |
| TRO | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange |
| BOE | Grey | Yellow | Grey | Green | Grey | Green | Green | Green | Yellow | Grey | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | | |
| WIL | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | Orange | |

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 June 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).

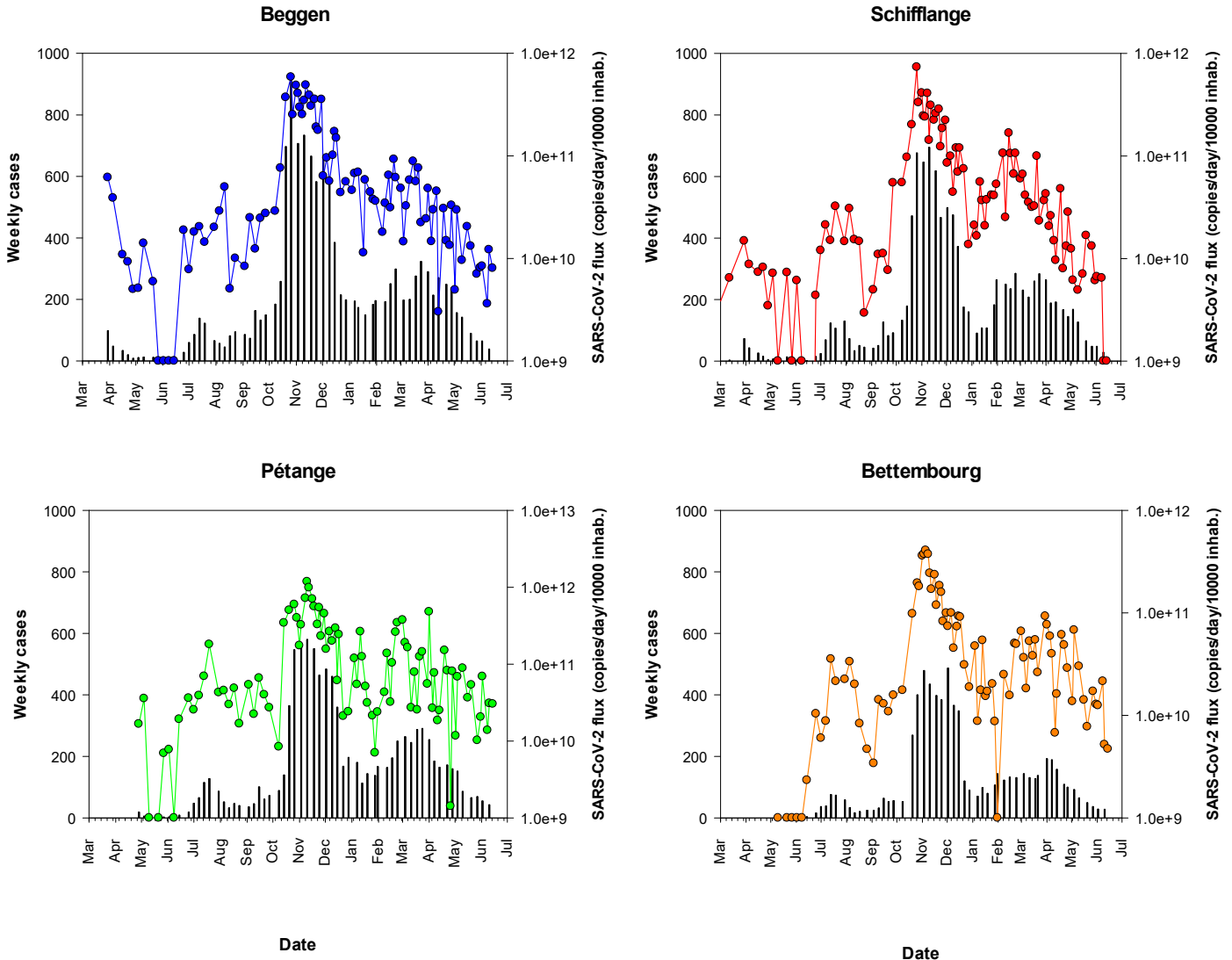


Figure 2b – Close-up of Figure 2a showing results from September 1st, 2020 on.

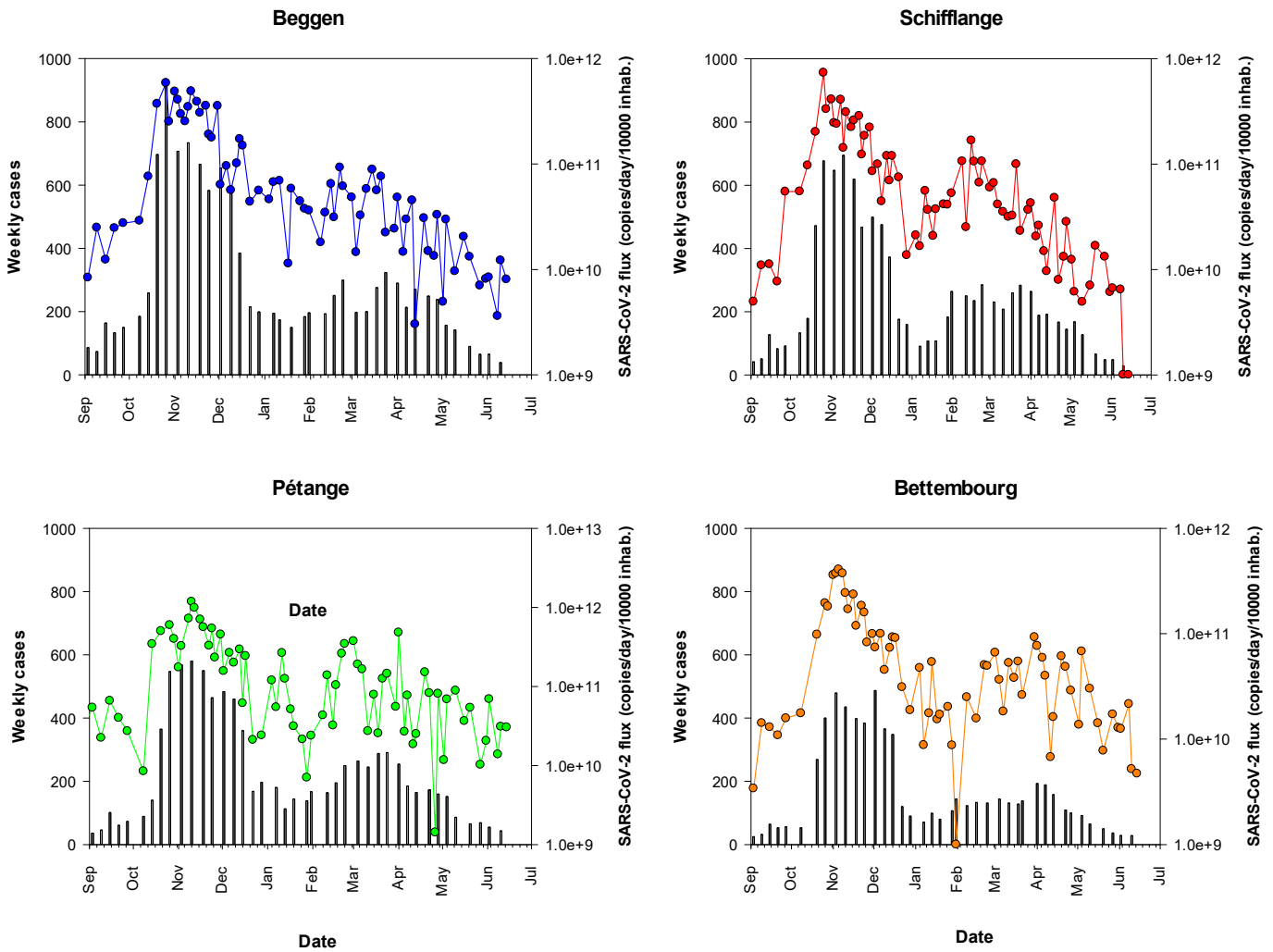


Figure 3a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Hespérange, Mersch and Boevange-sur-Attert wastewater treatment plants from March 2020 to June 2021. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux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

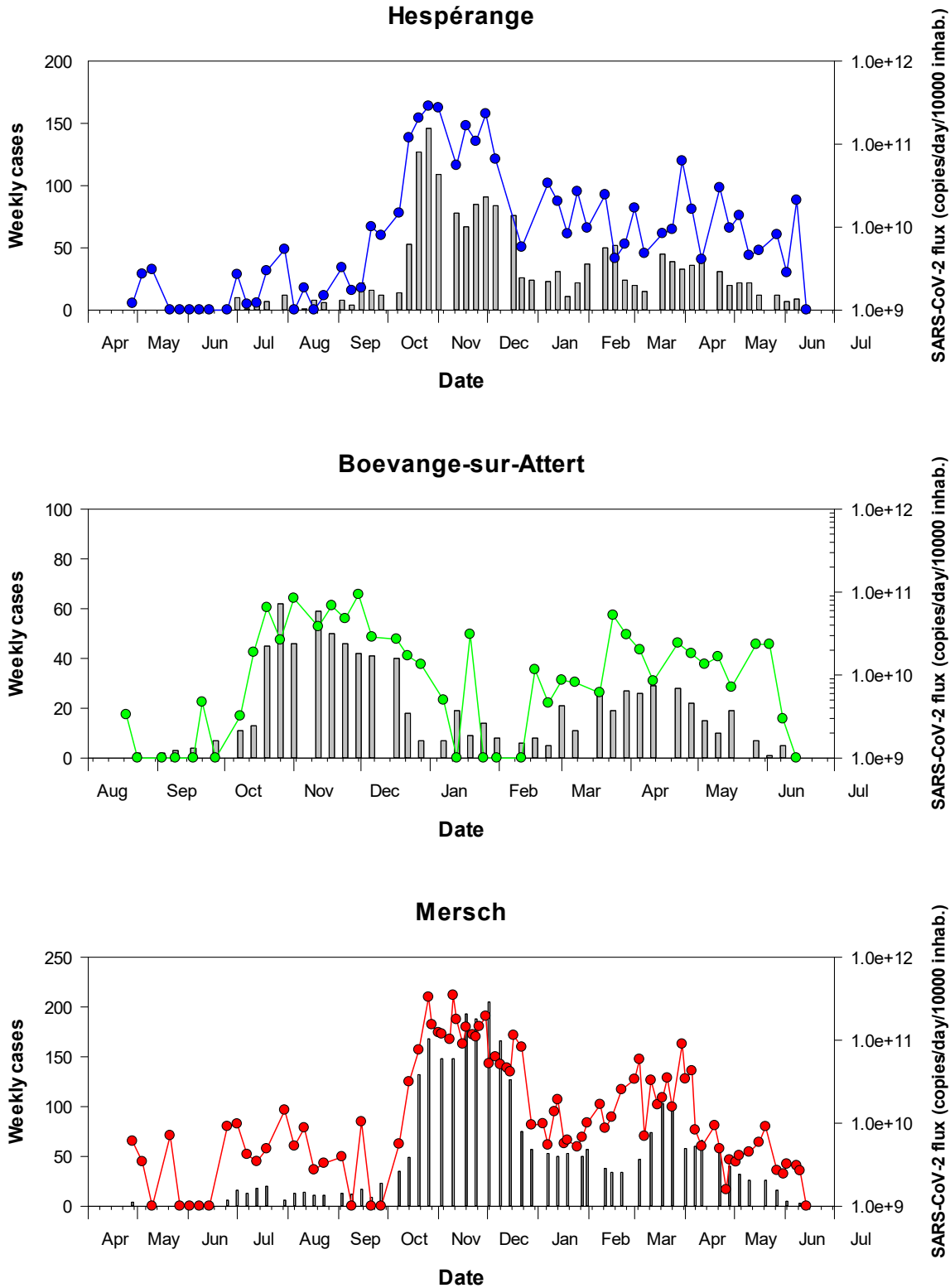


Figure 3b – Close-up of Figure 3a showing results from September 1st, 2020 on.

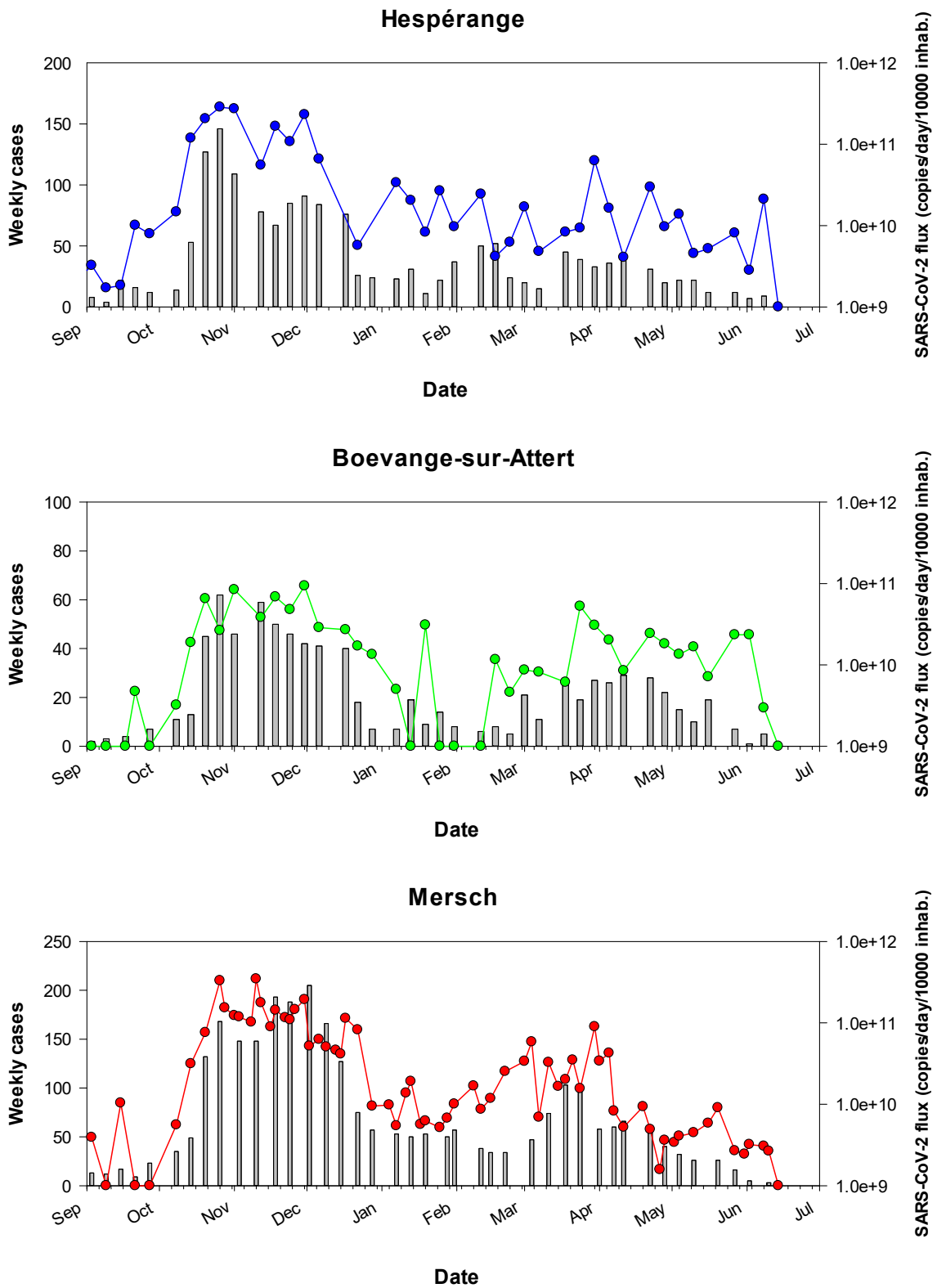


Figure 4a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from March 2020 to June 2021. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent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

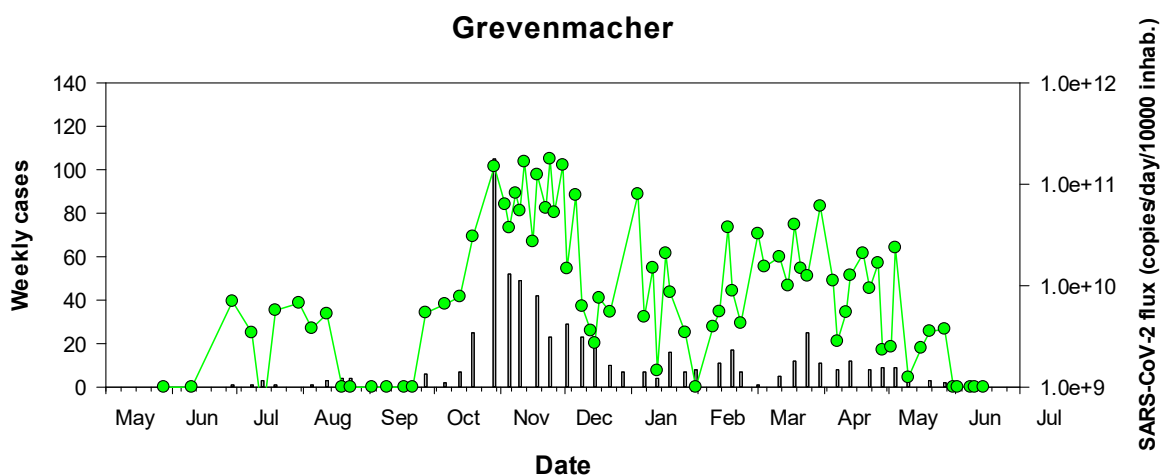
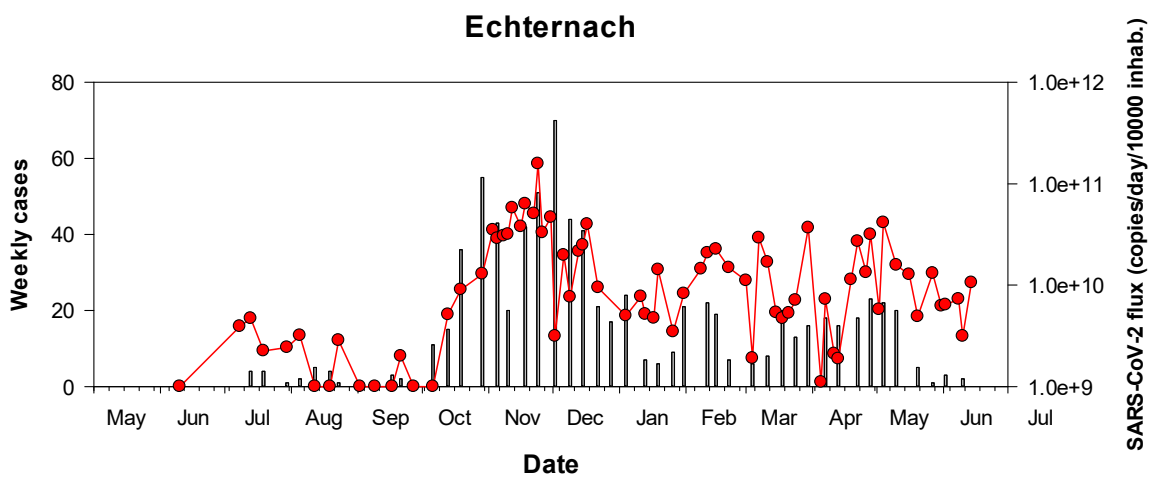
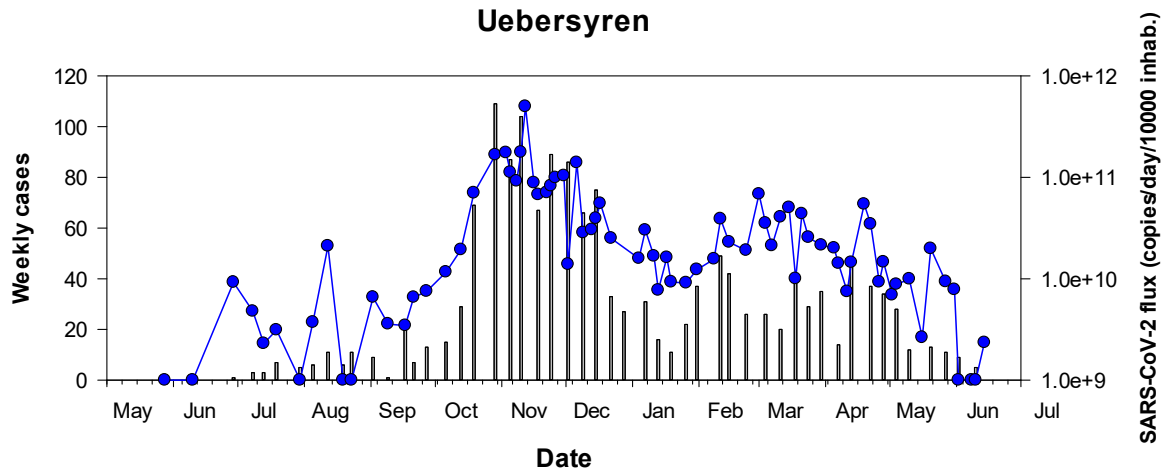


Figure 4b – Close-up of Figure 4a showing results from September 1st, 2021 on.

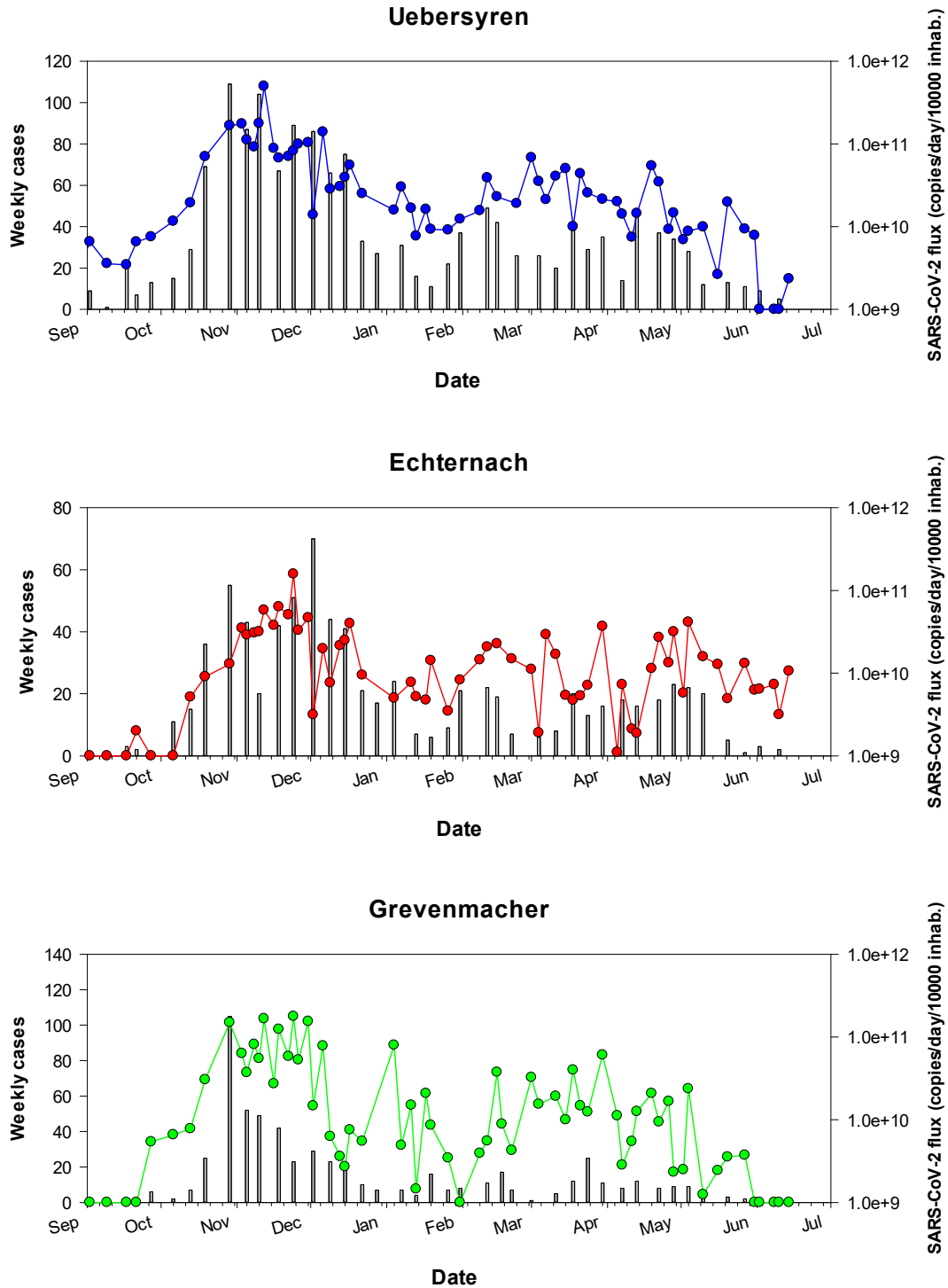


Figure 5a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from March 2020 to June 2021. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent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

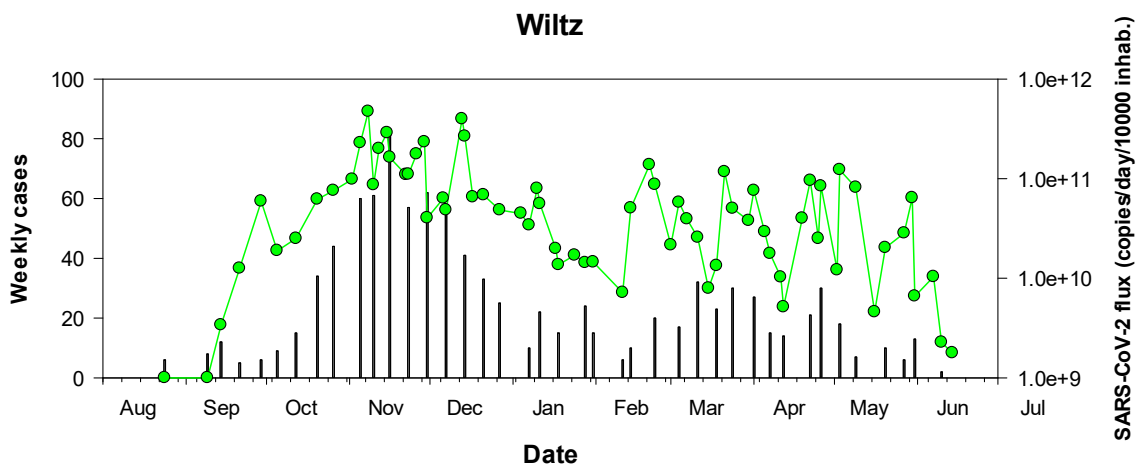
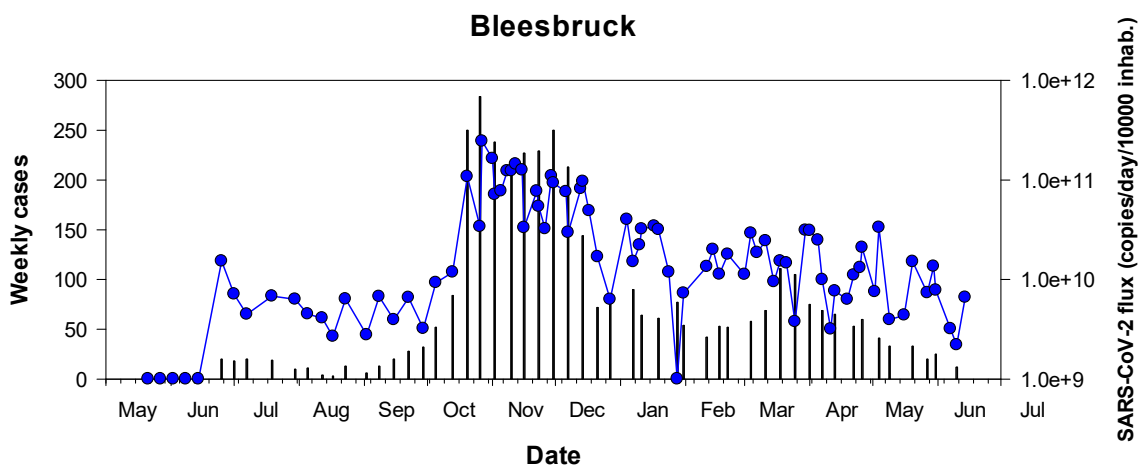
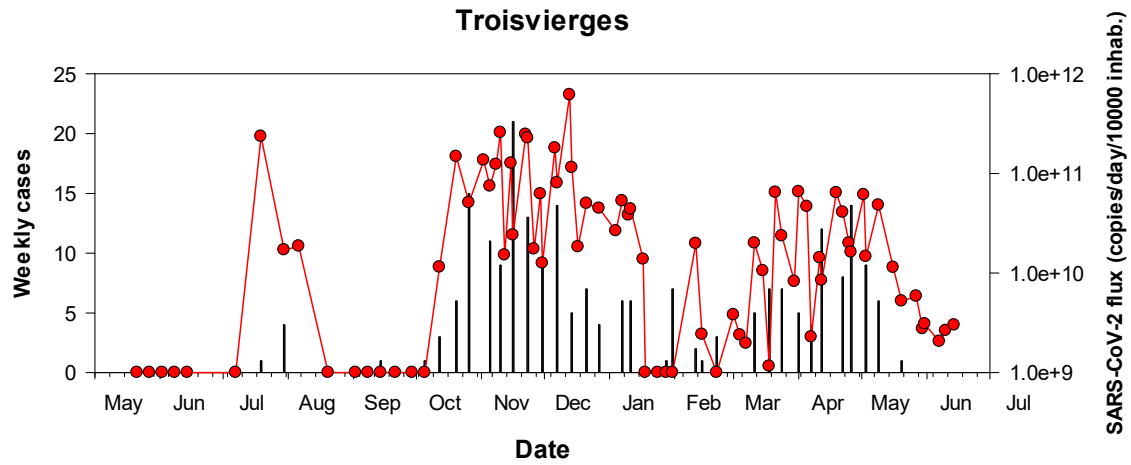
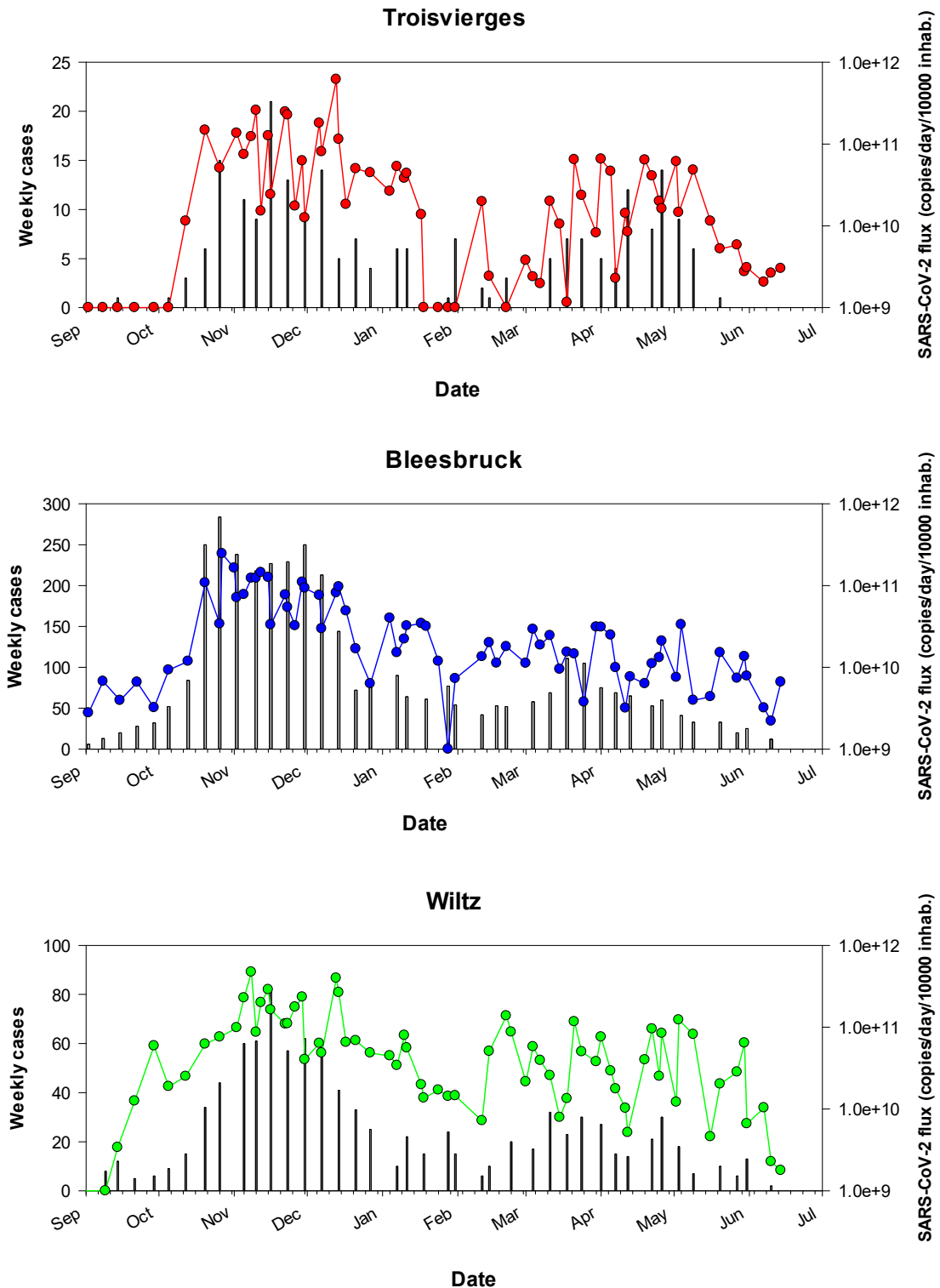


Figure 5b – Close-up of Figure 5a showing results from September 1st, 2021 on.



Materials and Methods

Sewage samples

From March 2020 to June 2021, 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 Vii7 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).

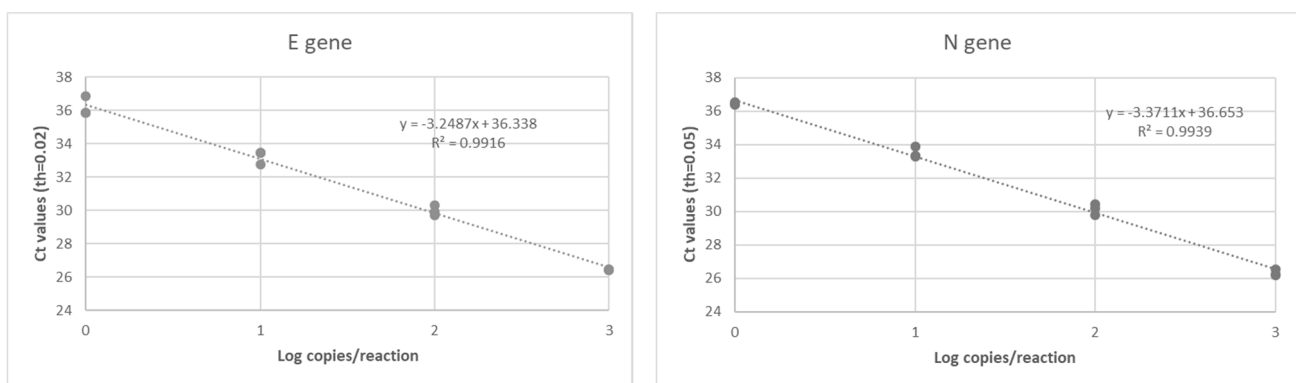


Figure 6 – RT-qPCR standard curves established for both target genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

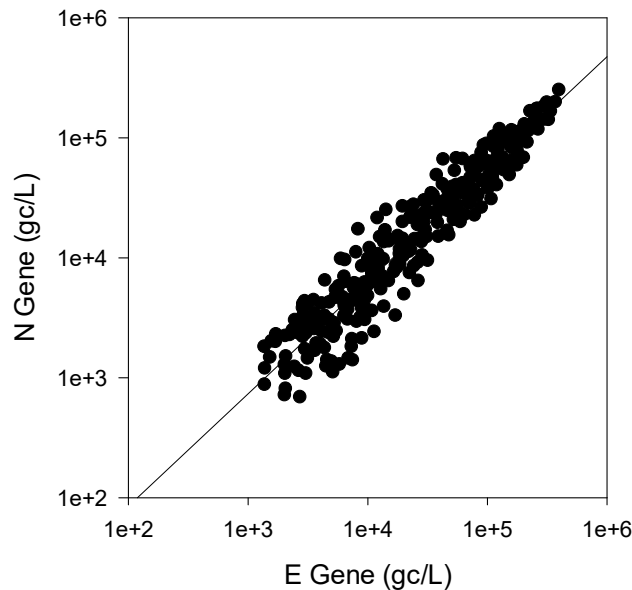
Data interpretation

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

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