CORONASTEP Report 194
(2024 – Weeks 41 and 42)
SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

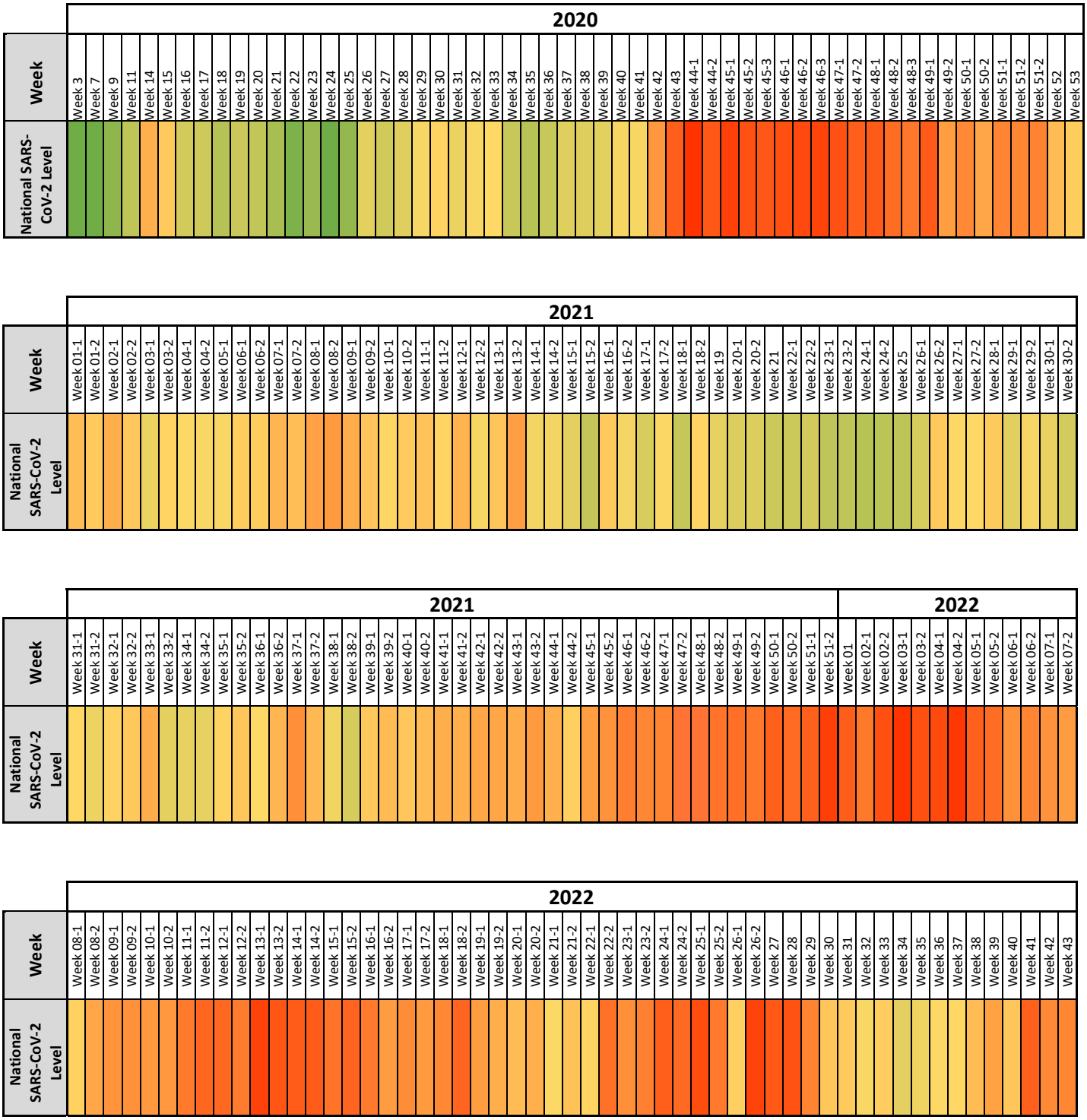
This report 194 presents the results of SARS-CoV-2 contamination of wastewater at the inlet of the 8 wastewater treatment plants (WWTPs) analysed during weeks 41 and 42 of 2024. The SARS-CoV-2 fluxes measured over the past two weeks appear to have stabilised at the high levels observed since the week 38, both nationally and regionally. National levels of SARS-CoV-2 measured ranged from approximately 2.1 to 2.7 x 10^{12} RNA copies per day per 100,000 population equivalents.

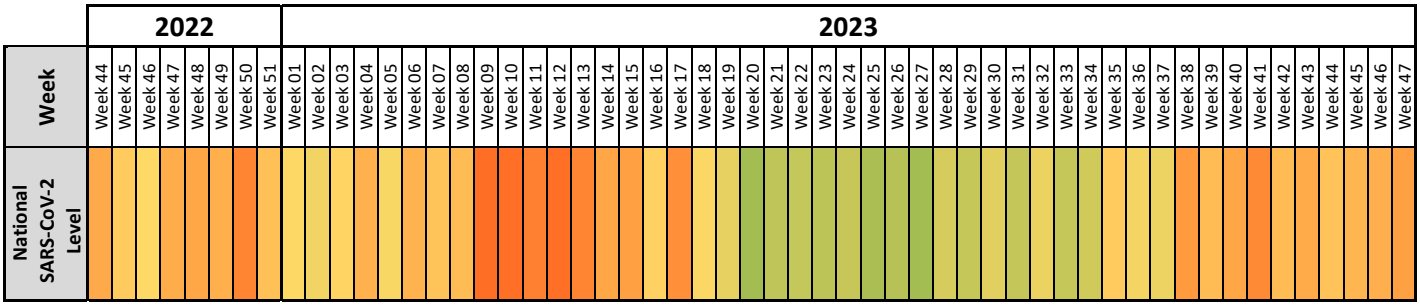
Remarks:

1. *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.*
2. *After a detailed analysis of the data collected to date, we demonstrated that it was possible to reduce the number of wastewater treatment plants (WWTPs) monitored without affecting the relevance and representativeness of the national data. The 8 WWTPs selected are as follows: Beggen, Schifflange, Bettembourg, Pétange, Mersch, Echternach, Ubersyren and Bleesbruck. From January 2024, only the results of these eight analyses will be presented in the fortnightly reports.*
3. ***As mentioned in the Materials and Methods (page 11), the protocol has changed and the report has been adapted. In Table 2, the values are no longer comparable to those obtained with the old protocol, as the new protocol has a higher concentration factor than the previous one. Table 2 has therefore been removed from the report.***

Table 1 – National level of SARS-CoV-2 contamination of wastewater 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).




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
Week 04
Week 05
Week 06
Week 07
Week 08
Week 09
Week 10
Week 11
Week 12
Week 13
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
Week 45
Week 46
Week 47
National SARS-CoV-2 Level

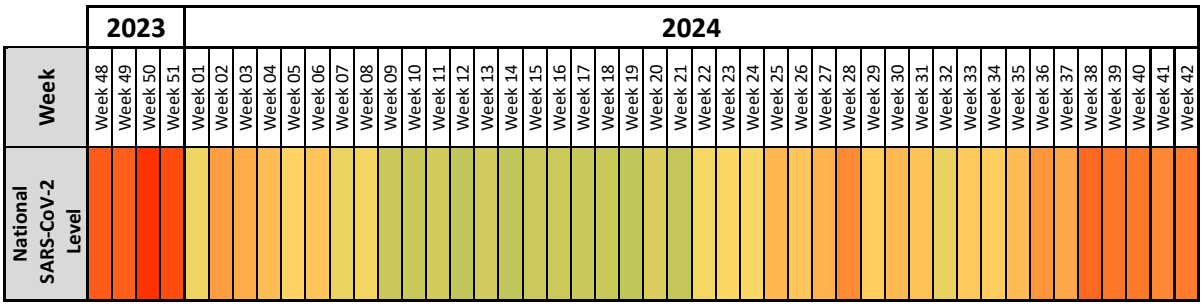
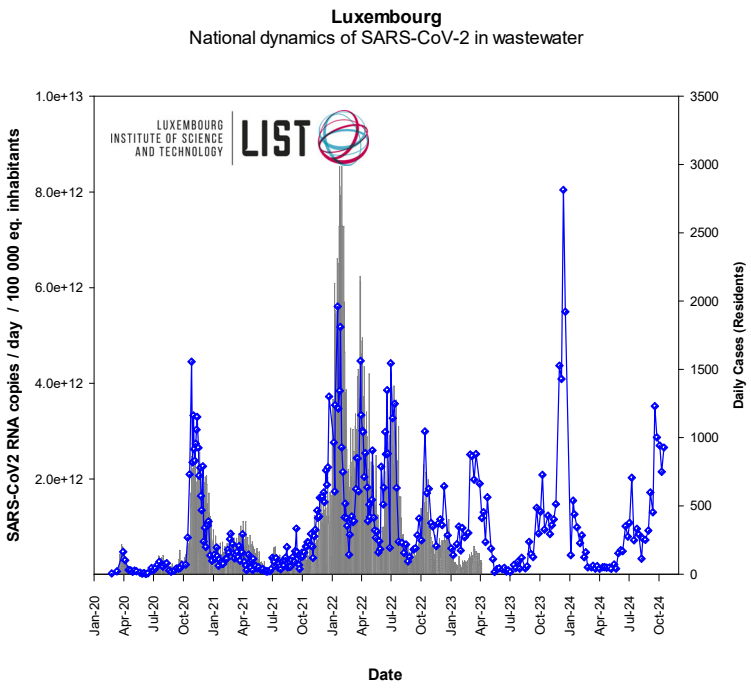

2023
2024
Week
Week 48
Week 49
Week 50
Week 51
Week 01
Week 02
Week 03
Week 04
Week 05
Week 06
Week 07
Week 08
Week 09
Week 10
Week 11
Week 12
Week 13
Week 14
Week 15
Week 16
Week 17
Week 18
Week 19
Week 20
Week 21
Week 22
Week 23
Week 24
Week 25
Week 26
Week 27
Week 28
Week 29
Week 30
Week 31
Week 32
Week 33
Week 34
Week 35
Week 36
Week 37
Week 38
Week 39
Week 40
Week 41
Week 42
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 October 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*

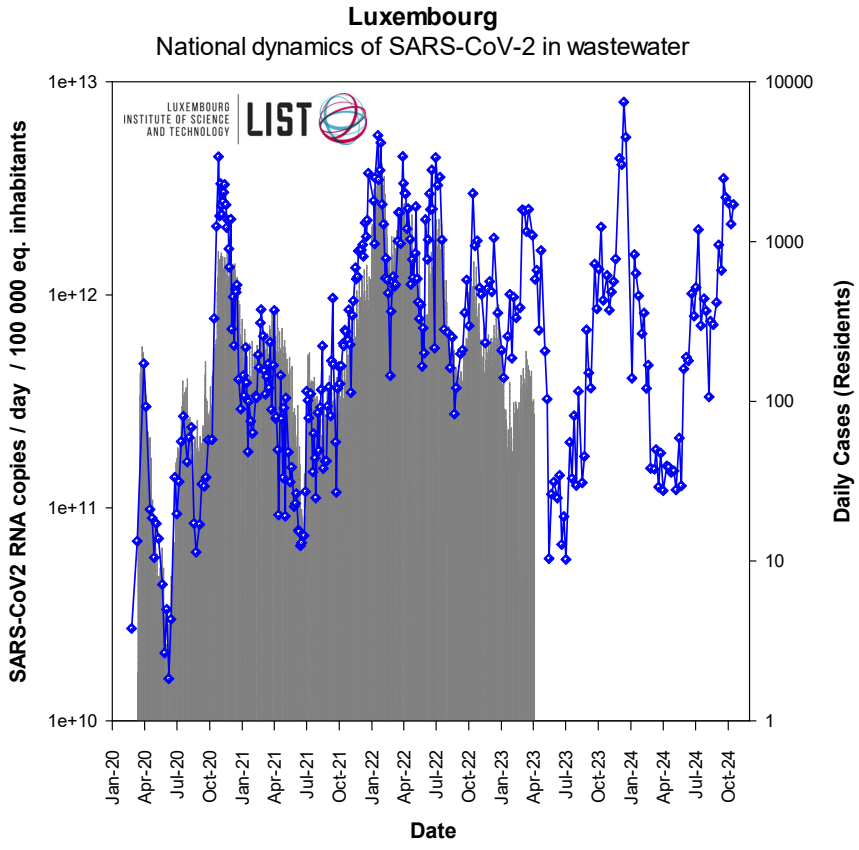
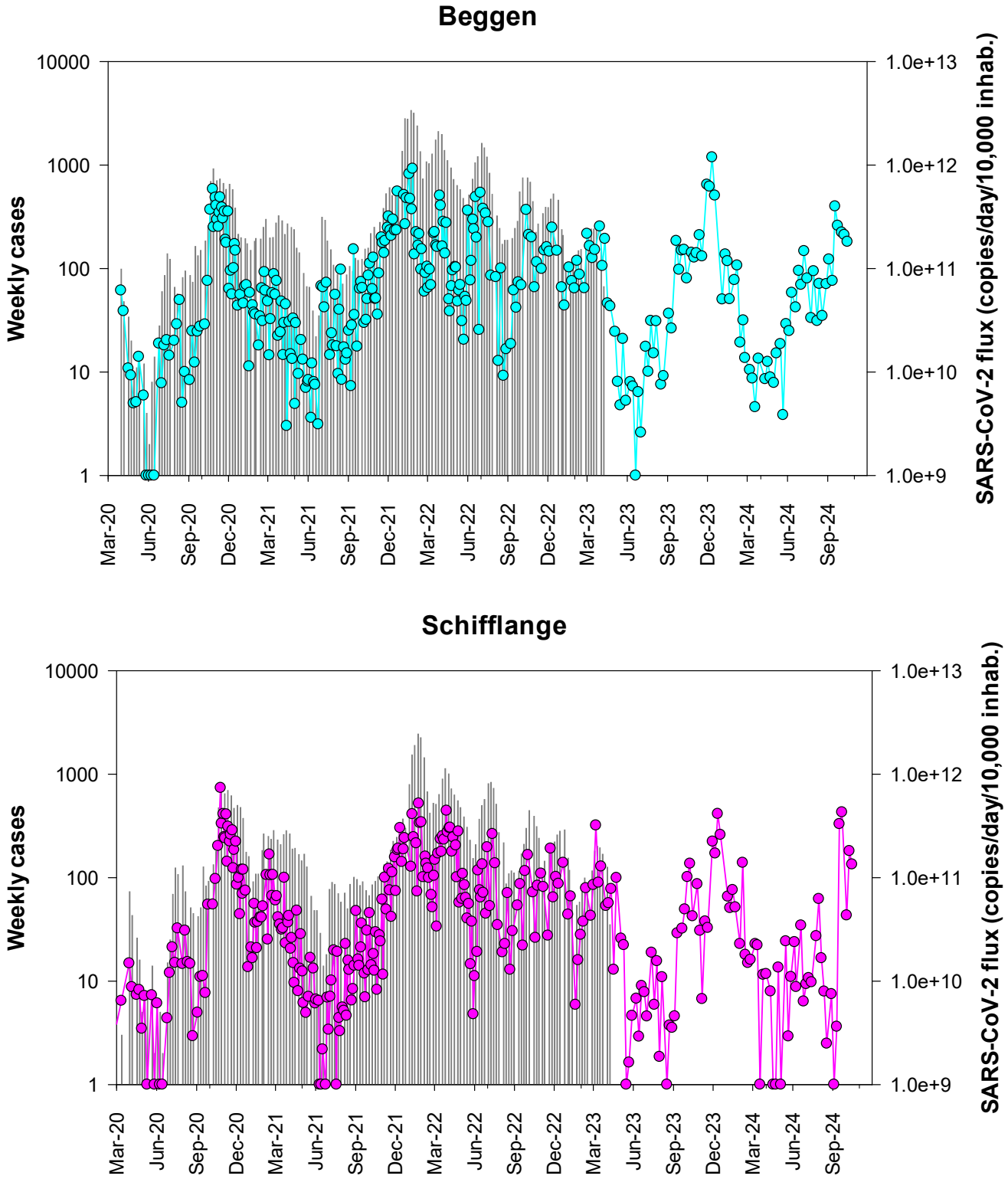
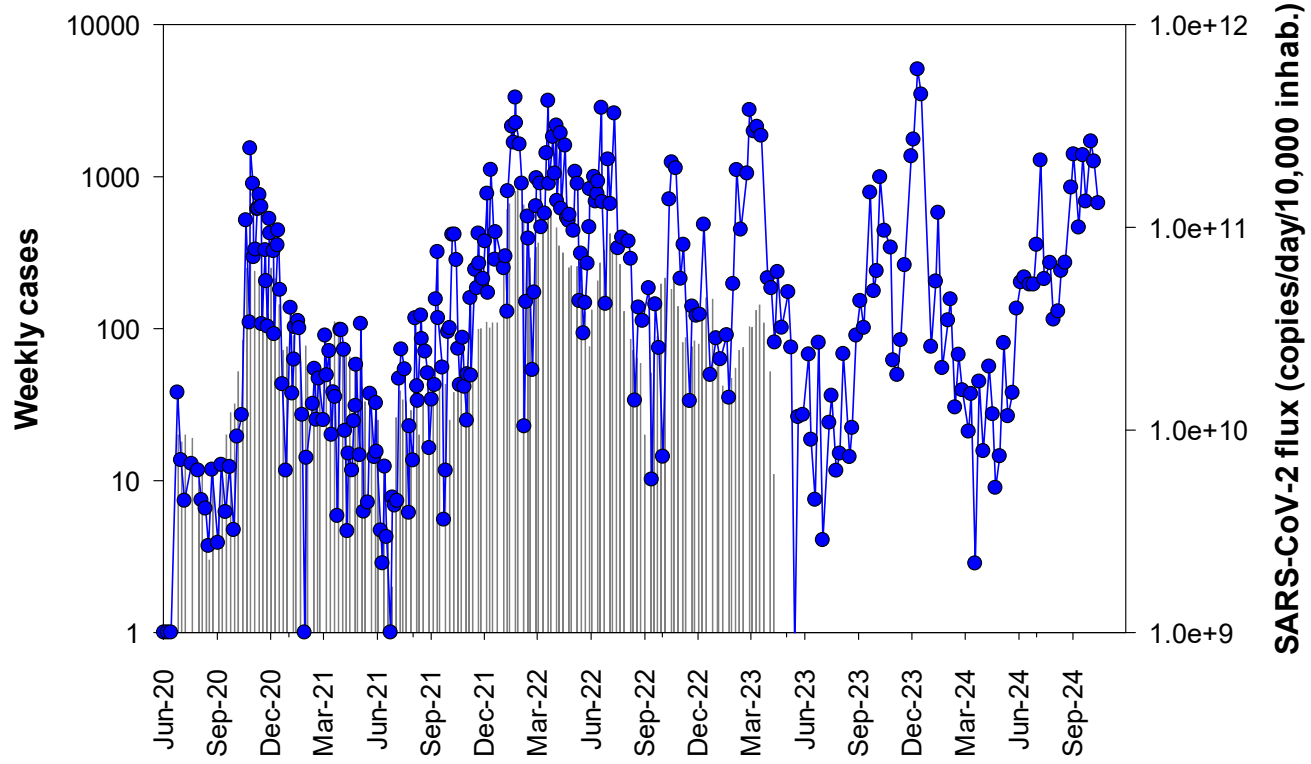


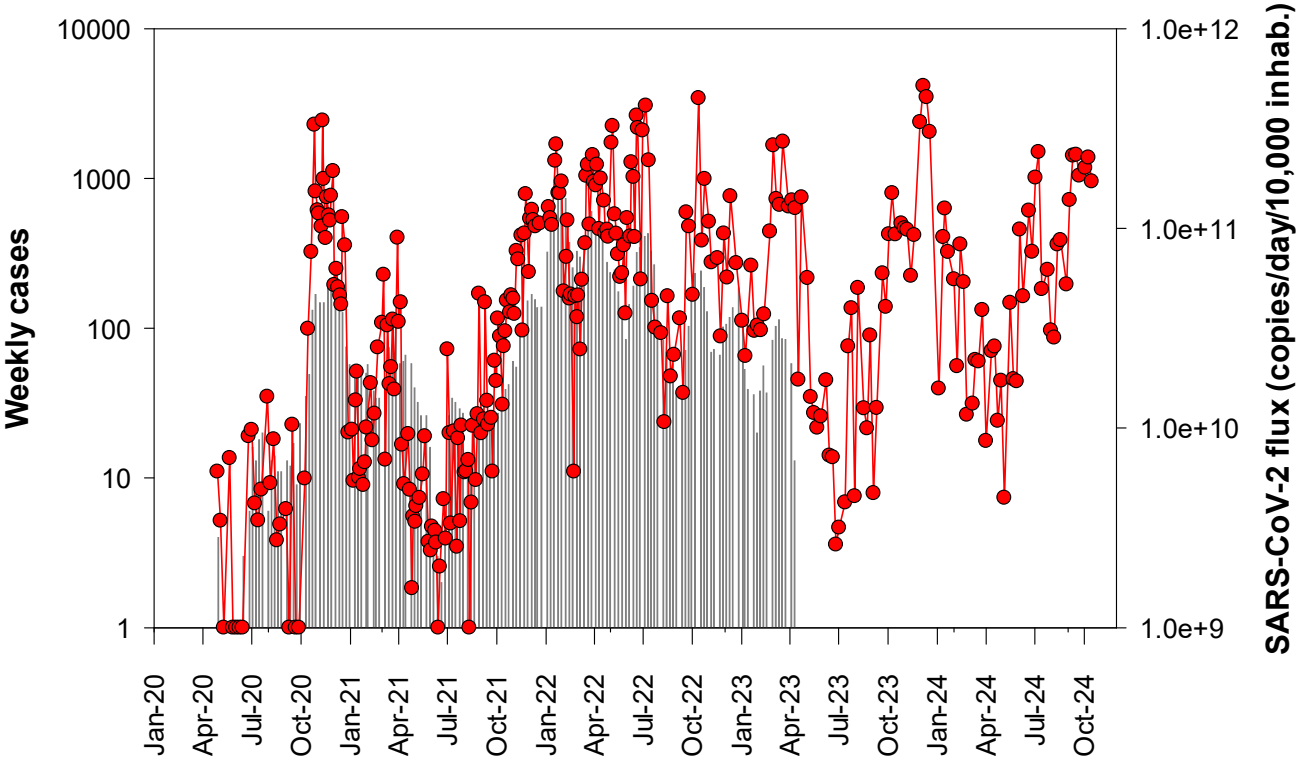
Figure 2 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in the eight selected wastewater treatment plants from March 2020 to October 2024. Grey squares: daily confirmed cases for the contributory area of each wastewater treatment plant, coloured dots: SARS-CoV-2 flux (RNA copies/day/10,000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

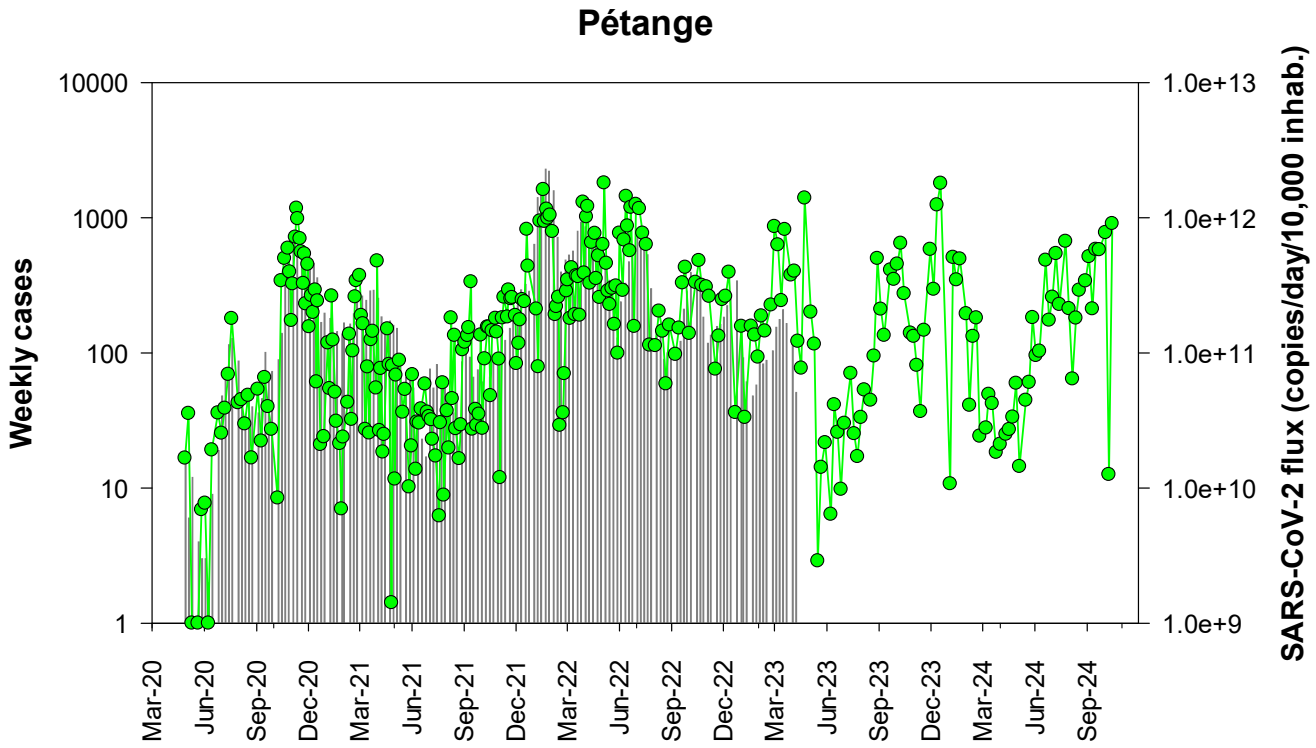


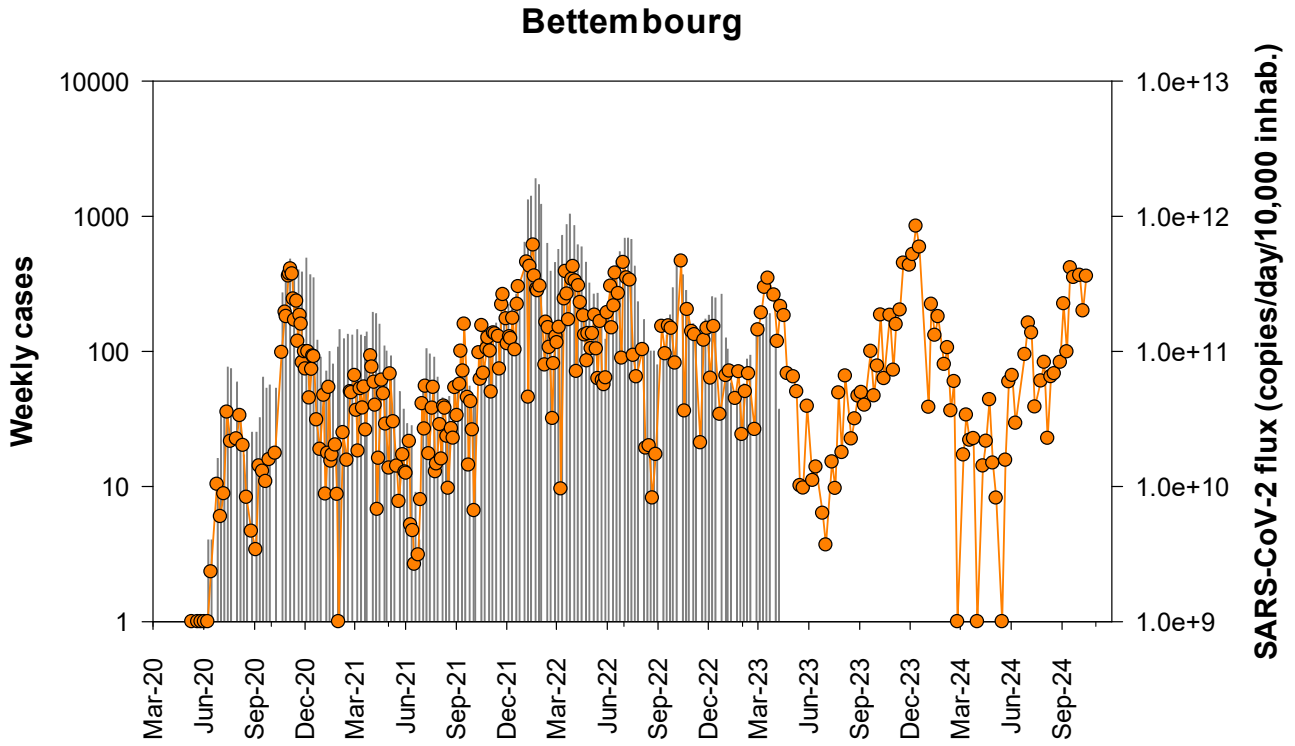
Bleesbruck



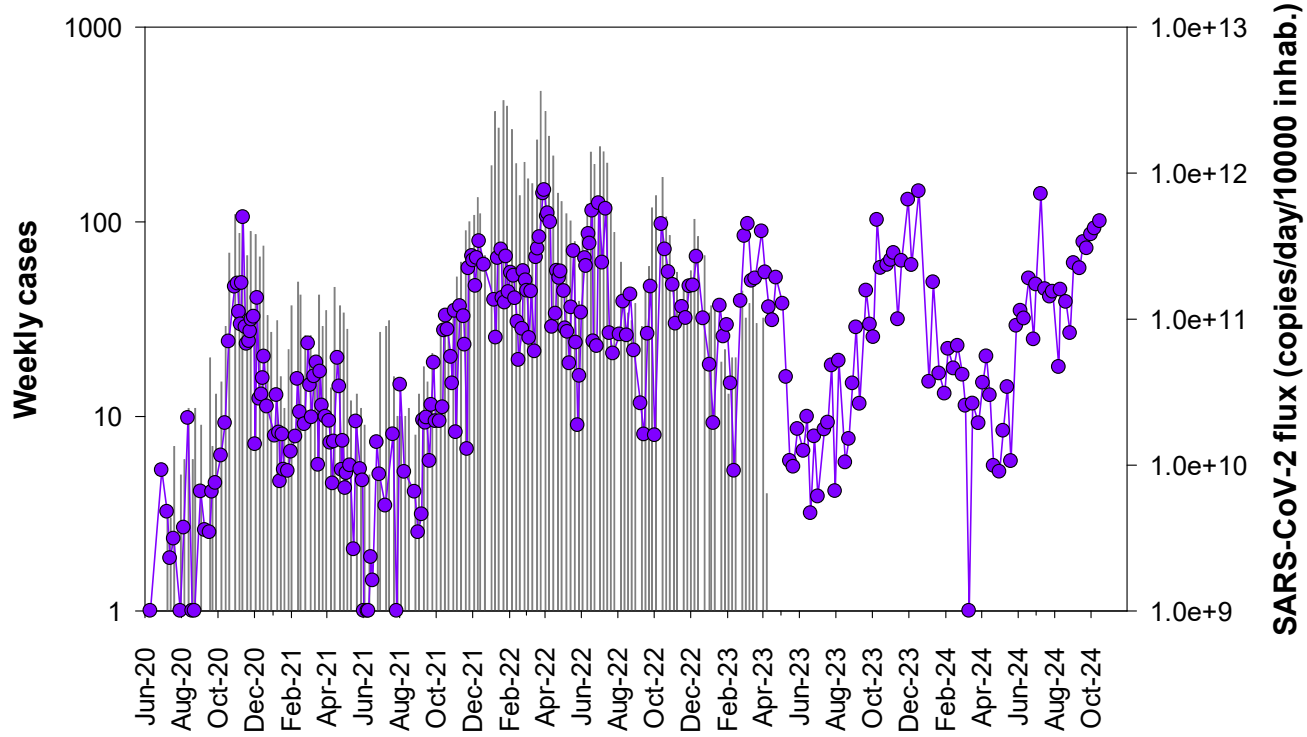
Mersch




Pétange
Weekly cases
SARS-CoV-2 flux (copies/day/10,000 inhab.)
10000
1000
100
10
1
1.0e+13
1.0e+12
1.0e+11
1.0e+10
1.0e+9
Mar-20
Jun-20
Sep-20
Dec-20
Mar-21
Jun-21
Sep-21
Dec-21
Mar-22
Jun-22
Sep-22
Dec-22
Mar-23
Jun-23
Sep-23
Dec-23
Mar-24
Jun-24
Sep-24


Bettembourg
Weekly cases
SARS-CoV-2 flux (copies/day/10,000 inhab.)
10000
1000
100
10
1
1.0e+13
1.0e+12
1.0e+11
1.0e+10
1.0e+9
Mar-20
Jun-20
Sep-20
Dec-20
Mar-21
Jun-21
Sep-21
Dec-21
Mar-22
Jun-22
Sep-22
Dec-22
Mar-23
Jun-23
Sep-23
Dec-23
Mar-24
Jun-24
Sep-24

Uebersyren



Echternach

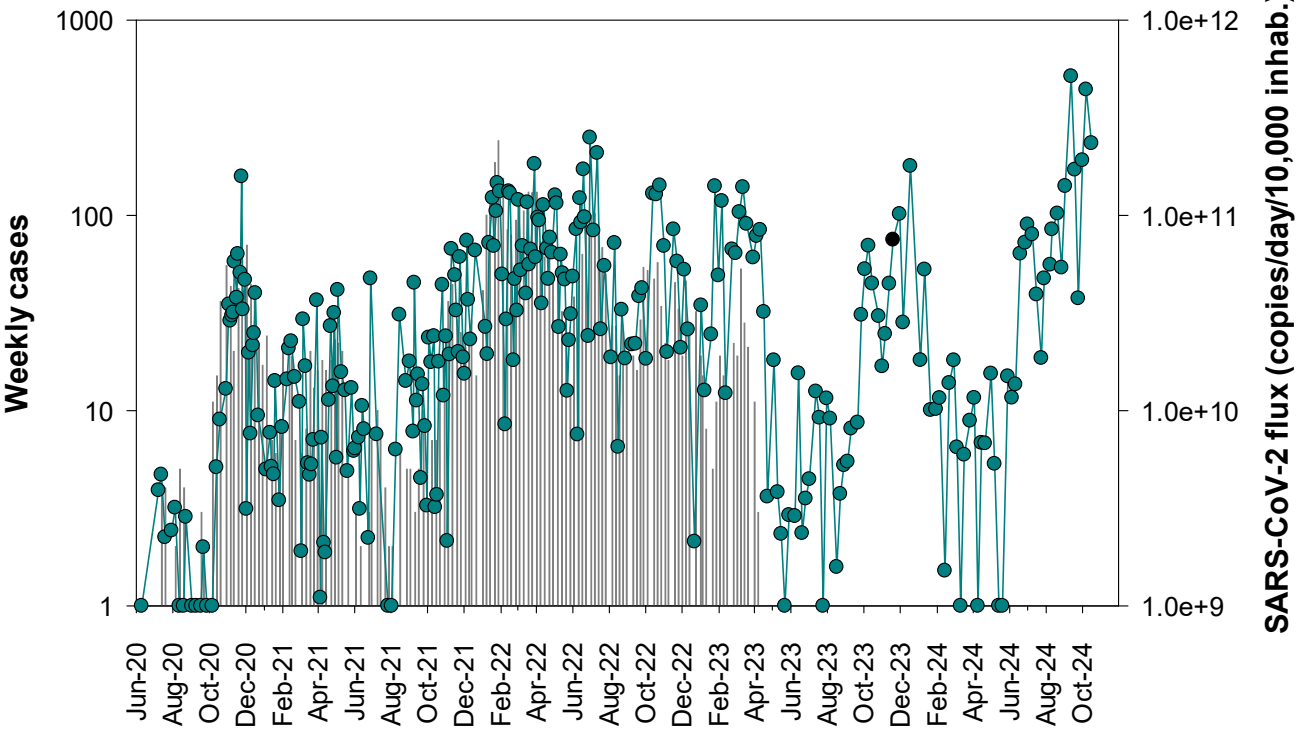


Table 2- Sewage sampling since the beginning of the CORONASTEP study

WWTP	2019	2020	2021	2022	2023	2024	Total
Beggen		52	92	77	50	41	**312**
Bettembourg		45	91	75	50	42	**303**
Schifflange	4	56	96	76	51	42	**325**
Bleesbrück		44	92	77	51	42	**306**
Mersch		47	91	77	51	42	**308**
Pétange	4	51	96	77	51	42	**321**
Hesperange		35	48	50	51	3	**187**
Echternach		36	79	77	50	41	**288**
Uebersyren		38	78	77	50	41	**284**
Grevenmacher		38	81	76	51	2	**248**
Troisvierges		38	92	77	51	3	**261**
Boevange sur Attert		20	48	51	51	3	**173**
Wiltz		28	92	77	51	3	**251**
Total	**8**	**528**	**1076**	**944**	**659**	**347**	**3562**

Materials and Methods

Sewage samples

From March 2020 to October 2024, up to thirteen wastewater treatment plants (WWTPs) were sampled at their inlet according to the planning presented in Table 3. Since January 2024, the number of WWTPs followed was reduced to eight. 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 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.

The concentration step and RNA extraction protocols were replaced by the PROMEGA - Maxwell® RSC Enviro Total Nucleic Acid Kit from the 22nd August 2024 campaign onwards. In summary, 45 mL of samples were treated with a protease solution for 30 minutes at room temperature. Larger particles were removed from the treated sample by centrifugation at 3,000 x g for 10 min at 4°C. Then 40 mL of the samples were concentrated in two steps, the first by column passage and the second by magnetic beads. The final elution of nucleic acids is performed in 80 µL DNase/RNase-free water.

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 COVID-19 pandemic, it can be assumed that only SARS-CoV-2 strains will be detected by this assay given that the 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 3 – 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

[1] https://www.cdc.gov/coronavirus/2019-ncov/downloads/rt-pcr-panel-primer-probes.pdf

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.

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 copy 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).

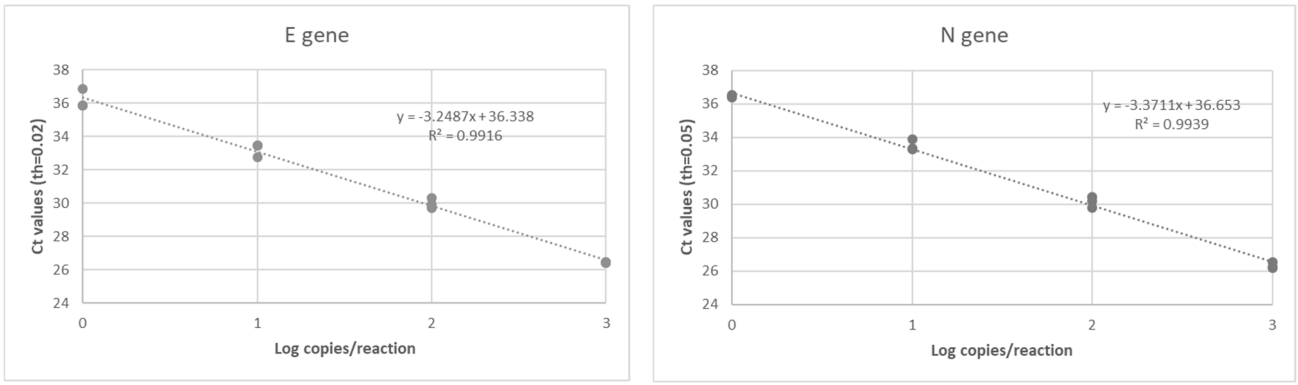


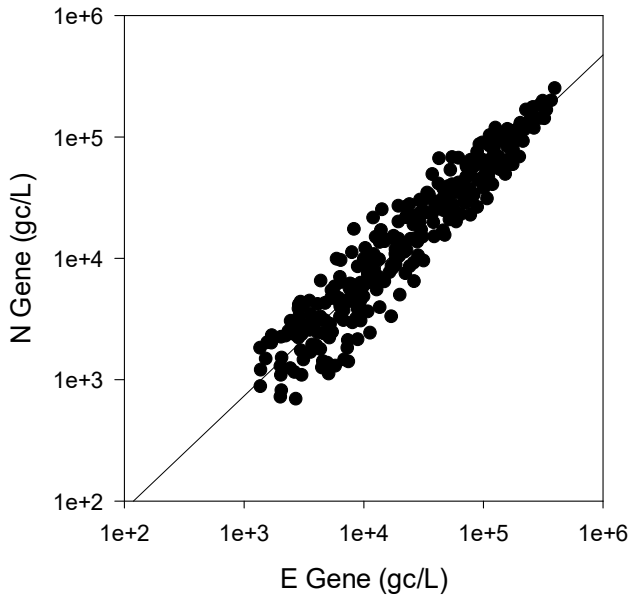
Figure 6 – RT-qPCR standard curves established for both target genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

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, the 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),



Acknowledgements

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, SIVEC, 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 scales.