Summary

This report 49 presents the results of SARS-CoV-2 contamination of wastewater at the entrance of 11 wastewater treatment plants at the beginning of the week 07 of 2021. Hespérance and Boevange-sur-Attert treatment plants were not analysed.

The SARS-CoV-2 RNA fluxes present in wastewater treatment plants at the beginning of the week 06 indicate a high prevalence of the virus in wastewater at national. A slight upward trend was perceptible again at the beginning of the week 07. However, we must remain cautious in our interpretation and wait for the results of the next analyses to be able to identify a real trend.

At the level of individual wastewater treatment plants, certain geographical disparities appear. An upward dynamic is notably observed at the Schifflange, Beggen and Grevenmacher wastewater treatment plants, while the RT-qPCR signal is constant for other treatment plants.

Table 1 – National level of SARS-CoV-2 contamination of wastewaters in Luxembourg.

<table>
<thead>
<tr>
<th>Week</th>
<th>National Contamination Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 3</td>
<td>Week 7</td>
</tr>
<tr>
<td>Week 4</td>
<td>Week 9</td>
</tr>
<tr>
<td>Week 5</td>
<td>Week 11</td>
</tr>
<tr>
<td>Week 6</td>
<td>Week 14</td>
</tr>
<tr>
<td>Week 7</td>
<td>Week 16</td>
</tr>
<tr>
<td>Week 8</td>
<td>Week 18</td>
</tr>
<tr>
<td>Week 9</td>
<td>Week 20</td>
</tr>
<tr>
<td>Week 10</td>
<td>Week 21</td>
</tr>
<tr>
<td>Week 11</td>
<td>Week 22</td>
</tr>
<tr>
<td>Week 12</td>
<td>Week 23</td>
</tr>
<tr>
<td>Week 13</td>
<td>Week 24</td>
</tr>
<tr>
<td>Week 14</td>
<td>Week 25</td>
</tr>
<tr>
<td>Week 15</td>
<td>Week 26</td>
</tr>
<tr>
<td>Week 16</td>
<td>Week 27</td>
</tr>
<tr>
<td>Week 17</td>
<td>Week 28</td>
</tr>
<tr>
<td>Week 18</td>
<td>Week 29</td>
</tr>
<tr>
<td>Week 19</td>
<td>Week 30</td>
</tr>
<tr>
<td>Week 20</td>
<td>Week 31</td>
</tr>
<tr>
<td>Week 21</td>
<td>Week 32</td>
</tr>
<tr>
<td>Week 22</td>
<td>Week 33</td>
</tr>
<tr>
<td>Week 23</td>
<td>Week 34</td>
</tr>
<tr>
<td>Week 24</td>
<td>Week 35</td>
</tr>
<tr>
<td>Week 25</td>
<td>Week 36</td>
</tr>
<tr>
<td>Week 26</td>
<td>Week 37</td>
</tr>
<tr>
<td>Week 27</td>
<td>Week 38</td>
</tr>
<tr>
<td>Week 28</td>
<td>Week 39</td>
</tr>
<tr>
<td>Week 29</td>
<td>Week 40</td>
</tr>
<tr>
<td>Week 30</td>
<td>Week 41</td>
</tr>
<tr>
<td>Week 31</td>
<td>Week 42</td>
</tr>
</tbody>
</table>
|       | 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).
Figure 1a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in Luxembourgish wastewater samples from December 2019 to February 2021. 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).

Figure 1b – Close-up of Figure 1a showing results from September 1st on.

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) Grey boxes: no data
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 February 2021. 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 September 1st 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 March 2020 to February 2021. 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 September 1st on.
Figure 4a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEST wastewater treatment plants from March 2020 to February 2021. 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 September 1st on.

Uebersyren

Grevenmacher

Echternach
Figure 5a – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in SIDEN wastewater treatment plants from March 2020 to February 2021. 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 September 1st on.

Troisvierges

Bleesbruck

Wiltz

Weekly cases

SARS-CoV-2 flux (copies/day/10000 inhab.)

Date

Weekly cases

SARS-CoV-2 flux (copies/day/10000 inhab.)

Date

Weekly cases

SARS-CoV-2 flux (copies/day/10000 inhab.)

Date
Table 3- Timing of sewage sampling since the beginning of the CORONASTEP study

<table>
<thead>
<tr>
<th>WWTP</th>
<th>Max capacity (eq. inhabitants)</th>
<th>WWTP capacity connected</th>
<th>Week 41</th>
<th>Week 43</th>
<th>Week 46</th>
<th>Week 51</th>
<th>Week 03</th>
<th>Week 05</th>
<th>Week 07</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beggen</td>
<td>210000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bettembourg</td>
<td>95000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Schiﬄange</td>
<td>90000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bliesbrück</td>
<td>80000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mersch</td>
<td>70000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pétange</td>
<td>50000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hesperange</td>
<td>36000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Echternach</td>
<td>36000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Uelsterven</td>
<td>35000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Grevenmacher</td>
<td>47000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Trondesges</td>
<td>50000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bevoie sur Attert</td>
<td>15000</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wiltz</td>
<td>16500</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>785500</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2- Pop Lux (2019) 613901
72.34%
Materials and Methods

Sewage samples
From March 2020 to February 2021, up to thirteen WWTPs were sampled at the inlet of the plant according to the planning presented in Table 3. 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 protocol1. 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

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E gene</td>
<td>E_Sarbeco_F1</td>
<td>5’-ACAGGTACGTTAATAGTTAATAGCGT-3</td>
<td>Corman et al., 2020</td>
</tr>
<tr>
<td></td>
<td>E_Sarbeco_R2</td>
<td>5’-ATATTGCAGCAGTACGCACACA-3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E_Sarbeco_P1</td>
<td>5’-FAM-ACACTAGCCAT CCTTACTGCGTTTCG-BHQ1</td>
<td></td>
</tr>
<tr>
<td>N gene</td>
<td>2019-nCoV_N1_Fw</td>
<td>5’-GAC CCC AAA ATC AGC GAA AT-3’</td>
<td>CDC</td>
</tr>
<tr>
<td></td>
<td>2019-nCoV_N1_Rv</td>
<td>5’-TCT GGT TAC TGC CAG TGG AAT CTG-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2019-nCoV_N1_Probe</td>
<td>5’-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3’</td>
<td></td>
</tr>
</tbody>
</table>

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 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 6).

Figure 6 – 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 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).

Acknowledgments

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 precious 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 scale.