Preliminary CORONASTEP Report 17
SARS-CoV-2 Sewage Surveillance in Luxembourg
(partial results)

Summary

On October 29th, samples from 7 wastewater treatment plants (WWTP) out of 13 have been received by LIST and analysed, among which the 3 mostly impacted WWTPs (Schifflange, Beggen, Bettembourg) . For the sake of clarity, only the WWTPs with new data available today are shown in the present report.

This restricted dataset does not allow to produce a reliable graph summarising the national prevalence of SARS-CoV-2 RNA in the wastewater. On October 30th, the rest of the WWTPs will be sampled and analysed and a complete report including the “National prevalence” graph will be delivered afterwards.

The current report shows that the flux of SARS-CoV-2 RNA in the wastewater treatment plants are

- **Decreasing in some WWTPs (Beggen, Schifflange, Mersch, Troisvierges)**
- **Still increasing in others (Bettembourg, Wiltz)**
- **Showing a sawtooth dynamics (Bleesbruck)**

These results show that there is no consistent trend in the contamination level and that it can vary significantly on a very short term when in the range of high values. The contamination level is still high, in general (except for Beggen) above the values of week 42 and in all stations largely above the values of the first wave. The overall trend is, however, downward rather than upward.

Table 1 - Level of SARS-CoV-2 contamination of each analysed wastewater treatment plant in Luxembourg. BEG: Beggen, BET: Bettembourg, SCH: Schifflange, BLE: Bleesbruck, 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)

	2020																								
							2nd wave																		
WWTP	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
BEG																									
BET																									
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TRO																									
BOE																									No data
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Figure 1 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in the three most impacted wastewater treatment plants from March to October 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant (only available until week 42 included), dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

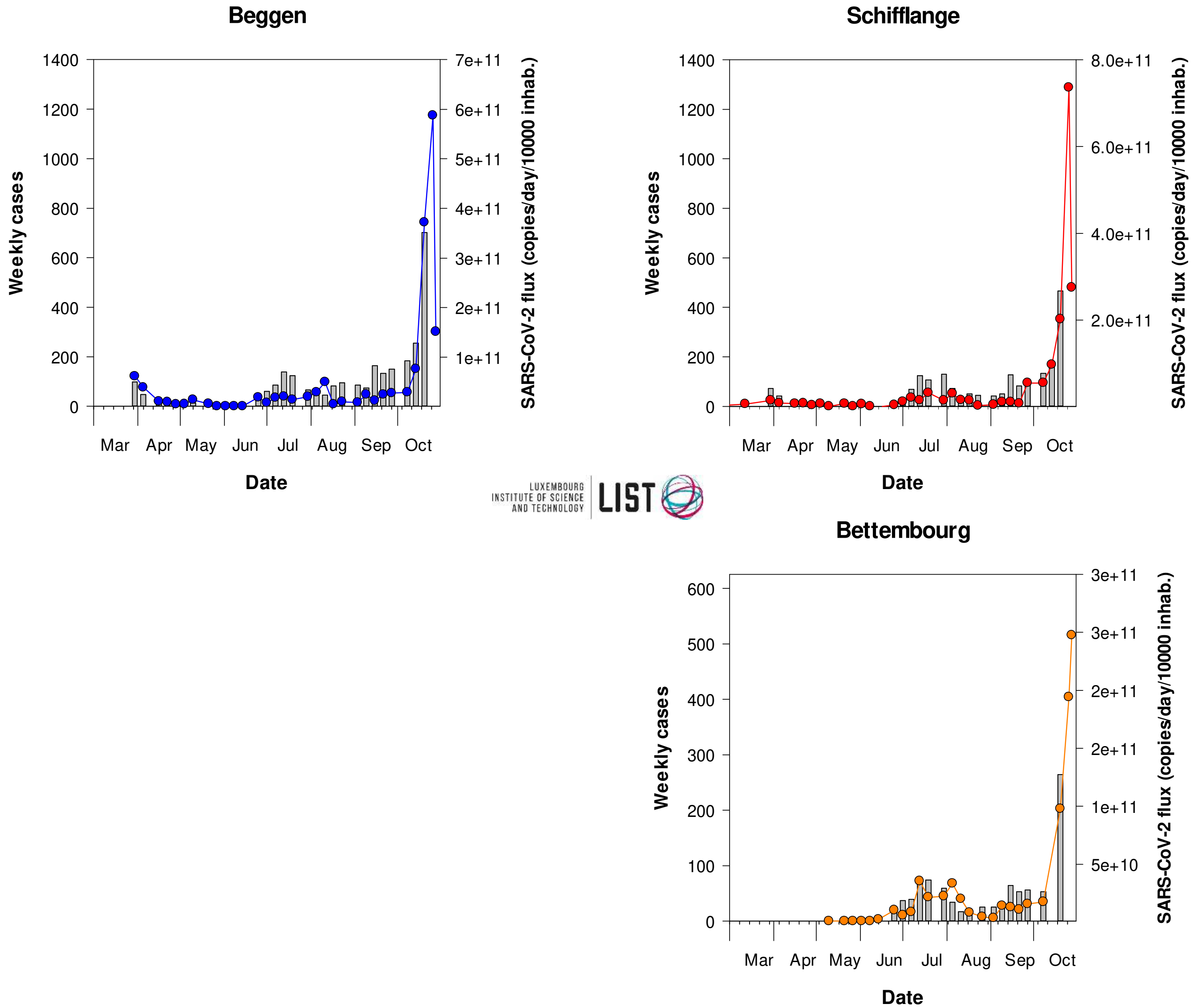


Figure 2 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Mersch wastewater treatment plants from March to October 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant (only available until week 42 included), dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).

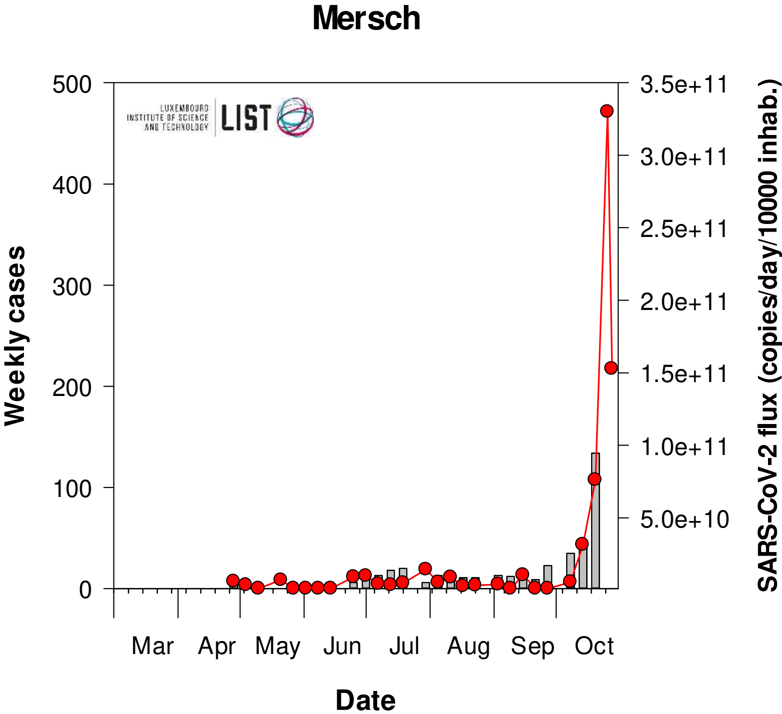
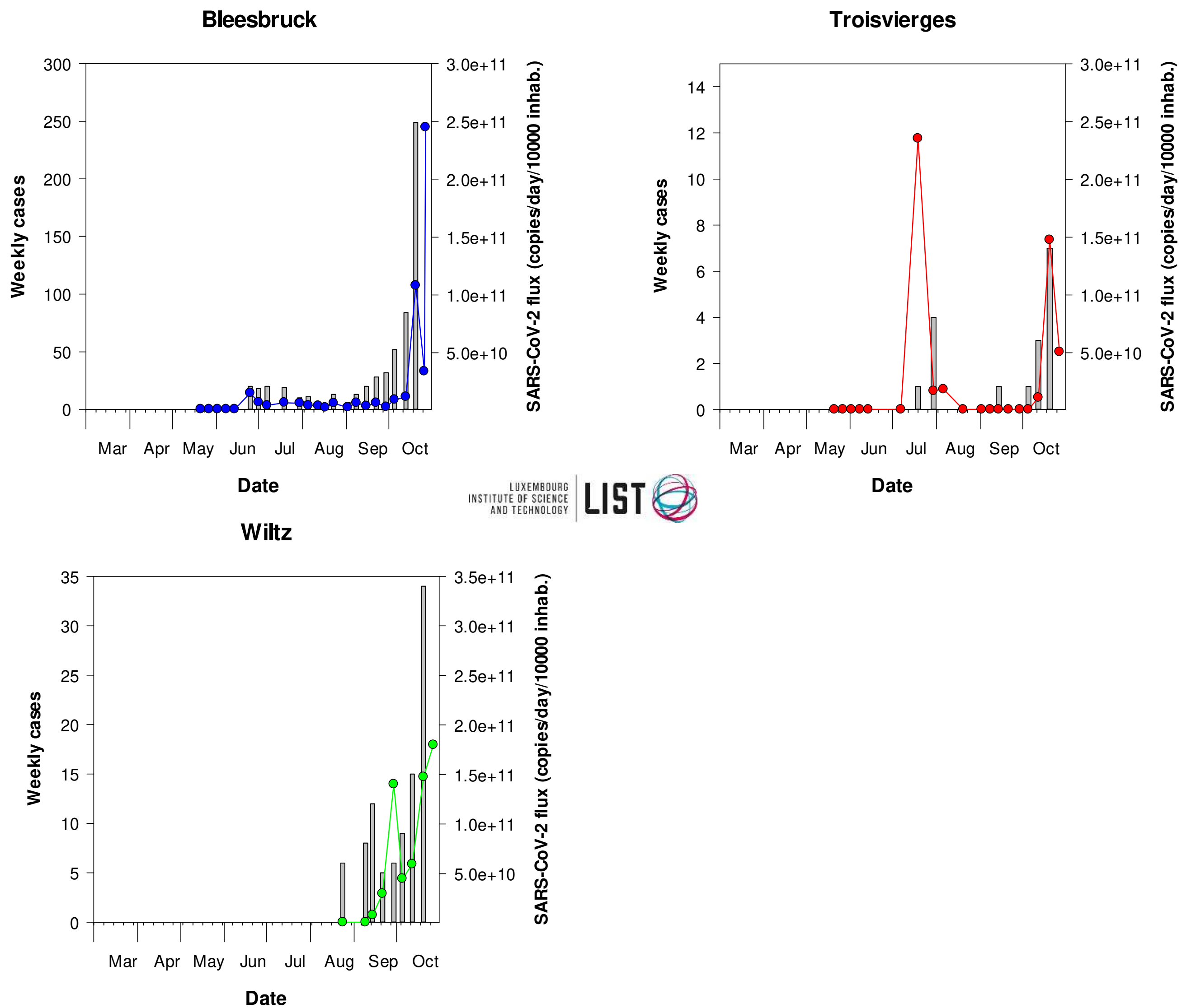


Table 1 – Details of the WWTP analysed on October 29th

Wastewater Treatment Plant	Nominal capacity (eq. inhabitants)	Inhabitants connected
Beggen	210,000	139,731
Bettembourg	95,000	53,606
Schifflange	90,000	68,143
Bleesbrück	80,000	30,930
Mersch	70,000	30,473
Troisvierges	5,000	3,411
Wiltz	16,500	6,944
Total	**566,500**	**333,238**

Figure 4 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from March to October 2020. Grey squares: daily-confirmed cases for the contributory area of each wastewater treatment plant (only available until week 42 included), dots: SARS-CoV-2 flux (RNA copies / day / 10 000 equivalent inhabitants).



Materials and Methods

Sewage samples

From March to October 2020, up to thirteen WWTPs were sampled at the inlet of the plant according to the planning presented in Table 2. The operators of the WWTPs sampled a 24-h composite sample of 96 samples according to your own 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 pelleting using 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 are screened for the presence of *Sarbecovirus* (*Coronaviridae, Betacoronaviruses*) and/or SARS-CoV-2 virus RNA by two distinct real-time one-step RT-PCR, one on the E gene (Envelope small membrane protein) and the second on 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[1]. The two primers/probe sets are presented in Table 3. The RT-qPCR protocols and reagents were all provided by the LIH.

Table 1 – 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-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 53°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).

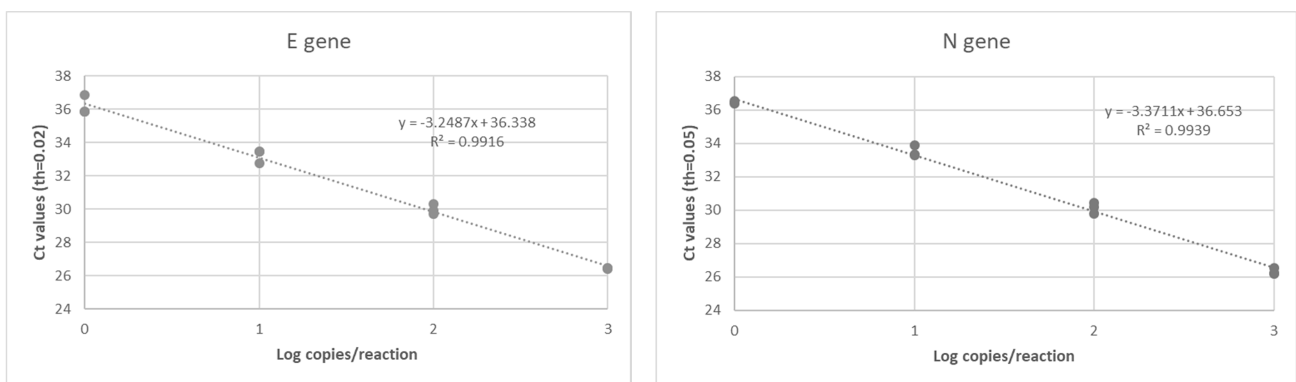


Figure 3 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

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 good linear relationship (R^2: 0.92) 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.

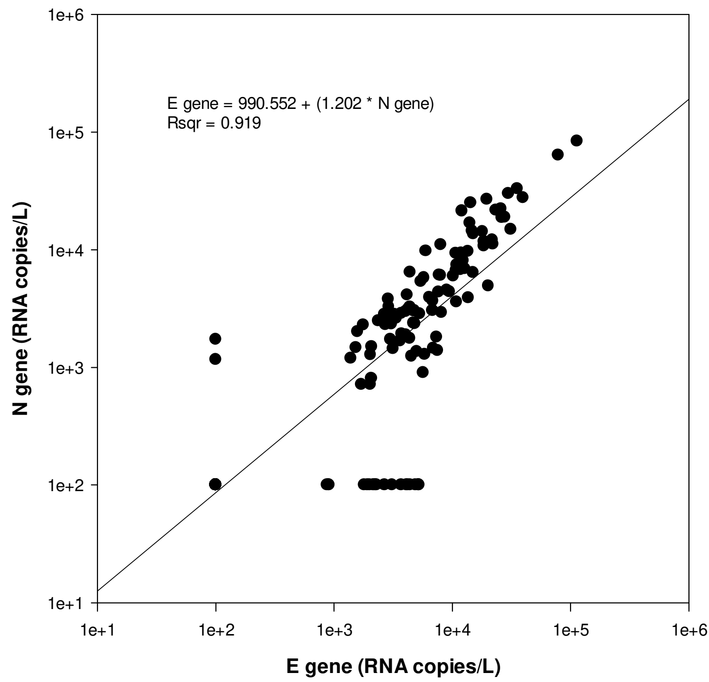


Figure 4 - Relationship between the SARS-CoV-2 RNA concentration (RNA copies / L of wastewater) estimated by the both distinct RT-qPCR systems targeting the E and N gene, respectively