CORONASTEP Report 28 July 2020
SARS-CoV-2 Sewage Surveillance in Luxembourg

Summary

The monitoring of SARS-CoV-2 in wastewater has been set on a weekly basis in Luxembourg from March 31st, 2020 on. Currently eleven wastewater treatment plants (WWTP) are monitored for SARS-CoV-2 in the inlet pipe (Table 1). For the WWTP of Schifflange, archived frozen samples have been analysed back to October 2019. The Figures 1 and 2 shows the complete set of data. The Figures 3 and 4 show the details from June 22nd, 2020.

From March 31st until today, the dynamics of the viral RNA copies in WWTP influents followed the dynamics of the COVID-19 active cases observed at the national level (https://msan.gouvernement.lu/fr/graphiques-evolution.html#sg) for 5 WWTPs: Beggen, Schifflange, Bettembourg, Pétange, Hespérange. This first group includes the largest WWTPs. Mersch WWTP displayed a rather similar trend but with higher values at the beginning of July. The results at Bleesbruck also show higher values at the beginning of the second wave, at the end of June. The other WWTPs have a fuzzier dynamics but this is partly due to a lower number of samples analysed. The very high value at Troisvierges on is noticeable. Only two samples have been analysed so far since the beginning of the second wave but this WWTP must certainly be analysed at a higher rate.

Table 1- Timing of sewage sampling

WWTP	Maximum capacity (equivalent inhabitants)	Inhabitants connected	08-Oct-19	20-Oct-19	12-Nov-19	17-Dec-19	14-Jan-20	12-Feb-20	25-Feb-20	12-Mar-20	30-Mar-20	05-Apr-20	16-Apr-20	22-Apr-20	28-Apr-20	04-May-20	10-May-20	21-May-20	27-May-20	02-Jun-20	08-Jun-20	14-Jun-20	25-Jun-20	01-Jul-20	07-Jul-20	13-Jul-20	19-Jul-20	Tested samples
Beggen	210000	139731									x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	17
Bettembourg	95000	53606															x	x	x	x	x	x	x	x	x	x	x	11
Schifflange	90000	68143	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	25
Bleesbrück	80000	30930																x	x	x	x	x	x	x	x		x	9
Mersch	70000	30473													x	x	x	x	x	x	x	x	x	x	x	x	x	13
Pétange	50000	59481	x	x	x	x	x	x	x	x					x	x	x	x	x	x	x	x	x	x	x	x	x	21
Hesperange	36000	15479													x	x	x	x	x	x	x	x	x	x	x	x	x	13
Echternach	36000	7499																			x				x	x	x	4
Uebersyren	35000	18600																	x		x		x		x	x	x	6
Grevenmacher	47000	9835																	x		x		x		x	x	x	6
Troisvierges	5000	3411																x	x	x	x	x			x		x	7
Total	**754000**	**437188**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**5**	**5**	**6**	**8**	**10**	**8**	**11**	**8**	**9**	**7**	**11**	**9**	**11**	**###**
Pop Lux (2019)		**613901**																										
		71.21%																										

Figure1- Evolution of the SARS-CoV-2 genes (E and N) in the wastewater treatments in Luxembourg (part 1). Grey areas correspond to periods without sampling.

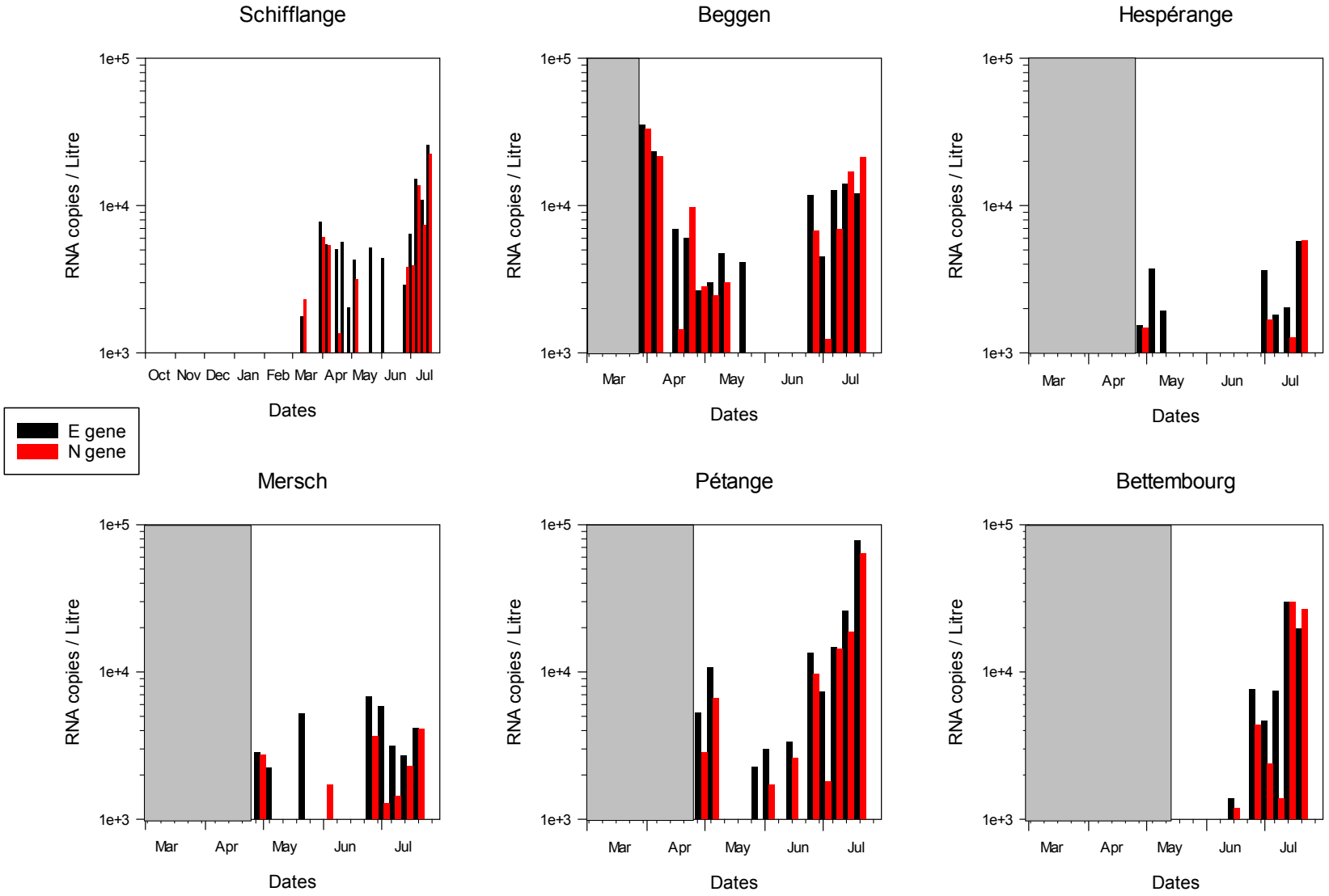


Figure 2- Evolution of the SARS-CoV-2 genes (E and N) in the wastewater treatments in Luxembourg (part 2). Grey areas correspond to periods without sampling.

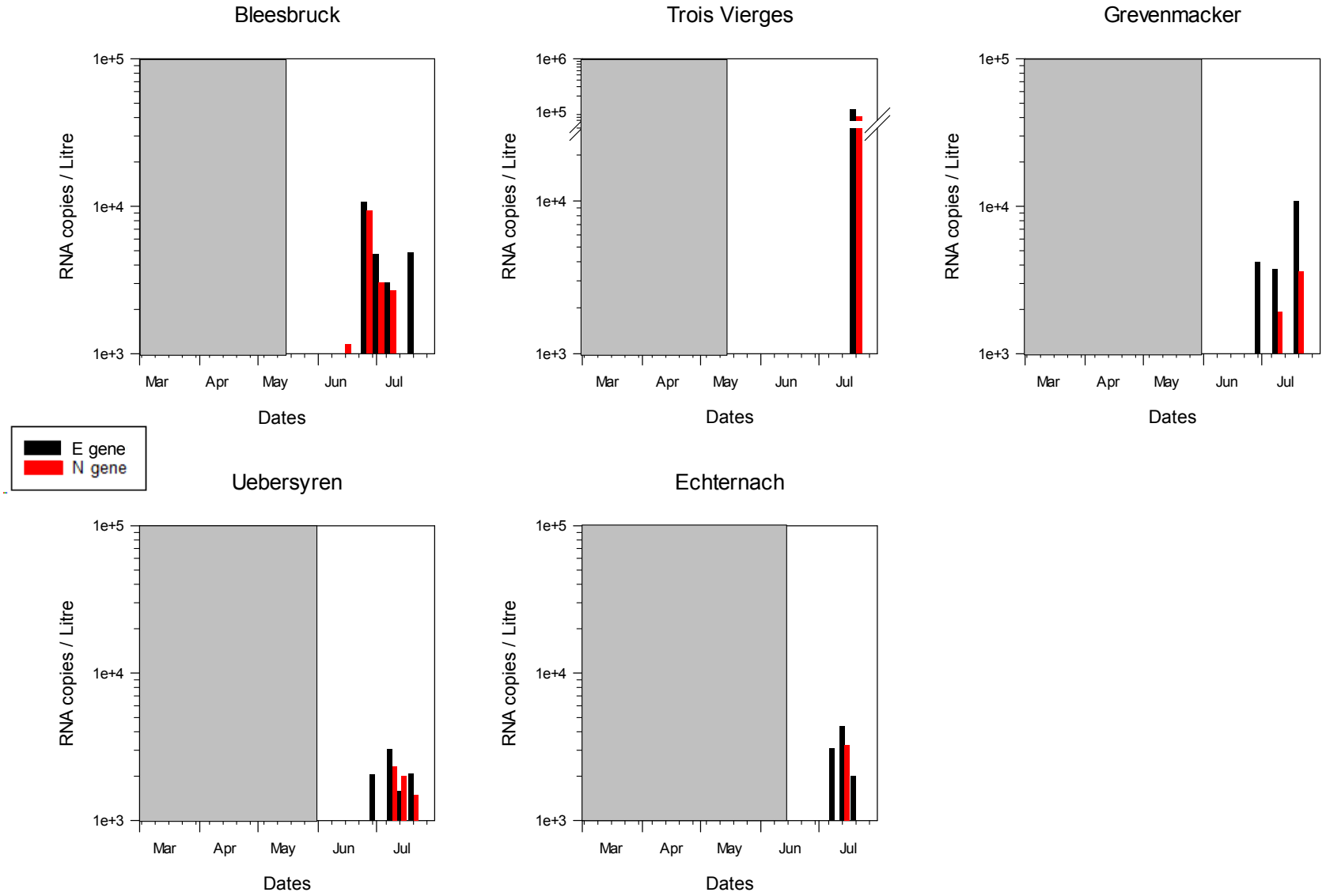


Figure 3- Detailed evolution of the SARS-CoV-2 genes (E and N) in the wastewater treatments in Luxembourg from June 22nd, 2020 (part 1).

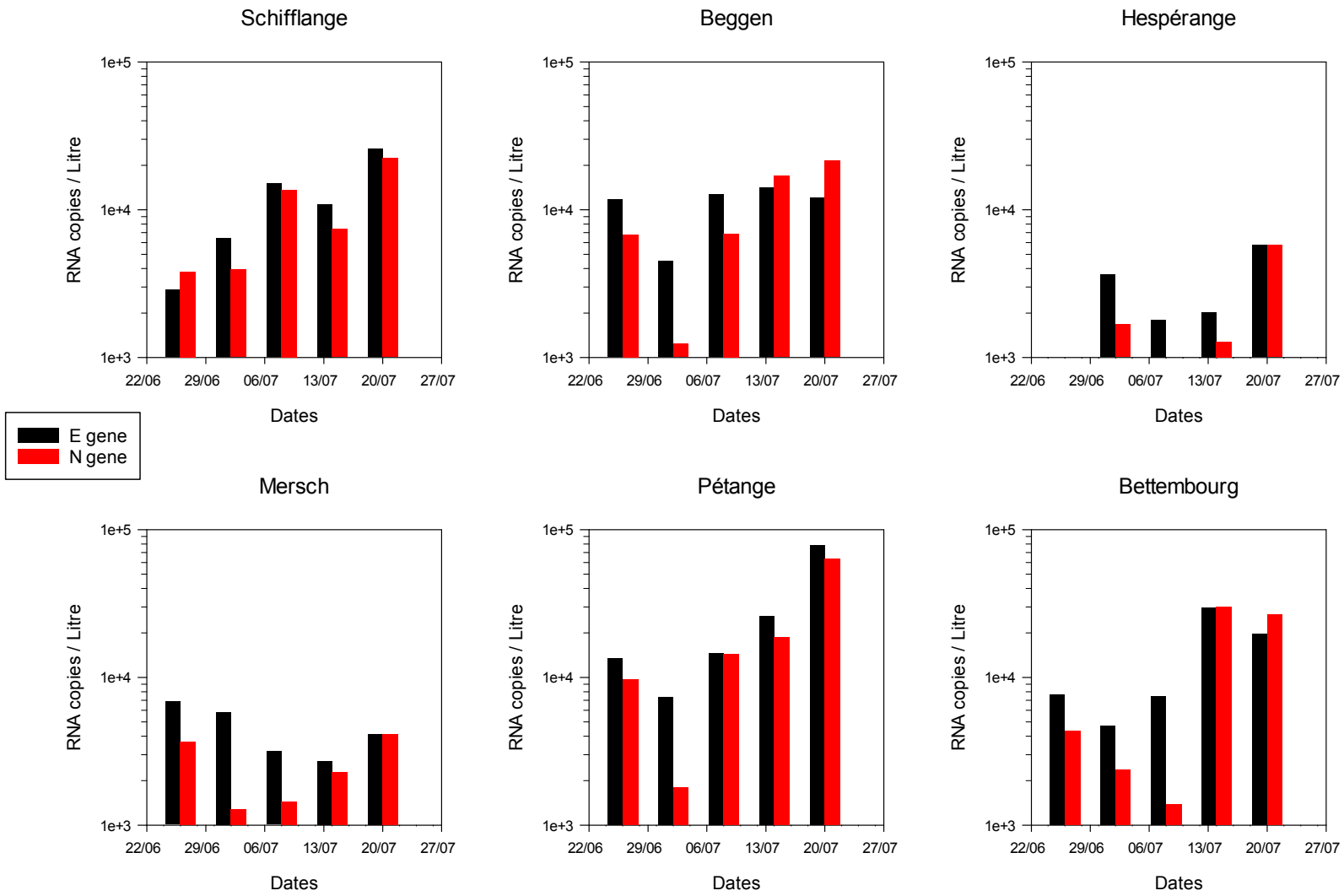
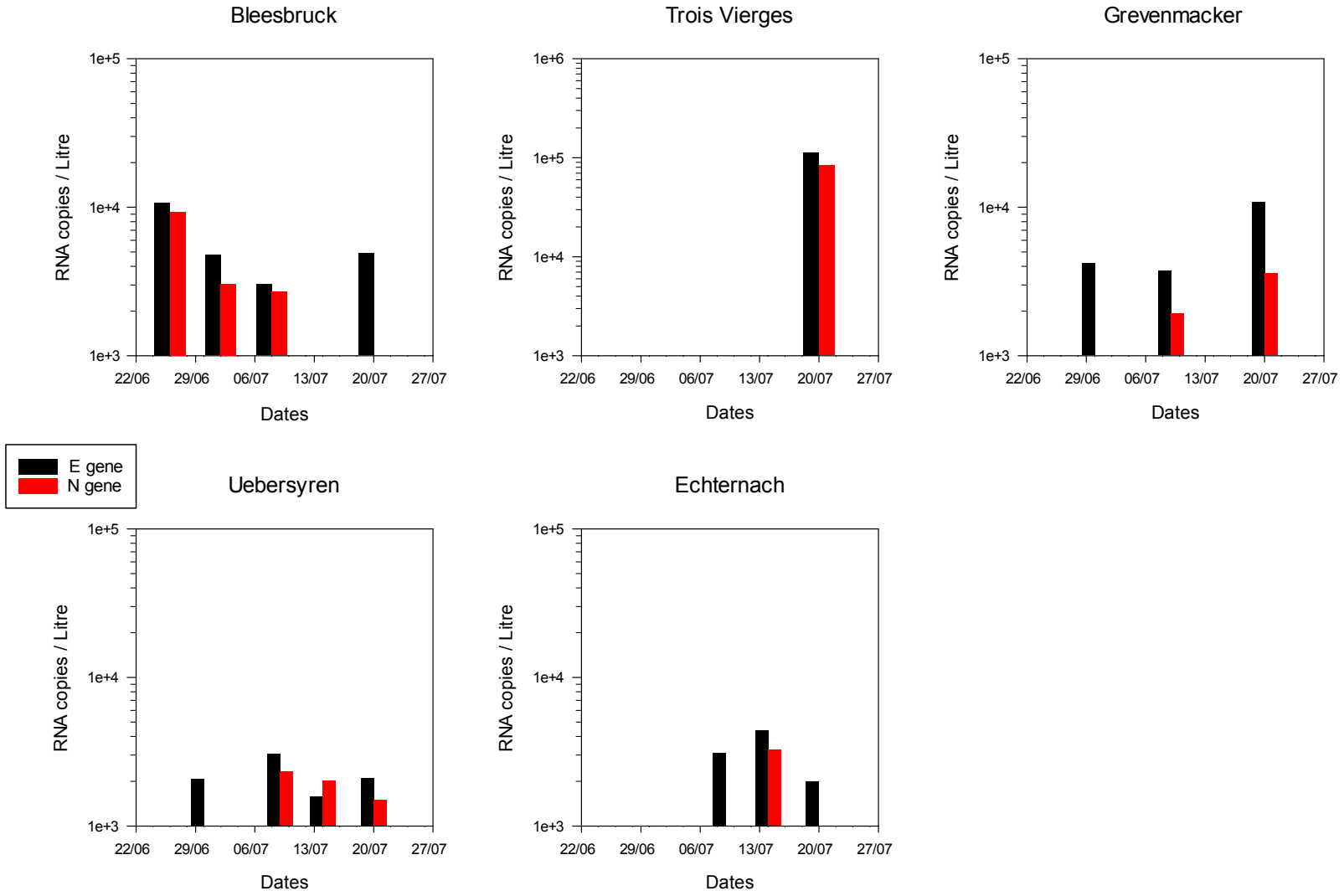


Figure 4 - Detailed evolution of the SARS-CoV-2 genes (E and N) in the wastewater treatments in Luxembourg from June 22nd, 2020 (part 2).



Materials and Methods

Sewage samples

From March 31st to July 14th 2020, up to eleven WWTPs were sampled at the inlet of the plant according to the planning presented in Table 1. 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 2. The RT-qPCR protocols and reagents were all provided by the LIH.

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.

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 1).

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

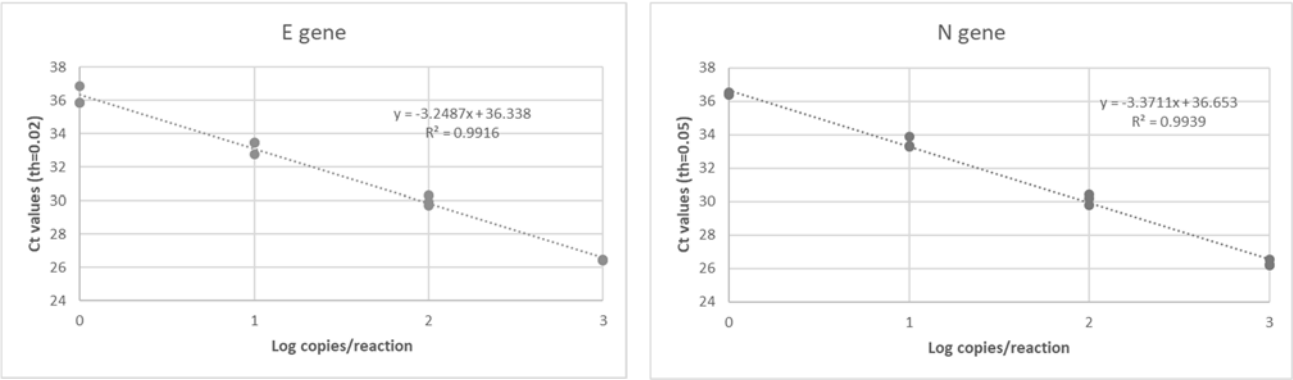


Figure 1 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

Table 2 – 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'	

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 for all of *Sarbecovirus* including SARS-CoV-2.

Table 3 – Summary of the screening of SARS-CoV-2 in 24-h composite samples of incoming wastewater at different WWTP in Luxembourg. Red: samples positive for SARS-CoV-2, Yellow: presumptive samples for SARS-CoV-2 (one of the gene below the quantification limit, the other above), Green: negative samples for SARS-CoV-2, white: not tested

WWTP	Maximun capacity (equivalent inhabitants)	Inhabitants connected	08-Oct-19	20-Oct-19	12-Nov-19	17-Dec-19	14-Jan-20	12-Feb-20	24-Feb-20	12-Mar-20	30-Mar-20	05-Apr-20	16-Apr-20	22-Apr-20	28-Apr-20	04-May-20	10-May-20	21-May-20	27-May-20	02-Jun-20	08-Jun-20	14-Jun-20	25-Jun-20	01-Jul-20	07-Jul-20	13-Jul-20	19-Jul-20
Beggen	210000	139731									+	+	+	+	+	+	+	+/-	-	-	-	-	+	+	+	+	+
Bettembourg	95000	53606															-	-	-	-	-	+/-	+	+	+	+	+
Bleesbrück	80000	30930																-	-	-	-	-	+	+	+	+	+
Schifflange	90000	68143	-	-	-	-	-	-	+/-	+	+	+	+	+	+	+	-	+/-	-	-	-	-	+	+	+		+
Mersch	70000	30473													+	+	-	+	-	-	-	-	+	+	+	+	+
Pétange	50000	59481	-	-	-	-	-	-	-	+					+	+	-	-	-	+	-	+	+	+	+	+	+
Hesperange	36000	15479													+/-	+	+/-	-	-	-	-	-	-	+	+/-	+	+
Trois Vierges	5000	3411																-	-	-	-	-			-		+
Grevenmacher	47000	9835																	-		-				+	+/-	+
Uebersyren	35000	18600																	-		-				+	+	+
Echternach	36000	7499																			-				+	+	+
Total	**754000**	**437188**																									