

CORONASTEP Report 175 (2024 – Weeks 01 and 02) SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

This report 175 presents the results of SARS-CoV-2 contamination of wastewater at the entrance of the 13 wastewater treatment plants (WWTPs) analysed during weeks 01 and 02 of 2024.

Nationally, the SARS-CoV-2 RNA flux measured in the WWTPS during weeks 01 and 02 of 2024 decreased significantly compared to this measured in December 2023. The national flux of SARS-CoV-2 was around 1.5×10^{12} RNA copies per day per 100,000 population equivalents over the last week. The downward trend was also clearly visible at the regional level, with no disparities between regions.

The data from the first week of 2024 has to be considered with caution due to very heavy rainfall on the day of sampling, which diluted the samples and resulted in SARS-CoV-2 concentrations close to, or even below, the detection limit.

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

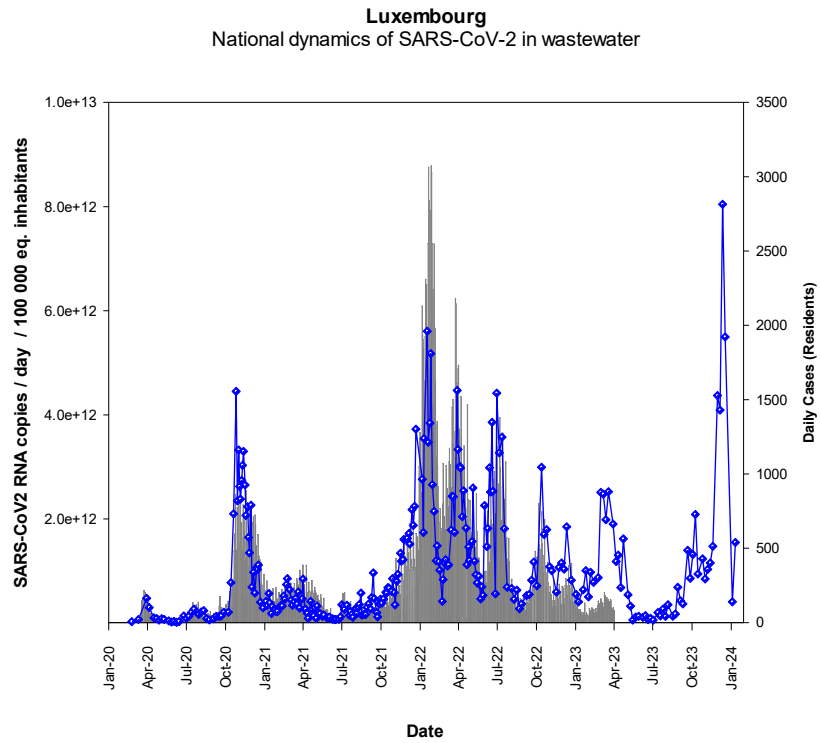
| 2021 | |
|---------------------------|-----------|
| National SARS-CoV-2 Level | Week |
| Dark Green | Week 01-1 |
| Dark Green | Week 01-2 |
| Dark Green | Week 02-1 |
| Dark Green | Week 02-2 |
| Dark Green | Week 03-1 |
| Dark Green | Week 03-2 |
| Dark Green | Week 04-1 |
| Dark Green | Week 04-2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

| 2021 | | 2022 | |
|---------------------------|-----------|---------------------------|-----------|
| National SARS-CoV-2 Level | Week | National SARS-CoV-2 Level | Week |
| Dark Green | Week 31-1 | Dark Green | Week 01 |
| Dark Green | Week 31-2 | Dark Green | Week 02-1 |
| Dark Green | Week 32-1 | Dark Green | Week 02-2 |
| Dark Green | Week 32-2 | Dark Green | Week 03-1 |
| Dark Green | Week 33-1 | Dark Green | Week 03-2 |
| Dark Green | Week 33-2 | Dark Green | Week 04-1 |
| Dark Green | Week 34-1 | Dark Green | Week 04-2 |
| Dark Green | Week 34-2 | Dark Green | Week 05-1 |
| Dark Green | Week 35-1 | Dark Green | Week 05-2 |
| Dark Green | Week 35-2 | Dark Green | Week 06-1 |
| Dark Green | Week 36-1 | Dark Green | Week 06-2 |
| Dark Green | Week 36-2 | Dark Green | Week 07-1 |
| Dark Green | Week 37-1 | Dark Green | Week 07-2 |
| Dark Green | Week 37-2 | Dark Green | Week 08-1 |
| Dark Green | Week 38-1 | Dark Green | Week 08-2 |
| Dark Green | Week 38-2 | Dark Green | Week 09-1 |
| Dark Green | Week 39-1 | Dark Green | Week 09-2 |
| Dark Green | Week 39-2 | Dark Green | Week 10-1 |
| Dark Green | Week 40-1 | Dark Green | Week 10-2 |
| Dark Green | Week 40-2 | Dark Green | Week 11-1 |
| Dark Green | Week 41-1 | Dark Green | Week 11-2 |
| Dark Green | Week 41-2 | Dark Green | Week 12-1 |
| Dark Green | Week 42-1 | Dark Green | Week 12-2 |
| Dark Green | Week 42-2 | Dark Green | Week 13-1 |
| Dark Green | Week 43-1 | Dark Green | Week 13-2 |
| Dark Green | Week 43-2 | Dark Green | Week 14-1 |
| Dark Green | Week 44-1 | Dark Green | Week 14-2 |
| Dark Green | Week 44-2 | Dark Green | Week 15-1 |
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| Dark Green | Week 46-2 | Dark Green | Week 17-1 |
| Dark Green | Week 47-1 | Dark Green | Week 17-2 |
| Dark Green | Week 47-2 | Dark Green | Week 18-1 |
| Dark Green | Week 48-1 | Dark Green | Week 18-2 |
| Dark Green | Week 48-2 | Dark Green | Week 19-1 |
| Dark Green | Week 49-1 | Dark Green | Week 19-2 |
| Dark Green | Week 49-2 | Dark Green | Week 20-1 |
| Dark Green | Week 50-1 | Dark Green | Week 20-2 |
| Dark Green | Week 50-2 | Dark Green | Week 21-1 |
| Dark Green | Week 51-1 | Dark Green | Week 21-2 |
| Dark Green | Week 51-2 | Dark Green | Week 22-1 |
| Dark Green | Week 01 | Dark Green | Week 22-2 |
| Dark Green | Week 02-1 | Dark Green | Week 23-1 |
| Dark Green | Week 02-2 | Dark Green | Week 23-2 |
| Dark Green | Week 03-1 | Dark Green | Week 24-1 |
| Dark Green | Week 03-2 | Dark Green | Week 24-2 |
| Dark Green | Week 04-1 | Dark Green | Week 25-1 |
| Dark Green | Week 04-2 | Dark Green | Week 25-2 |
| Dark Green | Week 05-1 | Dark Green | Week 26-1 |
| Dark Green | Week 05-2 | Dark Green | Week 26-2 |
| Dark Green | Week 06-1 | Dark Green | Week 27 |
| Dark Green | Week 06-2 | Dark Green | Week 28 |
| Dark Green | Week 07-1 | Dark Green | Week 29 |
| Dark Green | Week 07-2 | Dark Green | Week 30 |

| 2022 | |
|---------------------------|-----------|
| National SARS-CoV-2 Level | Week |
| Dark Green | Week 08-1 |
| Dark Green | Week 08-2 |
| Dark Green | Week 09-1 |
| Dark Green | Week 09-2 |
| Dark Green | Week 10-1 |
| Dark Green | Week 10-2 |
| Dark Green | Week 11-1 |
| Dark Green | Week 11-2 |
| Dark Green | Week 12-1 |
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| Dark Green | Week 31 |
| Dark Green | Week 32 |
| Dark Green | Week 33 |
| Dark Green | Week 34 |
| Dark Green | Week 35 |
| Dark Green | Week 36 |
| Dark Green | Week 37 |
| Dark Green | Week 38 |
| Dark Green | Week 39 |
| Dark Green | Week 40 |
| Dark Green | Week 41 |
| Dark Green | Week 42 |
| Dark Green | Week 43 |

Figure 1 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (*E* gene) in Luxembourgish wastewater samples from December 2019 to January 2024. 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

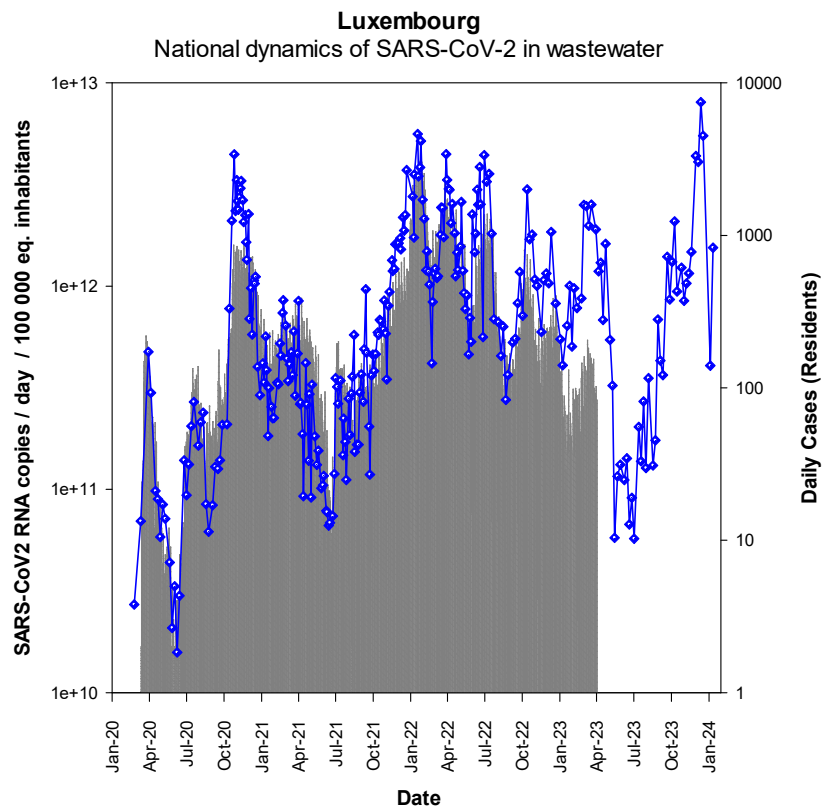


Figure 2a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in the four most impacted wastewater treatment plants from March 2020 to January 2024. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

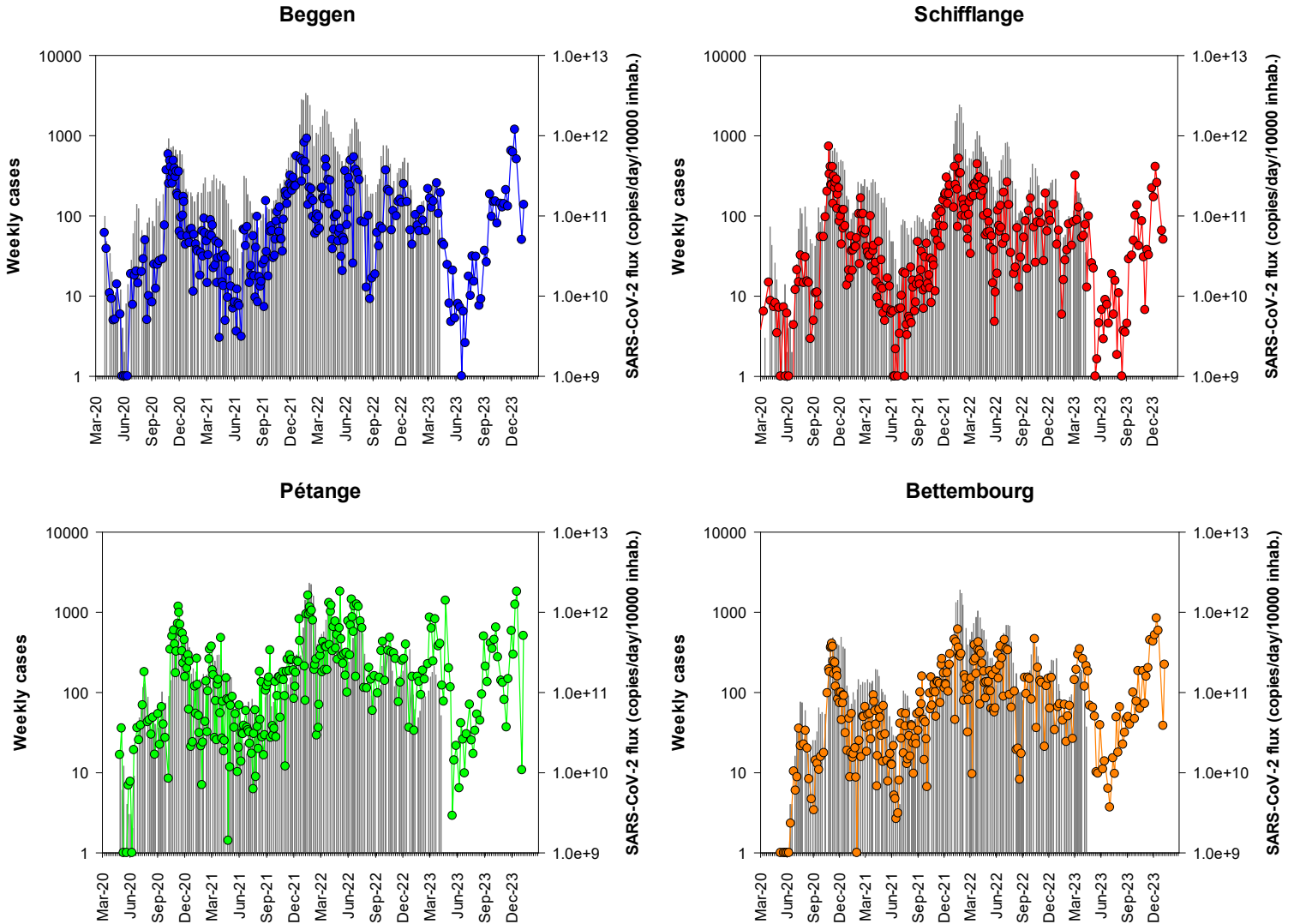


Figure 2b – Close-up of Figure 2a showing results from January 1st, 2022 on.

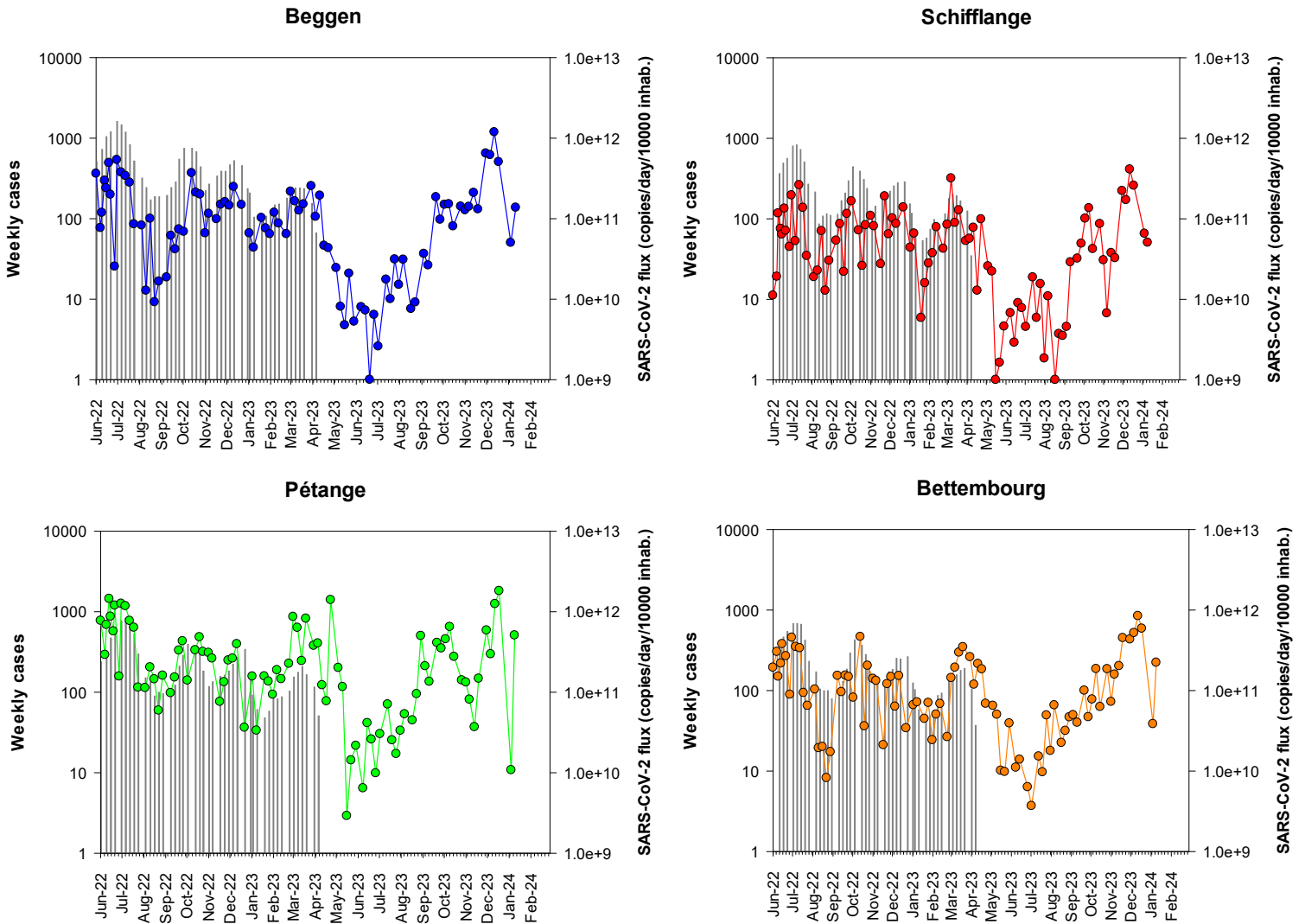


Figure 3a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (*E* gene) in Hesperange, Mersch and Boevange-sur-Attert wastewater treatment plants from May 2020 to January 2024. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

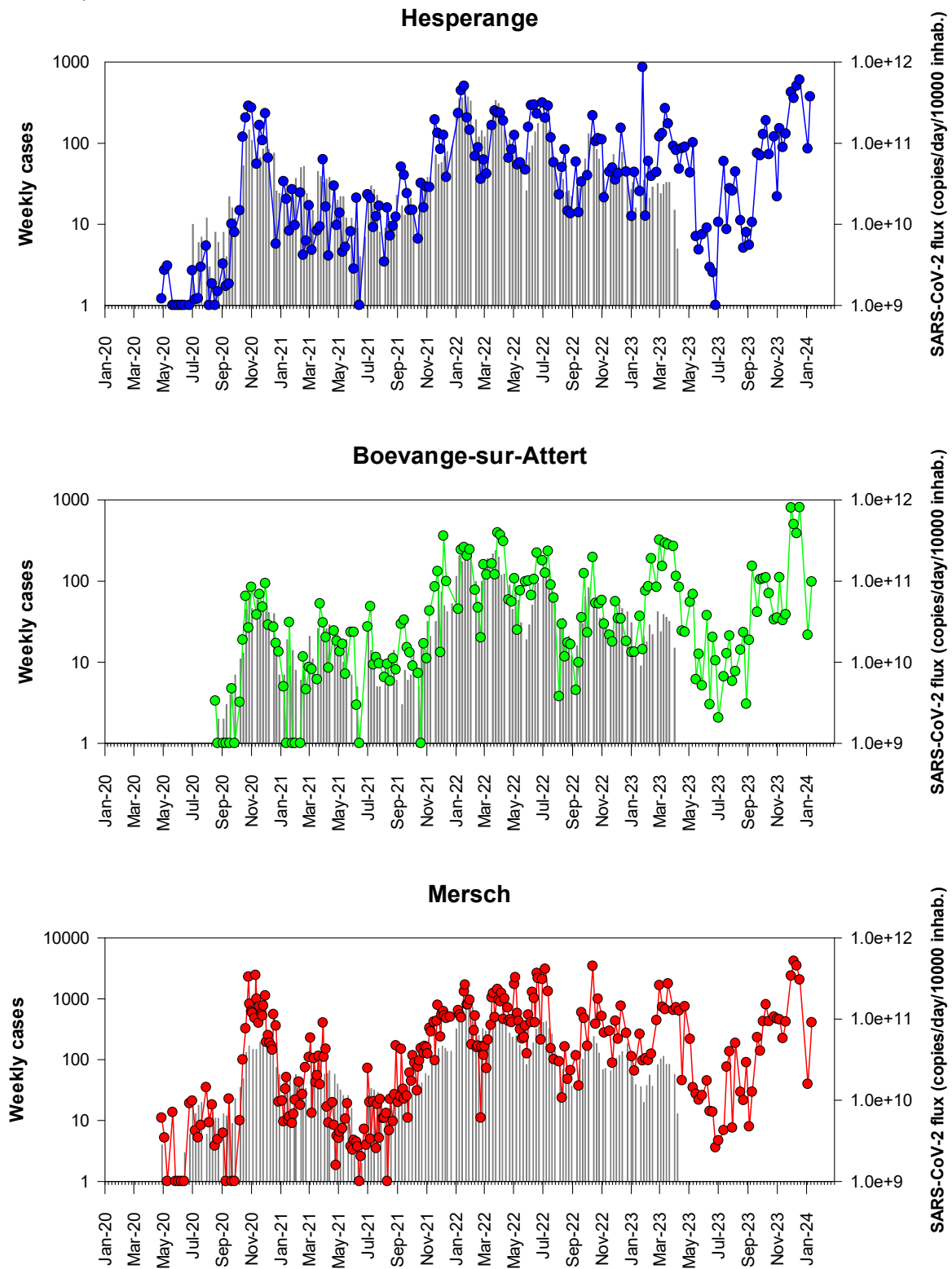


Figure 3b – Close-up of Figure 3a showing results from January 1st, 2022 on.

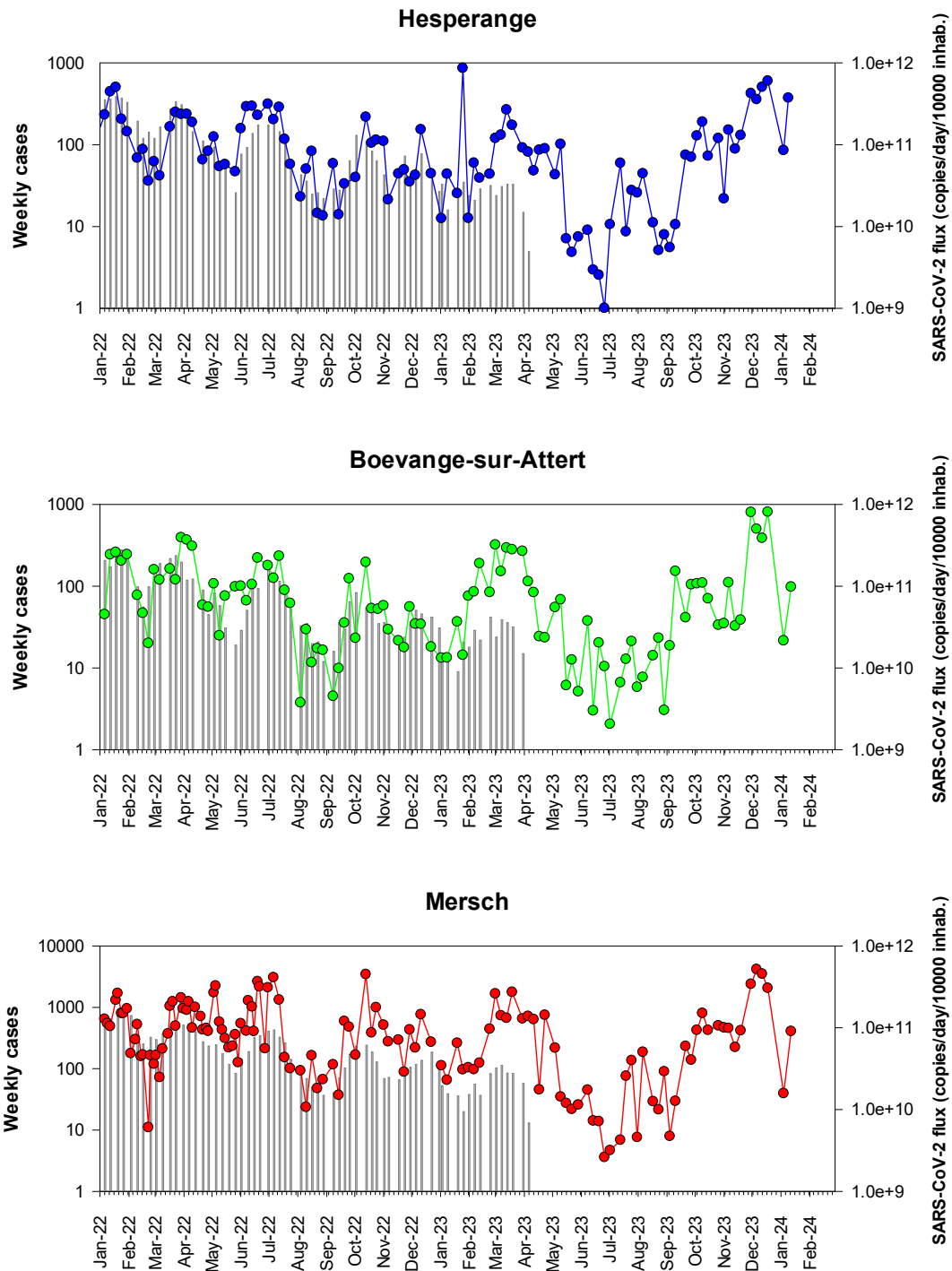


Figure 4a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from May 2020 to January 2024. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

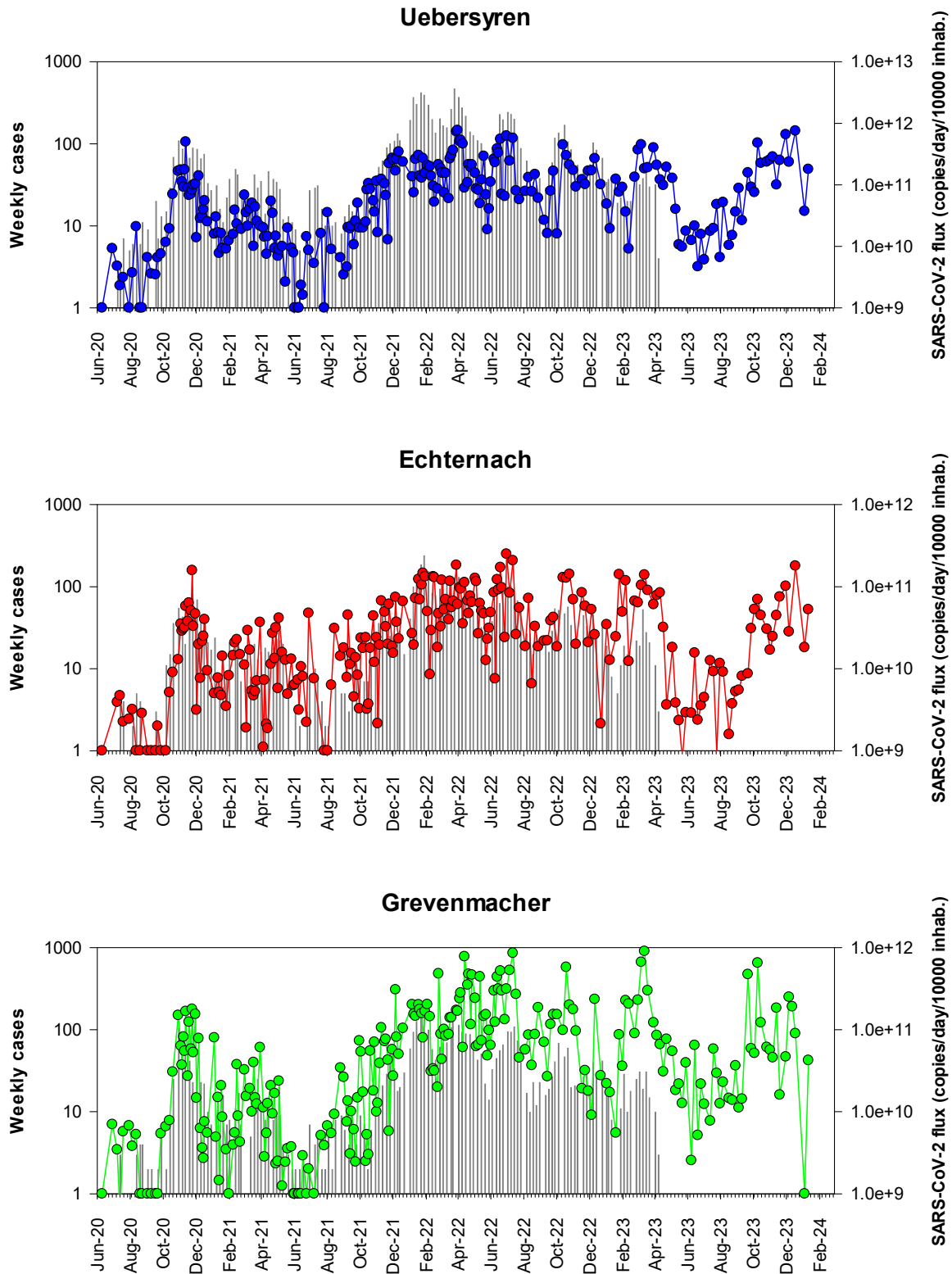


Figure 4b – Close-up of Figure 4a showing results from January 1st, 2022 on.

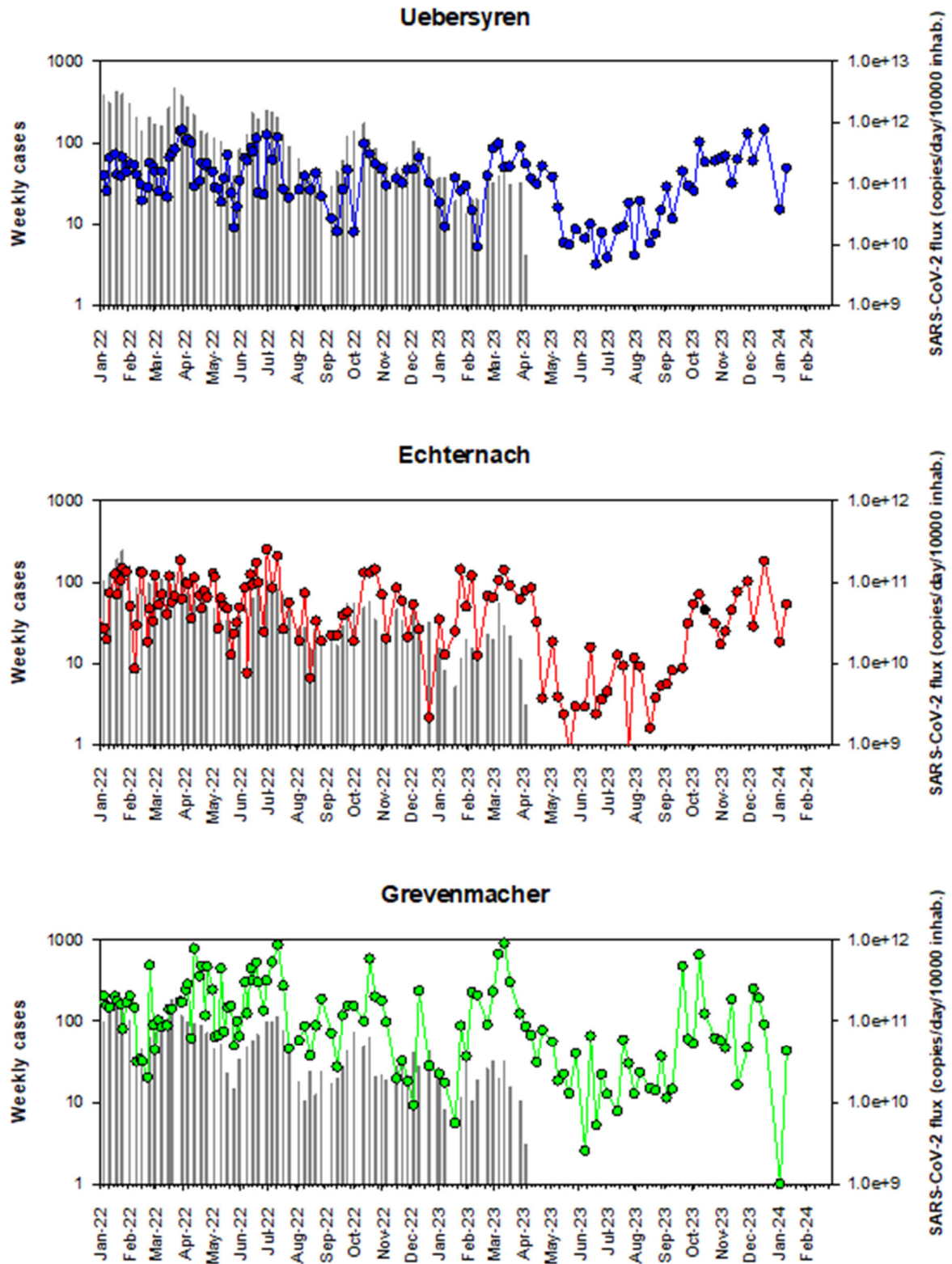


Figure 5a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from May 2020 to January 2024. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

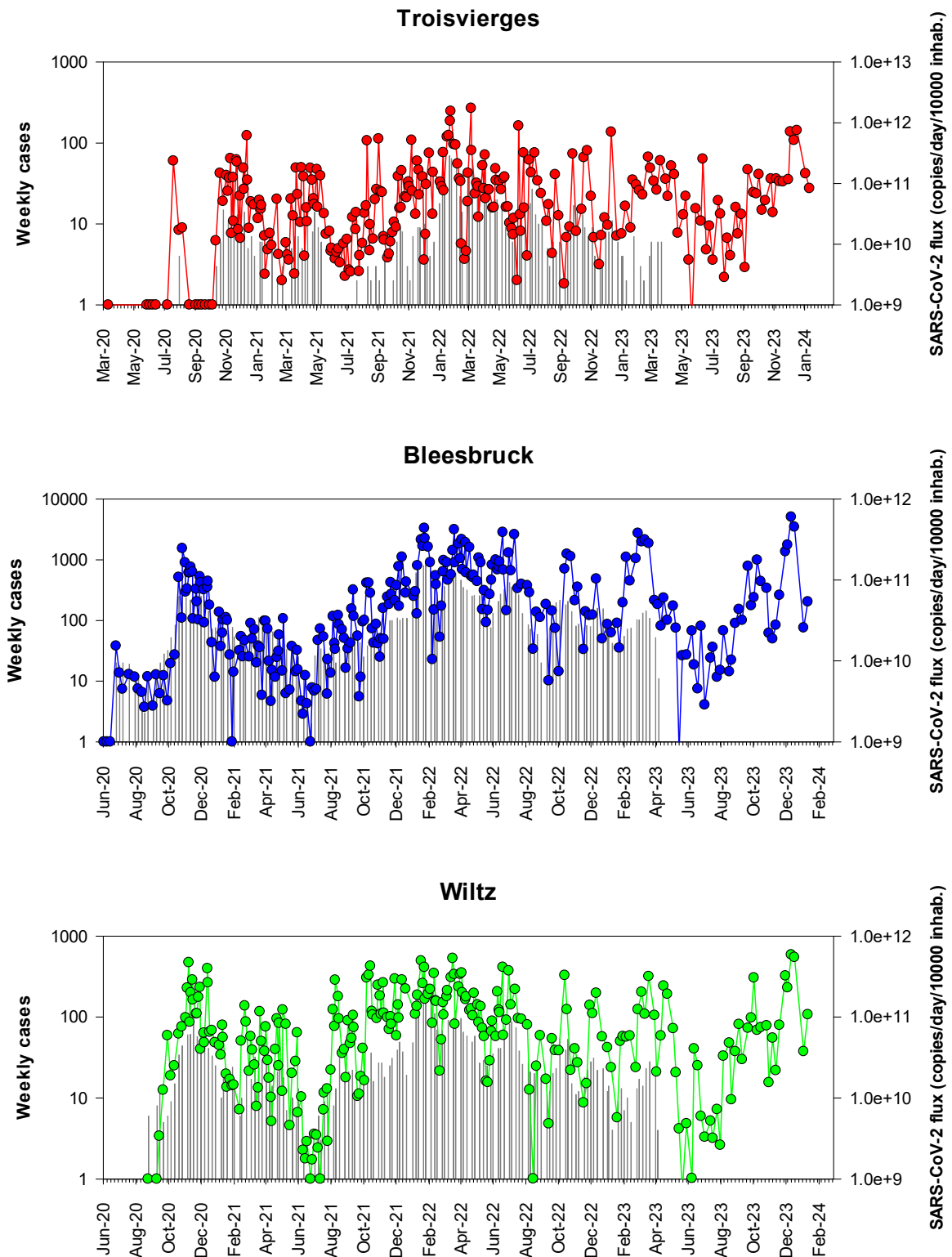


Figure 5b – Close-up of Figure 5a showing results from January 1st, 2022 on.

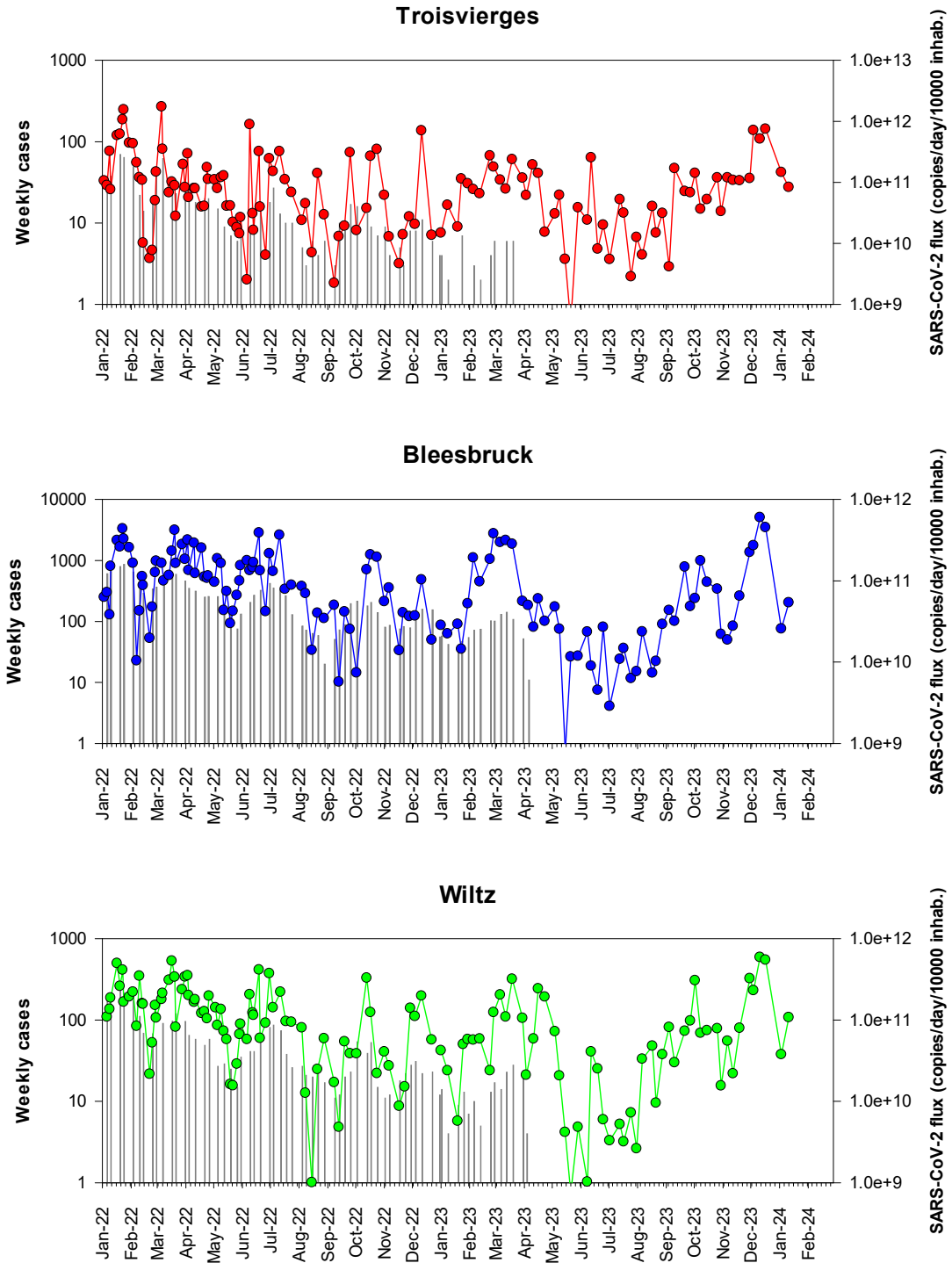


Table 3- Sewage sampling since the beginning of the CORONASTEP study

| WWTP | 2019 | 2020 | 2021 | 2022 | 2023 | 2024 | Total |
|---------------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------|
| Beggen | | 52 | 92 | 77 | 50 | 2 | 273 |
| Bettembourg | | 45 | 91 | 75 | 50 | 2 | 266 |
| Schiffflange | 4 | 56 | 96 | 76 | 51 | 2 | 285 |
| Bleesbrück | | 44 | 92 | 77 | 51 | 2 | 266 |
| Mersch | | 47 | 91 | 77 | 51 | 2 | 268 |
| Pétange | 4 | 51 | 96 | 77 | 51 | 2 | 281 |
| Hesperange | | 35 | 48 | 50 | 51 | 2 | 186 |
| Echternach | | 36 | 79 | 77 | 50 | 2 | 244 |
| Uebersyren | | 38 | 78 | 77 | 50 | 2 | 245 |
| Grevenmacher | | 38 | 81 | 76 | 51 | 2 | 248 |
| Troisvierges | | 38 | 92 | 77 | 51 | 2 | 260 |
| Boevange sur Attert | | 20 | 48 | 51 | 51 | 2 | 172 |
| Wiltz | | 28 | 92 | 77 | 51 | 2 | 250 |
| Total | 8 | 528 | 1076 | 944 | 659 | 26 | 3241 |

Materials and Methods

Sewage samples

From March 2020 to January 2024, 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-hour composite sample according to their routine sampling procedure. Composite samples were 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 sampling day. Larger particles (debris, bacteria) were removed from the samples by centrifugation at 2,400 x g for 20 min at 4°C. A 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).

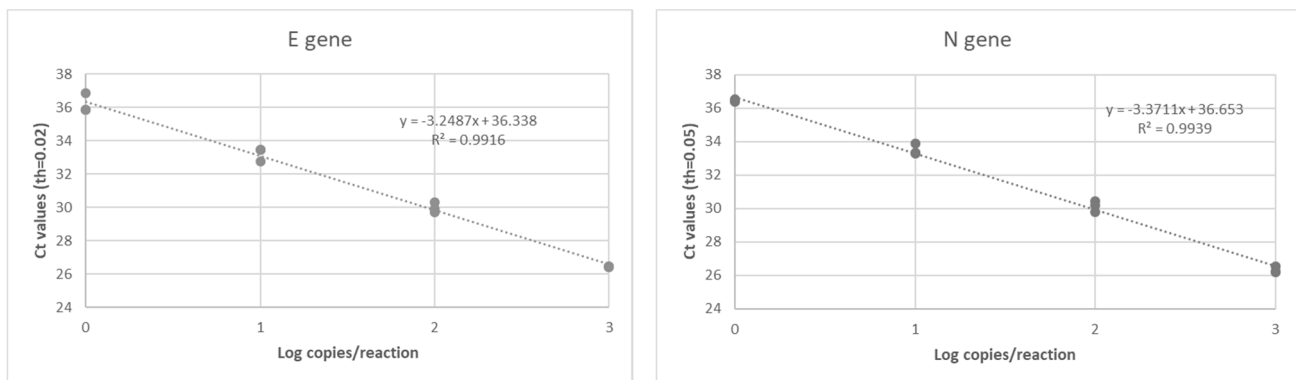


Figure 6 – RT-qPCR standard curves established for both target genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

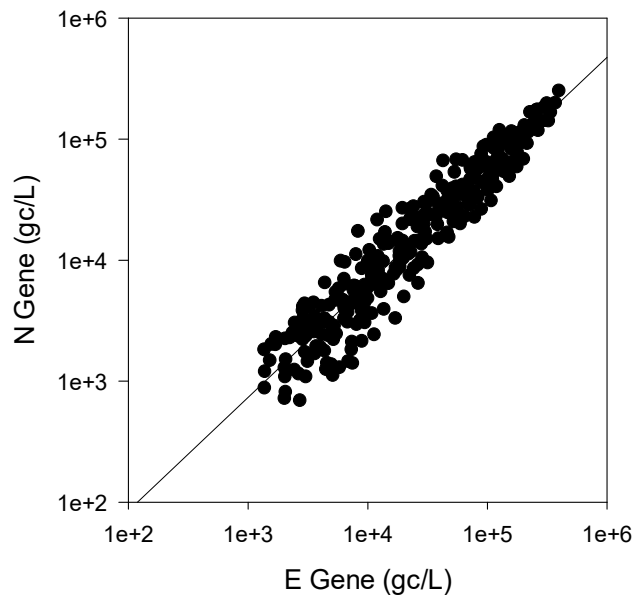
Data interpretation

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

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