CORONASTEP Report 17 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 are monitored for SARS-CoV-2 in the inlet pipe. From March 31st until today, the dynamics of the viral RNA copies in wastewater influents followed the dynamics of the COVID-19 active cases observed at the national level. A decreasing phase of SARS-CoV-2 in wastewater has been observed until the beginning of May, followed by a period of negative samples during the exit phase until June 14th. **A resurgence of current increase in SARS-CoV-2 RNA concentration observed from June 25th on confirms the re-emergence of infection in the population of Luxembourg.** The monitoring of SARS-CoV-2 using wastewater proved to be very sensitive and allows to have a positive signal when between 20 and 30 people are detected positive in the contributory areas of the wastewater treatment plants.

The Table 3 gives a summary of the SARS-CoV-2 dynamics in wastewater treatment plants in Luxembourg.

Introduction – Context – Objectives

In case of pandemics, a clear image of the prevalence in the population is essential to manage the containment, particularly with regard to its release or in case of a second wave. Tracking mild or asymptomatic cases that do not require care or testing in the general population is costly, logistically difficult and presents a delay in implementation at the onset of a pandemic of a new nature. However, as soon as the pathogen is excreted in significant amounts in faeces, a monitoring of the wastewater is proving to be an effective way to obtain a detailed dynamics of the viral prevalence. This strategy is particularly recommended by WHO in the context of the global poliovirus eradication. Recent reports show that SARS-CoV-2 has been detected in stool of COVID-19 cases worldwide [1-6] as well as in sewage [7-12]. **The collection of information on the occurrence and fate of this new virus in sewage is important to determine the extent to which sewage surveillance can be used to monitor the circulation of SARS-CoV-2 in a population to complement current clinical surveillance and ultimately serve as early warning of (re)-emergence of COVID-19 at the national level**.
The Environmental Microbiology Group of the LIST has long been involved in monitoring of viruses in wastewater [13-16]. As soon as the SARS-CoV-2 outbreak spread outside of China, discussions were held between the country's major microbiology research groups, highlighting great interest in a nationwide molecular epidemiology study. Through coordination with the staff of the wastewater treatment plants (WWTPs), LIST began to set SARS-CoV-2 monitoring in wastewater on March 31st in the frame of a self-funded internal project called CORONASTEP. From June 1st on, the CORONASTEP project has been extended until the end of November 2020 thanks to a funding by FNR through the COVID-19 call. This extension called CORONASTEP+ involves LIH, LCSB and LNS for aspects as data treatment or sequencing.

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).

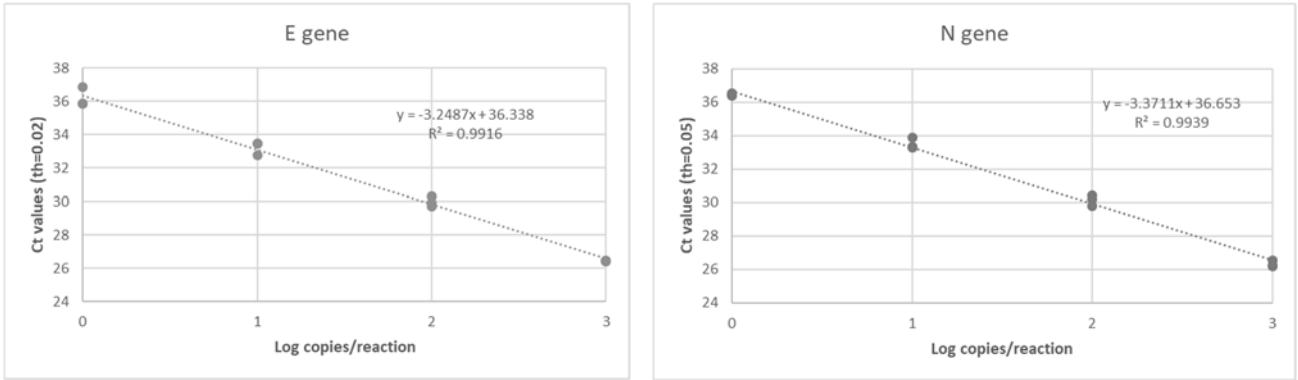

Figure 1 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).

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

Table 1- Timing of sewage sampling

			Before Containment								Containment							Exit phase										
			Before First Covid-19 case							After First Covid-19 case																		
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	02-Jul-20	08-Jul-20	14-Jul-20	Tested samples
Beggen	210000	139731									x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16
Bettembourg	95000	53606															x	x	x	x	x	x	x	x	x	x	10
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	24
Bleesbrück	80000	30930																x	x	x	x	x	x	x	x		8
Mersch	70000	30473													x	x	x	x	x	x	x	x	x	x	x	x	12
Pétange	50000	59481	x	x	x	x	x	x	x	x					x	x	x	x	x	x	x	x	x	x	x	x	20
Hesperange	36000	15479													x	x	x	x	x	x	x	x	x	x	x	x	12
Echternach	36000	7499																			x				x	x	1
Uebersyren	35000	18600																	x		x		x		x		2
Grevenmacher	47000	9835																	x		x		x		x	x	2
Troisvierges	5000	3411																x	x	x	x	x			x		5
Total	**754000**	**437188**	2	2	2	2	2	2	2	2	2	2	2	2	5	5	6	8	10	8	11	8	9			8	**112**
Pop Lux (2019)		**613901**																									
		71.21%																									

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.

Results

Qualitative results – The Table 3 presents an overview of all results gathered to date.

Prior to containment: All samples collected prior to the first confirmed Covid-19 case and tested so far are scored negative for the detection of the SARS-CoV-2 (Ct value higher than 38), with the exception of the sample collected on February 25th at the Schifflange wastewater treatment plant. This sample was suspected to be positive for the SARS-CoV-2 because it showed a positive signal for the E gene only, with a Ct value above the LOD but below the LOQ. A supplementary analysis using the Allplex 2019-nCoV Assay (Seegene) was recently performed by LNS and has confirmed the presence of SARS-CoV-2 in this sample (E gene: Ct value = 35, RdRp gene: Ct value = 36.5 and N gene: Ct value = 38). **Our date indicates therefore that the first positive sample found in Luxembourg dates back to February 25th, 2020.**

- During containment: All samples (100%, n=18) collected after the first confirmed case of COVID-19, and between March 11th and May 4th, are declared positive for the presence of SARS-CoV-2 (Ct values between 31 and 37). A clear decrease is observed in the SARS-CoV-2 in wastewater as containment progressed and the number of active cases decreased.

- During the exit phase: From May 10th to June 14th, corresponding to the first part of the exit phase, the viral RNA was no longer detectable in most of the tested wastewater treatment plants. **From June 25th on, the vast majority of the analysed samples proved to be positive again in the Luxembourg's major wastewater treatment plants (with a number of connected people superior to 15,000 inhabitants), indicating a resurgence of cases in the connected population. This resurgence has been observed a bit in advance compared to the increase in confirmed active cases, confirming the ability of wastewater testing to constitute an efficient pre-alert system.**

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

		Before First Covid-19 case											**After First Covid-19 case**																										
		Before Containment											**Containment**									**Exit Phase**																		
WWTP	**Inhabitants connected**	08-Oct-19	20-Oct-19	12-Nov-19	17-Dec-19	14-Jan-20	12-Feb-20	24-Feb-20	11-Mar-20	Tested samples	Positive samples	**Positive rate (%)**	30-Mar-20	05-Apr-20	16-Apr-20	22-Apr-20	28-Apr-20	04-May-20	Tested samples	Positive samples	**Positive rate (%)**	10-May-20	21-May-20	27-May-20	02-Jun-20	08-Jun-20	14-Jun-20	25-Jun-20	02-Jul-20	08-Jul-20	14-Jul-20	Tested samples	Positive samples	**Positive rate (%)**
Beggen	139731									0	0		+	+	+	+	+	+	6	6	**100**	+	±	-	-	-	-	+	+	+	+	10	6	**60**
Bettembourg	53606									0	0								0	0		-	-	-	-	-	±	+	+	+	+	10	5	**50**
Schifflange	68143	-	-	-	-	-	-	±	+	8	2	**25**	+	+	+	+	+	+	6	6	**100**	-	±	-	-	-	-	+	+	+	+	10	5	**50**
Bleesbrück	30930									0	0								0	0			-	-	-	-	-	+	+	+		8	3	**38**
Mersch	30473									0	0						+	+	2	2	**100**	-	+	-	-	-	-	+	+	+	+	10	5	**50**
Pétange	59481	-	-	-	-	-	-	-	+	8	1	**13**					+	+	2	2	**100**	-	-	-	+	-	+	+	+	+	+	10	6	**60**
Hesperange	15479									0	0						±	+	2	2	**100**	±	-	-	-	-	-	-	+	±	+	10	4	**40**
Echternach	7499									0	0								0	0						-				±	+	3	2	**67**
Uebersyren	18600									0	0								0	0				-		-			+	+	+	5	3	**60**
Grevenmacher	9835									0	0								0	0				-		-			±	+	±	5	3	**60**
Troisvierges	3411									0	0								0	0			-	-	-	-	-			-		6	0	**0**
Total	**437188**									**16**	**3**	**19**							**18**	**18**	**100**											**87**	**42**	**48**

Detailed quantitative Results – Dynamics of the concentration of E and N genes in each monitored wastewater treatment plants are shown in Figures 2 and 3, respectively, compared to the dynamics of active cases (Red curve with red open circles: data from Luxembourg's government site; https://coronavirus.gouvernement.lu/fr.html)

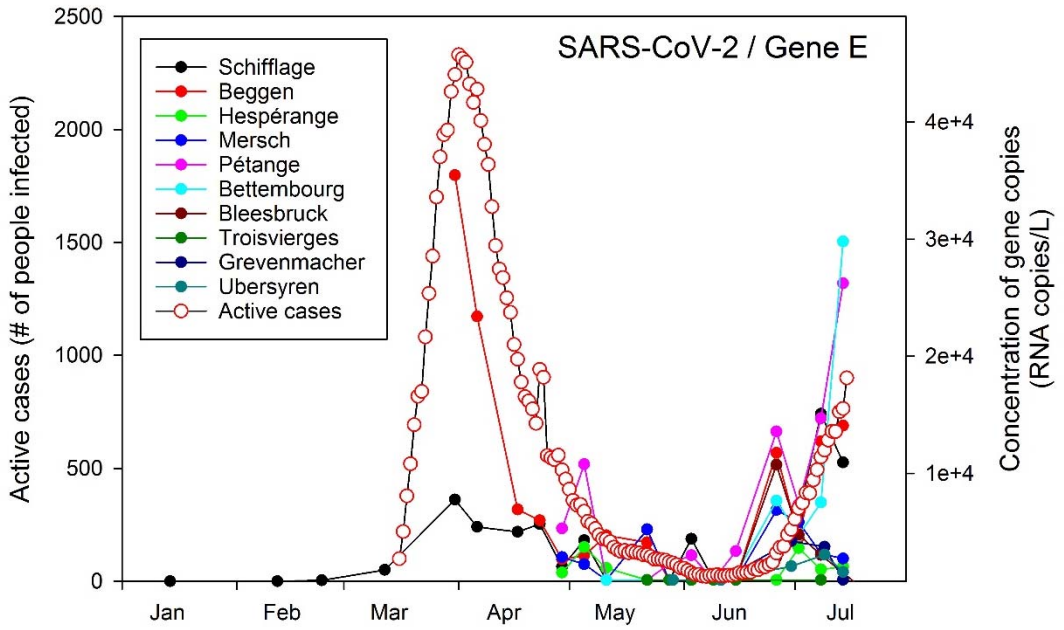

Figure 2 - RT-qPCR quantification time-course monitoring of SARS-CoV-2 (gene E) in samples taken at the inlet of the wastewater treatment plant (from March 31st to July 14th)

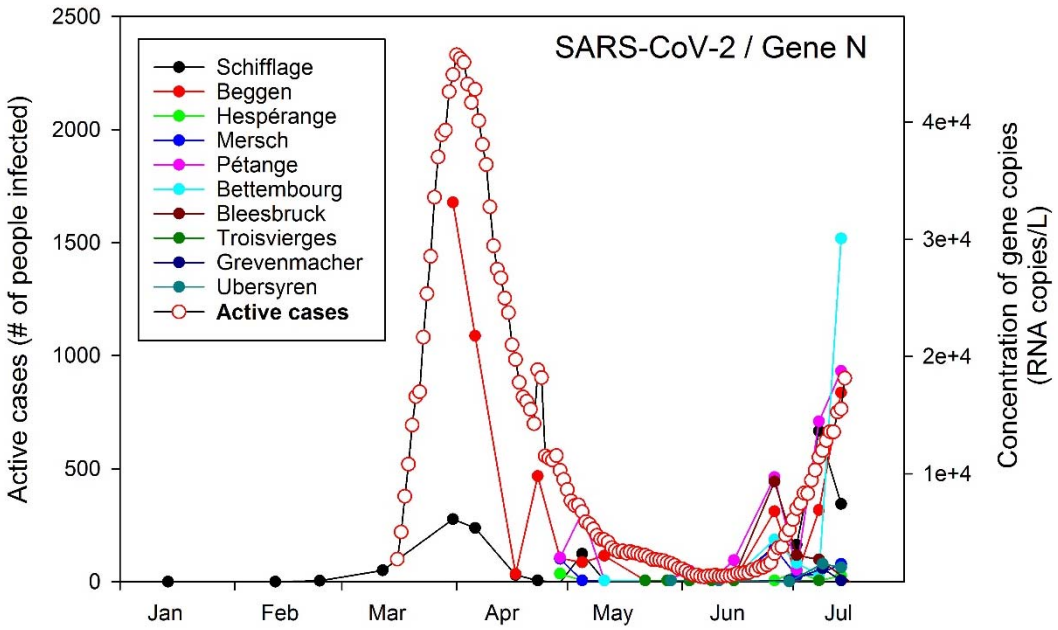

Figure 3 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (gene N) in samples taken at the inlet of the wastewater treatment plant (from March 31st to July 14th)

We compared the average level of SARS-CoV-2 genome in wastewater samples over time with the number of confirmed COVID-19 active cases in Luxembourg. As assumed, we have confirmed that the dynamics of the viral RNA copies in wastewater influents followed the dynamics of the COVID-19 active cases observed at the national level (Figures 2 and 3). The monitoring of SARS-CoV-2 using wastewater is very sensitive and allows us to have a positive signal when between 20 and 30 people are detected positive in the contributory areas of the wastewater treatment plants. The relationship between the concentrations of SARS-CoV-2 in wastewater and number of active cases is however not linear and needs further calibration.

In conclusion, the work undergone so far has demonstrated that quantitative monitoring of SARS-CoV-2 RNA in wastewater influents can provide additional relevant information for a better monitoring and surveillance of the circulation of SARS-CoV-2 at the national level. **The current increase in SARS-CoV-2 RNA concentration observed from June 25th on confirms the resurgence of infection in the population of Luxembourg.**

Our results show that sewage contamination and viral genome detection could take place before the start of the exponential growth of the epidemic. These interesting results argue, in particular, in favor of long-term wastewater monitoring, which would make it possible to alert the authorities to the occurrence of a possible epidemic and the emergence of new viruses circulating in the population.

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