

## CORONASTEP Report 169 (2023 - Weeks 40 and 41) SARS-CoV-2 Sewage Surveillance in Luxembourg

### Summary

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This report 169 presents the results of SARS-CoV-2 contamination of wastewater at the entrance of the 13 wastewater treatment plants (WWTPs) analysed during the weeks 40 and 41 of 2023. All WWTPs were tested on a weekly basis during this period.

On a national scale, the SARS-CoV-2 RNA flux measured in WWTPS during the weeks 40 and 41 of 2023 continue to show an increase of the prevalence of the viral RNA in the samples analysed. The national flux of SARS-CoV-2 ranged from  $1.3 \times 10^{12}$  to  $2.0 \times 10^{12}$  RNA copies per day per 100,000 population equivalents. The upward trend observed over the last few weeks continues to progress. The increase in the SARS-CoV-2 fluxes in WWTPs observed over the last few weeks seems to be generalized across the country, even if this progression seems to be slower in the north of the country.

*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

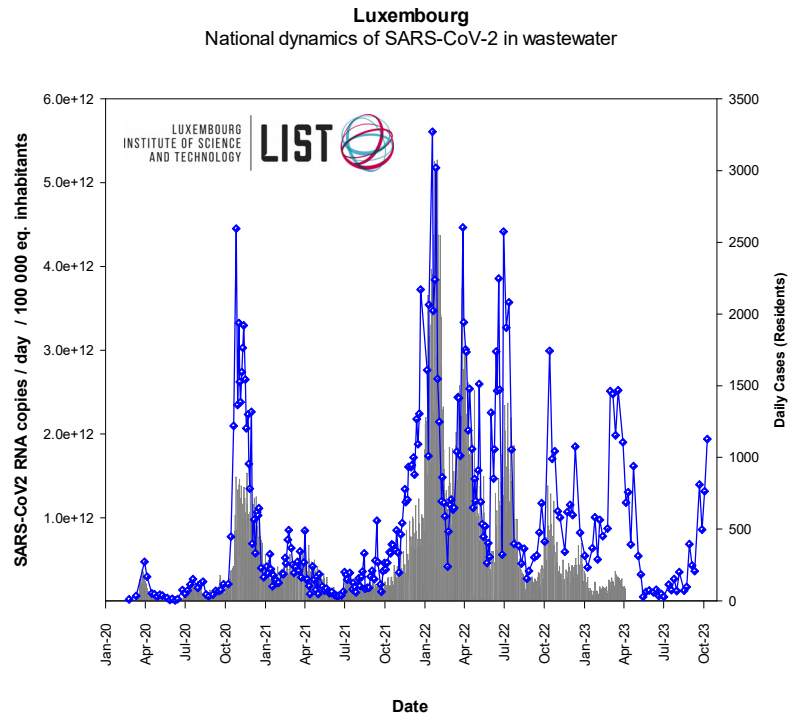
2021		2022	
National SARS-CoV-2 Level	Week	National SARS-CoV-2 Level	Week
Yellow	Week 31-1	Orange	Week 01
Yellow	Week 31-2	Orange	Week 02-1
Yellow	Week 32-1	Orange	Week 02-2
Yellow	Week 32-2	Orange	Week 03-1
Yellow	Week 33-1	Orange	Week 03-2
Yellow	Week 34-1	Orange	Week 04-1
Yellow	Week 34-2	Orange	Week 04-2
Yellow	Week 35-1	Orange	Week 05-1
Yellow	Week 35-2	Orange	Week 05-2
Yellow	Week 36-1	Orange	Week 06-1
Yellow	Week 36-2	Orange	Week 06-2
Yellow	Week 37-1	Orange	Week 07-1
Yellow	Week 37-2	Orange	Week 07-2
Yellow	Week 38-1	Orange	Week 08-1
Yellow	Week 38-2	Orange	Week 08-2
Yellow	Week 39-1	Orange	Week 09-1
Yellow	Week 39-2	Orange	Week 09-2
Yellow	Week 40-1	Orange	Week 10-1
Yellow	Week 40-2	Orange	Week 10-2
Yellow	Week 41-1	Orange	Week 11-1
Yellow	Week 41-2	Orange	Week 11-2
Yellow	Week 42-1	Orange	Week 12-1
Yellow	Week 42-2	Orange	Week 12-2
Yellow	Week 43-1	Orange	Week 13-1
Yellow	Week 43-2	Orange	Week 13-2
Yellow	Week 44-1	Orange	Week 14-1
Yellow	Week 44-2	Orange	Week 14-2
Yellow	Week 45-1	Orange	Week 15-1
Yellow	Week 45-2	Orange	Week 15-2
Yellow	Week 46-1	Orange	Week 16-1
Yellow	Week 46-2	Orange	Week 16-2
Yellow	Week 47-1	Orange	Week 17-1
Yellow	Week 47-2	Orange	Week 17-2
Yellow	Week 48-1	Orange	Week 18-1
Yellow	Week 48-2	Orange	Week 18-2
Yellow	Week 49-1	Orange	Week 19-1
Yellow	Week 49-2	Orange	Week 19-2
Yellow	Week 50-1	Orange	Week 20-1
Yellow	Week 50-2	Orange	Week 20-2
Yellow	Week 51-1	Orange	Week 21-1
Yellow	Week 51-2	Orange	Week 21-2
Yellow	Week 01	Orange	Week 22-1
Yellow	Week 02-1	Orange	Week 22-2
Yellow	Week 02-2	Orange	Week 23-1
Yellow	Week 03-1	Orange	Week 23-2
Yellow	Week 03-2	Orange	Week 24-1
Yellow	Week 04-1	Orange	Week 24-2
Yellow	Week 04-2	Orange	Week 25-1
Yellow	Week 05-1	Orange	Week 25-2
Yellow	Week 05-2	Orange	Week 26-1
Yellow	Week 06-1	Orange	Week 26-2
Yellow	Week 06-2	Orange	Week 27
Yellow	Week 07-1	Orange	Week 28
Yellow	Week 07-2	Orange	Week 29
Yellow		Orange	Week 30
Yellow		Orange	Week 31
Yellow		Orange	Week 32
Yellow		Orange	Week 33
Yellow		Orange	Week 34
Yellow		Orange	Week 35
Yellow		Orange	Week 36
Yellow		Orange	Week 37
Yellow		Orange	Week 38
Yellow		Orange	Week 39
Yellow		Orange	Week 40
Yellow		Orange	Week 41
Yellow		Orange	Week 42
Yellow		Orange	Week 43

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



Figure 1 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (*E* gene) in Luxembourgish wastewater samples from December 2019 to October 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)  $\text{Log}_{10}$  scale

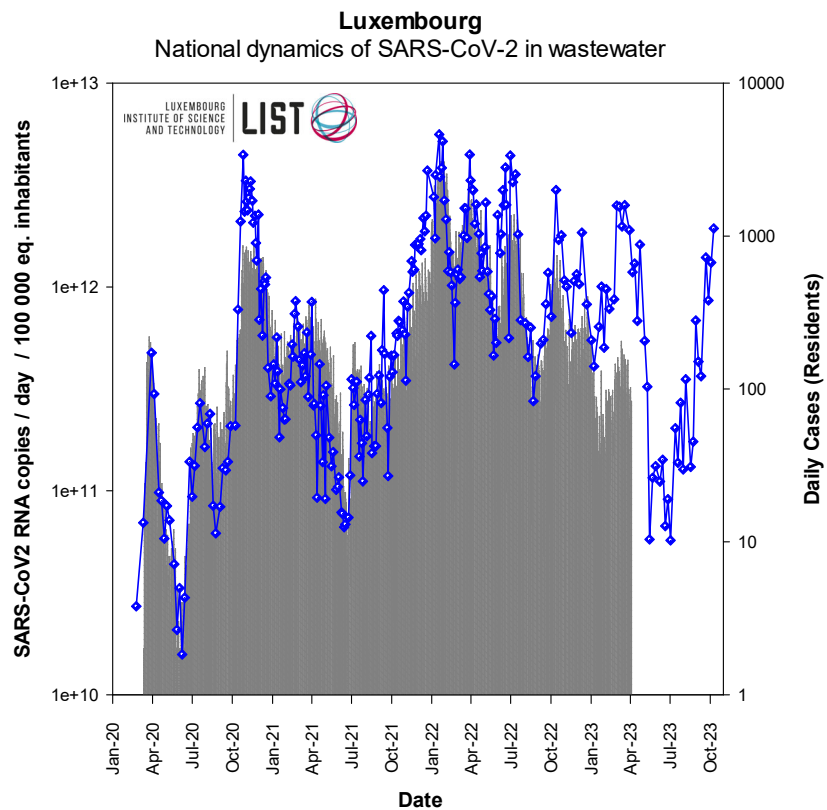




Figure 2a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in the four most impacted wastewater treatment plants from March 2020 to October 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies /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

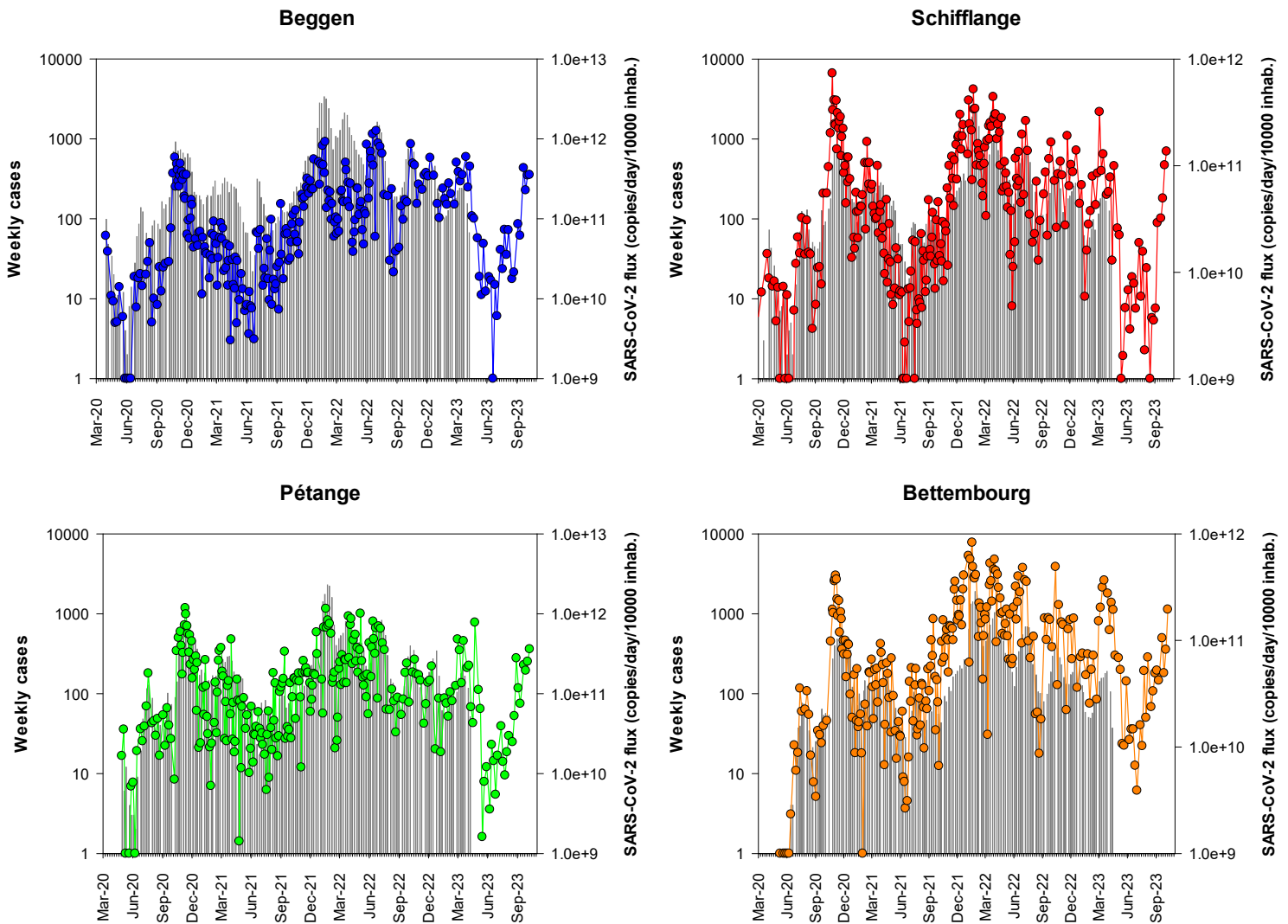


Figure 2b – Close-up of Figure 2a showing results from January 1<sup>st</sup>, 2022 on.

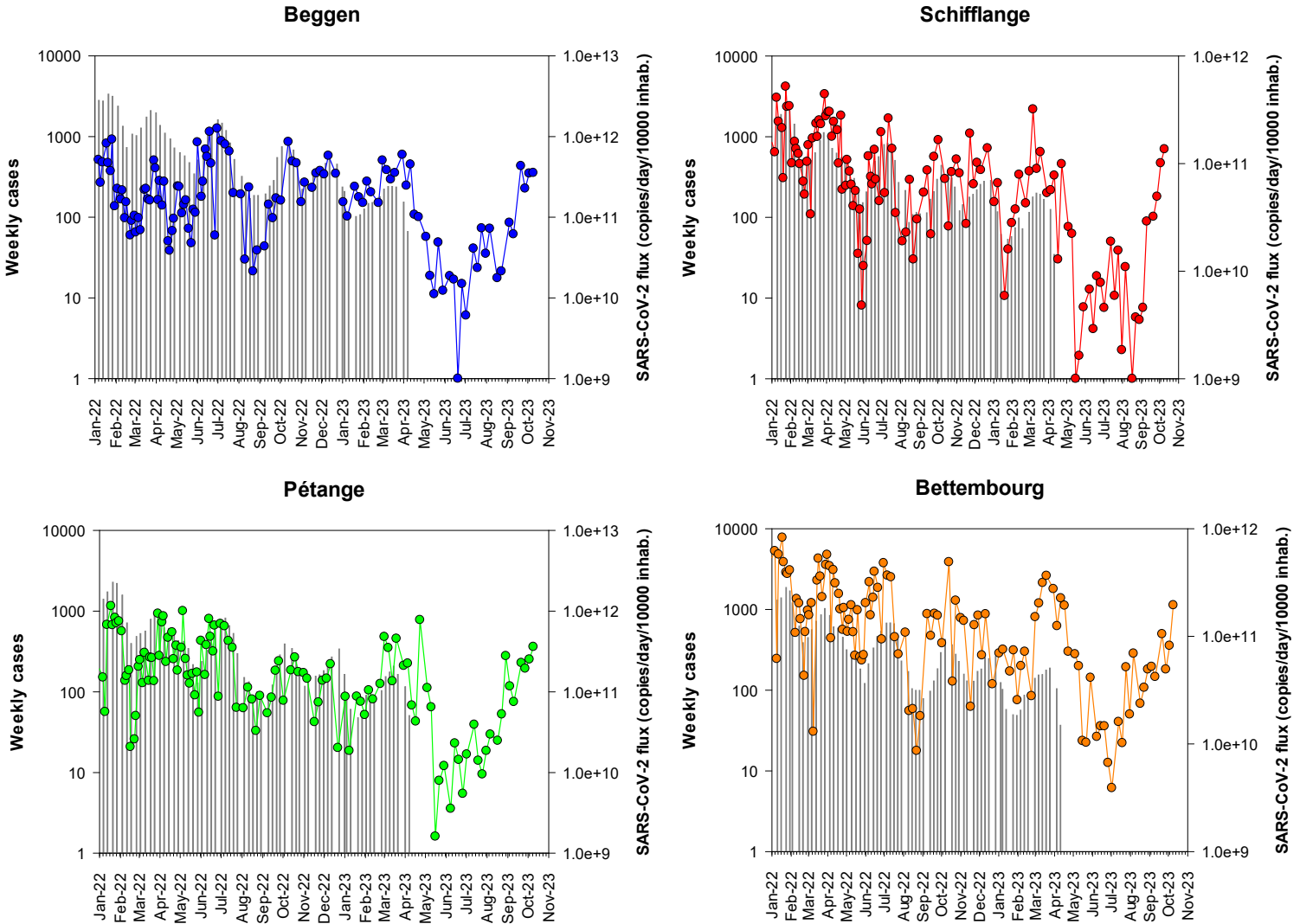


Figure 3a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (*E* gene) in Hesperange, Mersch and Boevange-sur-Attert wastewater treatment plants from May 2020 to October 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2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

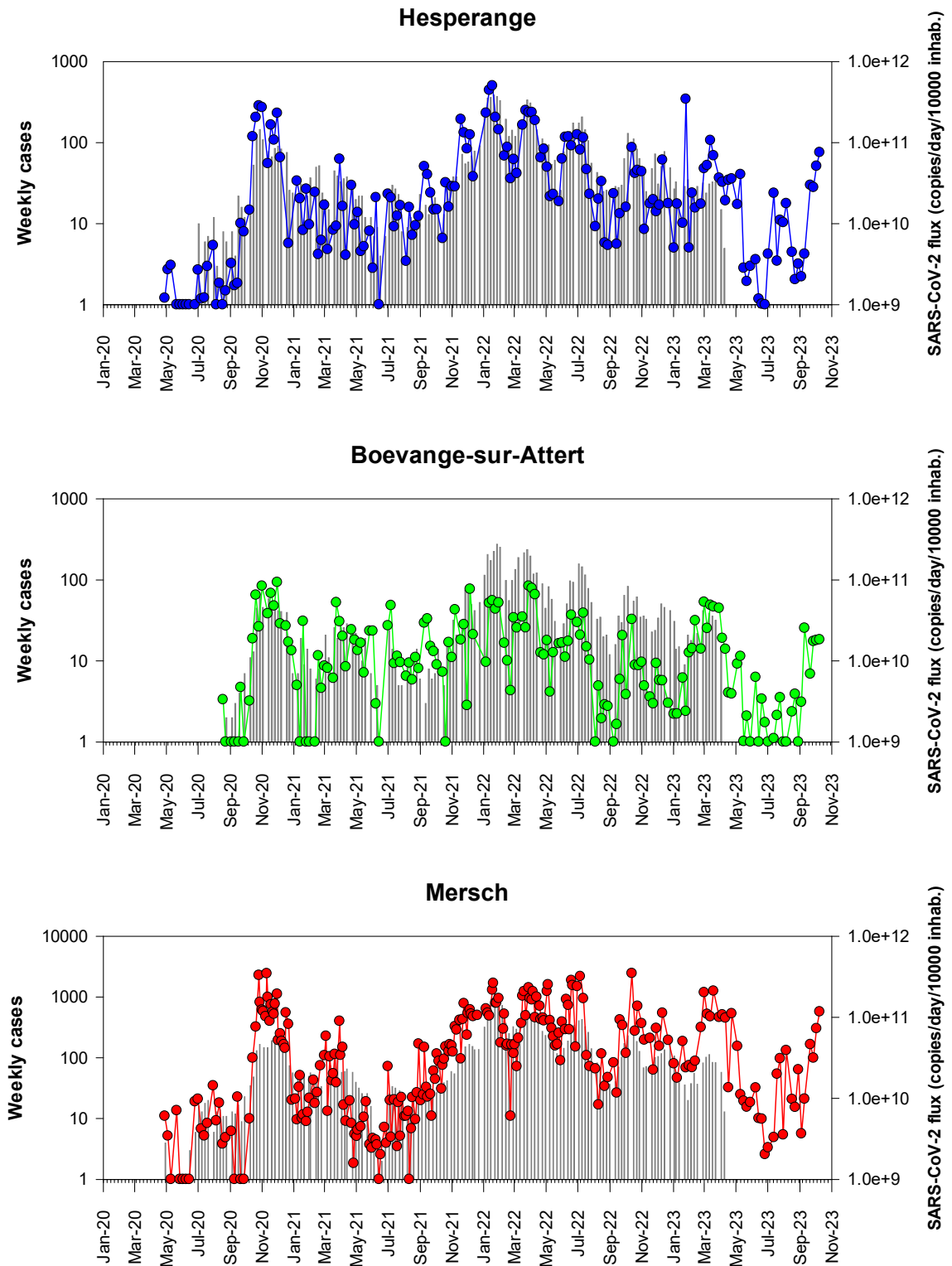




Figure 3b – Close-up of Figure 3a showing results from January 1<sup>st</sup>, 2022 on.

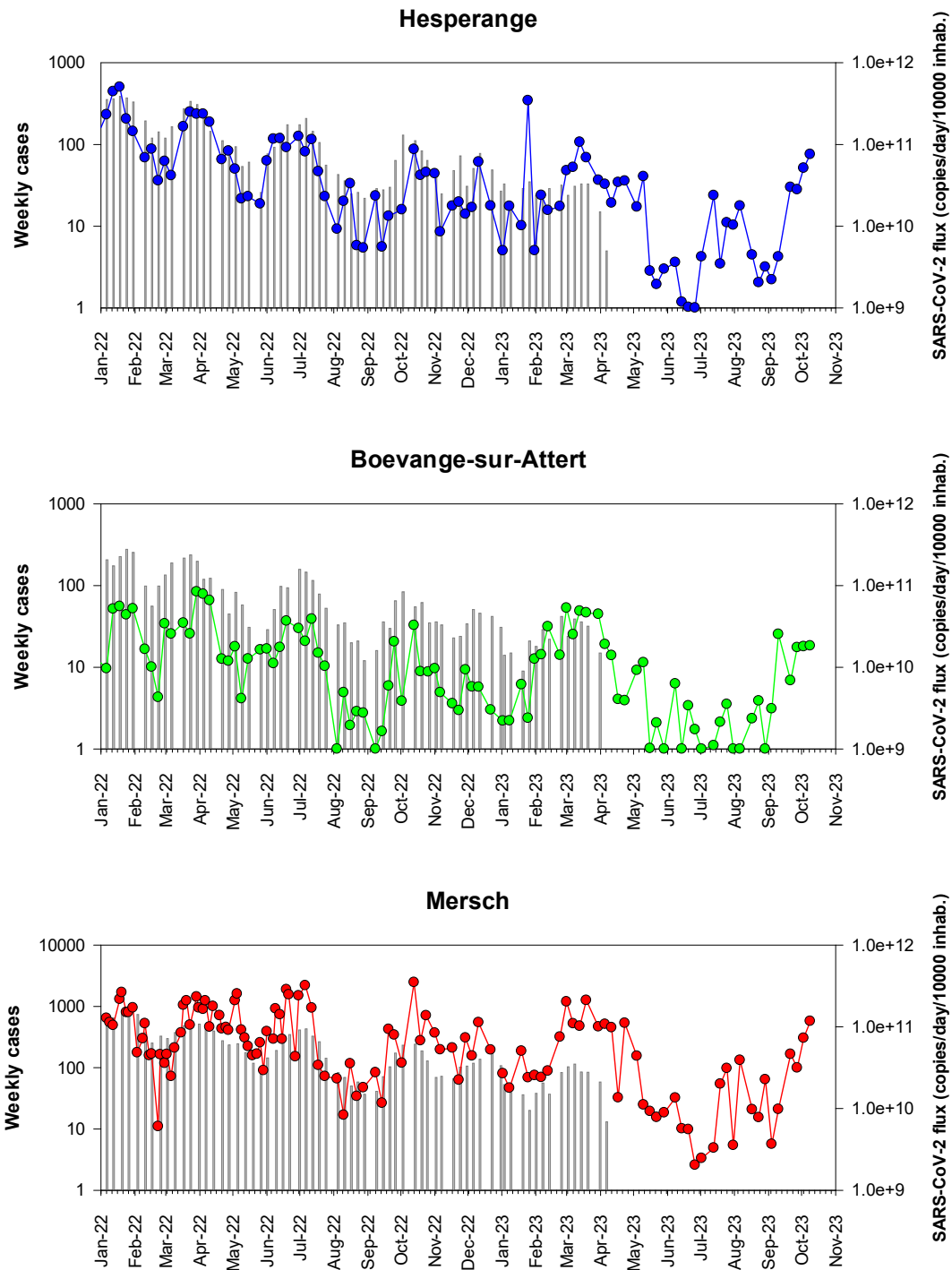


Figure 4a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from May 2020 to October 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent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

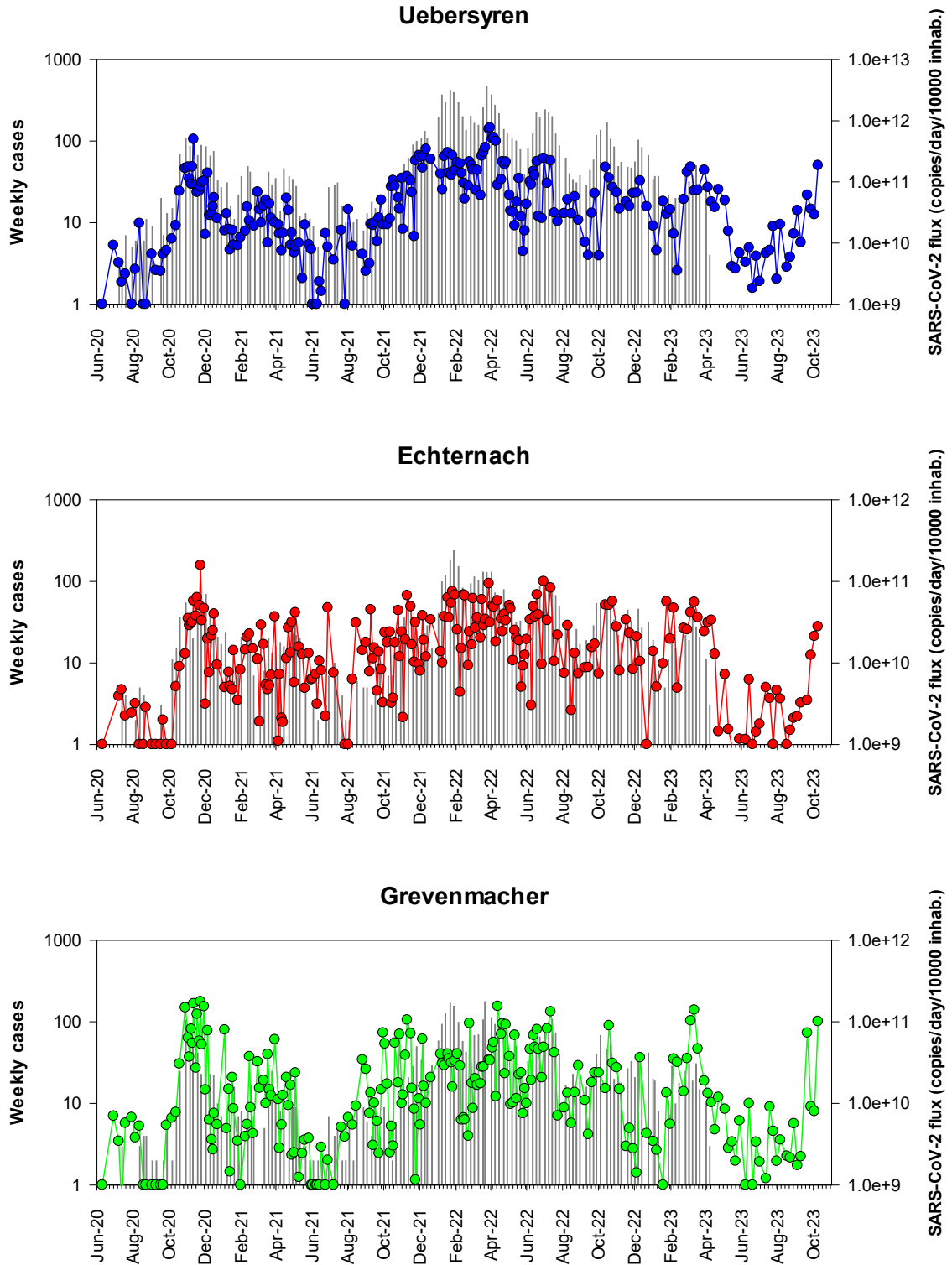


Figure 4b – Close-up of Figure 4a showing results from January 1<sup>st</sup>, 2022 on.

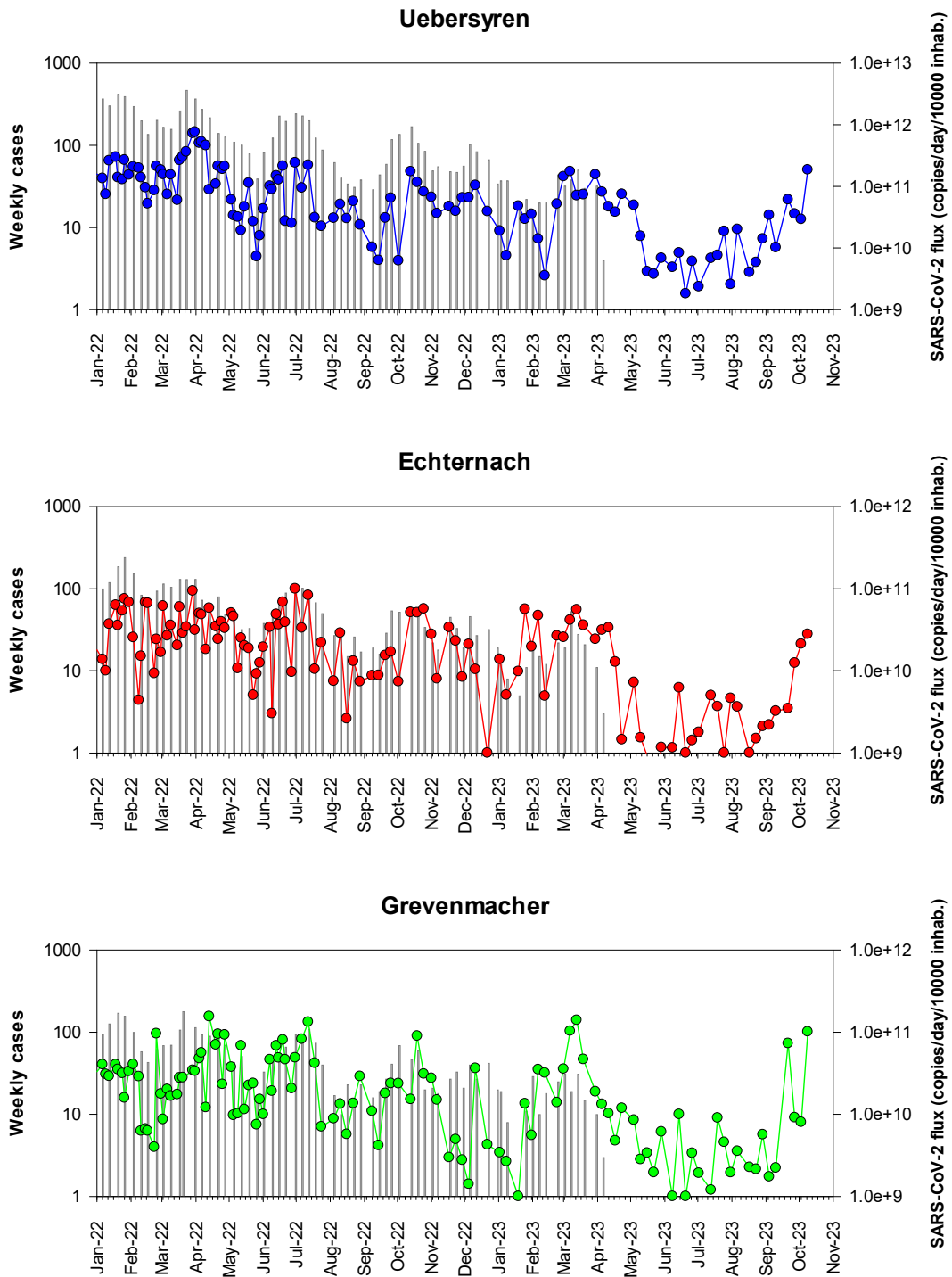


Figure 5a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from May 2020 to October 2023. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent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

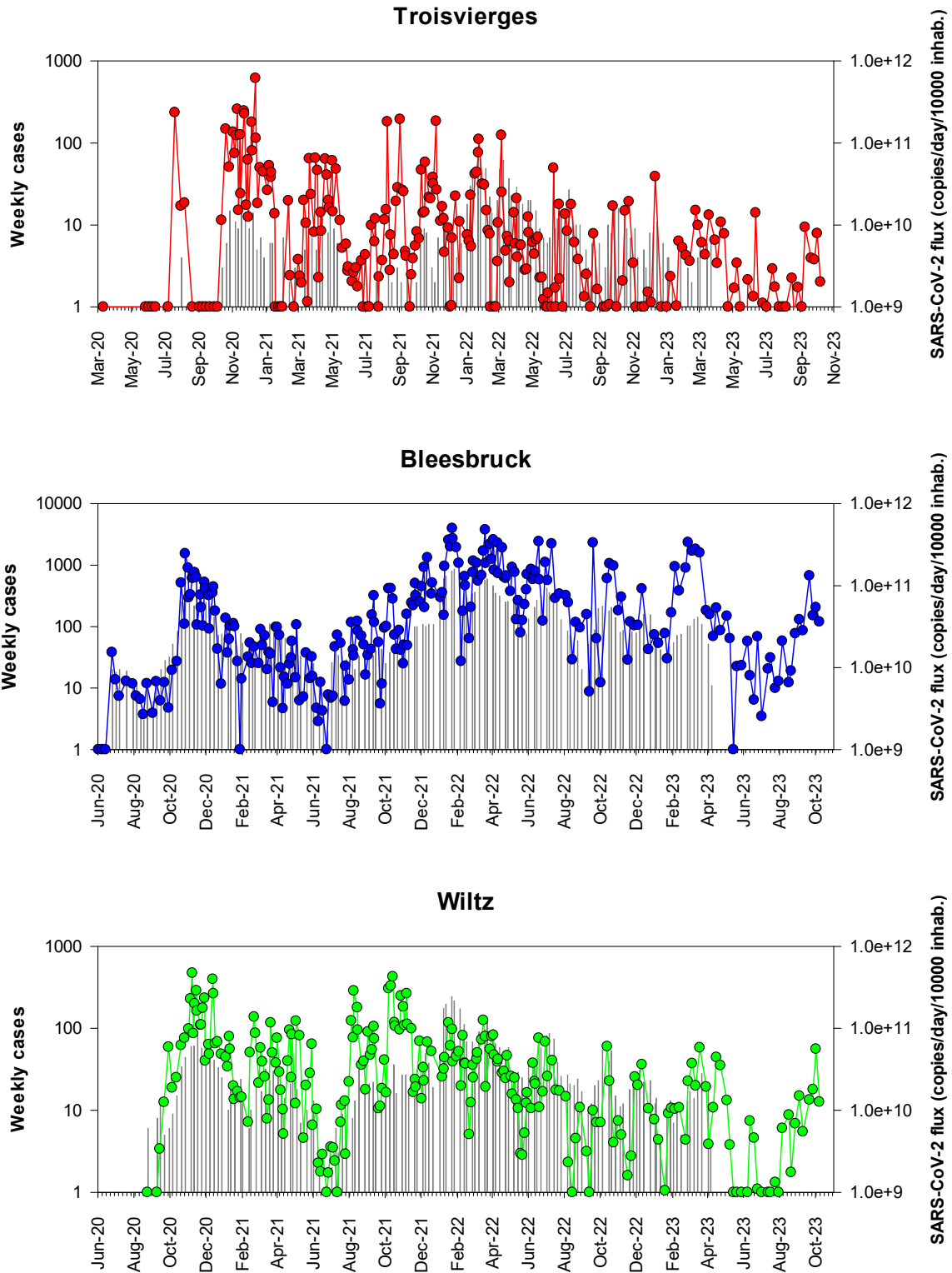


Figure 5b – Close-up of Figure 5a showing results from January 1<sup>st</sup>, 2022 on.

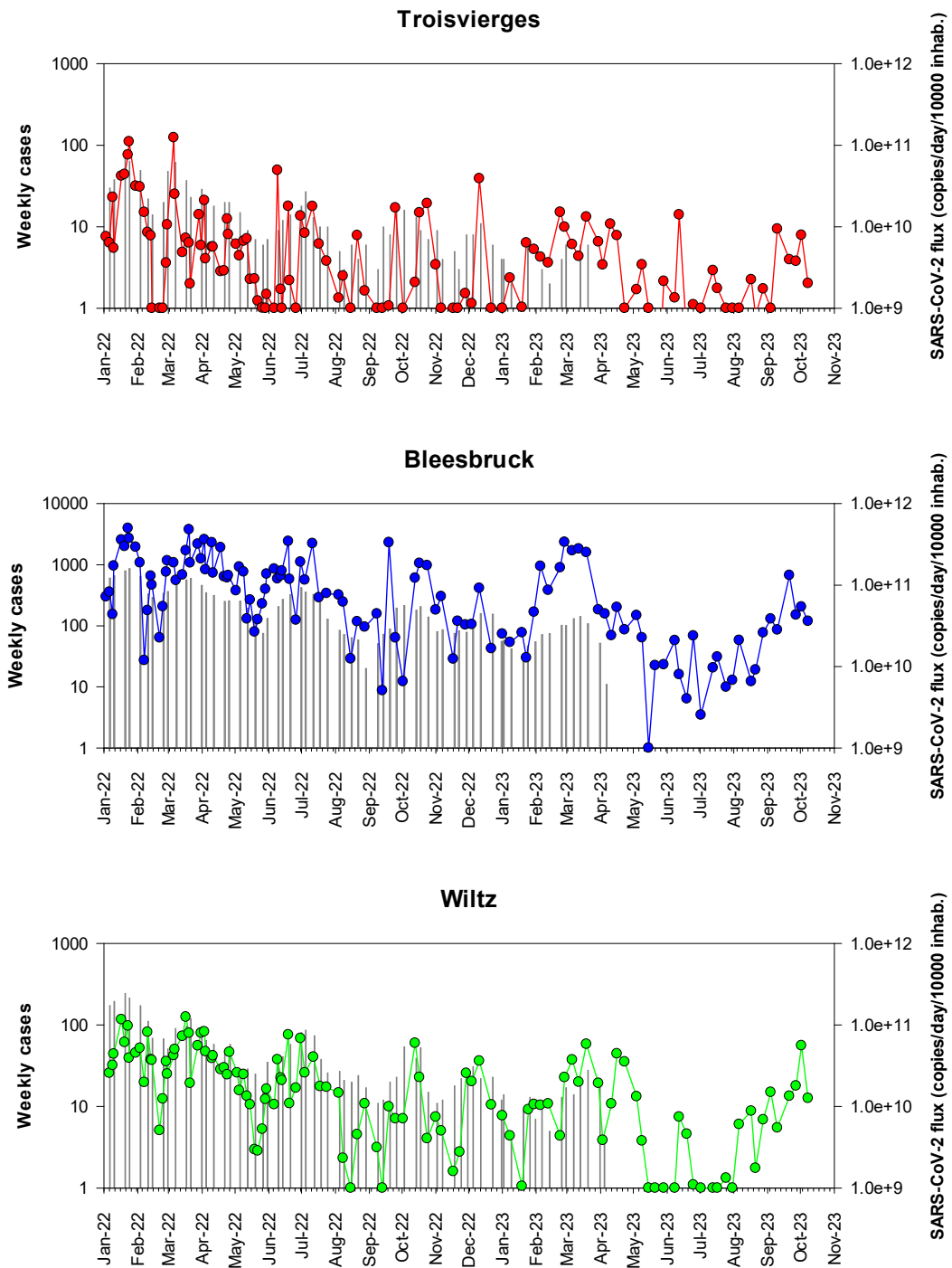


Table 3- Sewage sampling since the beginning of the CORONASTEP study

WWTP	2019	2020	2021	2022	2023	Total
Beggen		52	92	77	40	261
Bettembourg		45	91	75	40	251
Schifflange	4	56	96	76	41	273
Bleesbrück		44	92	77	41	254
Mersch		47	91	77	41	256
Pétange	4	51	96	77	41	269
Hesperange		35	48	50	41	174
Echternach		36	79	77	41	233
Uebersyren		38	78	77	41	234
Grevenmacher		38	81	76	41	236
Troisvierges		38	92	77	41	248
Boevange sur Attert		20	48	51	41	160
Wiltz		28	92	77	41	238
<b>Total</b>	<b>8</b>	<b>528</b>	<b>1076</b>	<b>944</b>	<b>531</b>	<b>3087</b>

## Materials and Methods

### Sewage samples

From March 2020 to October 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<sup>1</sup>. 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.

<sup>1</sup> <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).

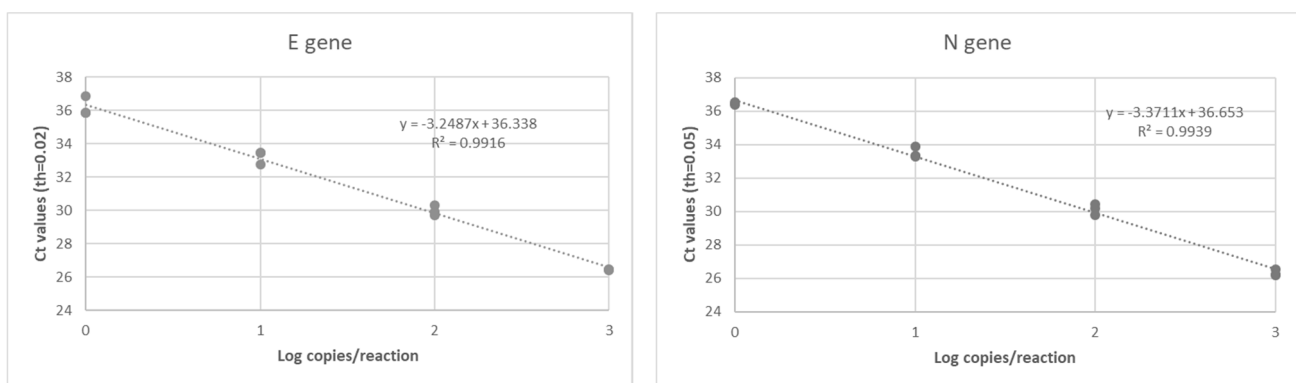


Figure 6 – RT-qPCR standard curves established for both target genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

## Data interpretation

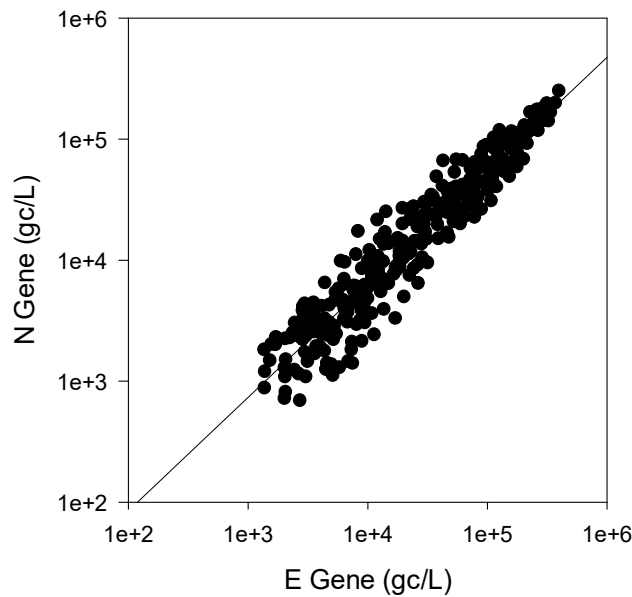
A sample is declared positive for the presence of SARS-CoV-2 if both targets (E and N gene) are detected with Ct values less than or equal to the LOQ. If only one target is detected or if target genes are detected with Ct values between the LOD and the LOQ, samples are reported as presumptive positive (+/-). 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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