# CORONASTEP Report 02
# SARS-CoV-2 Sewage Surveillance 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. 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 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 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 institutes, 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.

## Materials and Methods

### Sewage samples

From March 31st to June 25th 2020, eleven WWTP were sampled at the inlet of the plant according to the planning presented in Table 1. The operators of the WWTP 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 were presented in Table 2. The RT-qPCR protocols and reagents were all provided by the LIH.

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

*Table 1- Sewage samples collected before and during the CORONASTEP study.*

| WWTP | Maximum capacity (equivalent inhabitant) | Inhabitant 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 | Tested samples |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Beggen | 210000 | 139731 | | | | | | | | | x | x | x | x | x | x | x | x | x | x | x | x | x | 13 |
| Bettembourg | 95000 | 53606 | | | | | | | | | | | | | | | x | x | x | x | x | x | x | 7 |
| Schifflange | 90000 | 68143 | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | 21 |
| Bleesbrück | 80000 | 30930 | | | | | | | | | | | | | | | | x | x | x | x | x | x | 6 |
| Mersch | 70000 | 30473 | | | | | | | | | | | | | x | x | x | x | x | x | x | x | x | 9 |
| Pétange | 50000 | 59481 | x | x | x | x | x | x | x | x | | | | | x | x | x | x | x | x | x | x | x | 17 |
| Hesperange | 36000 | 15479 | | | | | | | | | | | | | x | x | x | x | x | x | x | x | x | 9 |
| Echternach | 36000 | 7499 | | | | | | | | | | | | | | | | | | | x | | | 1 |
| Uebersyren | 35000 | 18600 | | | | | | | | | | | | | | | | | x | | x | | | 2 |
| Grevenmacher | 14000 | 9835 | | | | | | | | | | | | | | | | | x | | x | | | 2 |
| Troisvierges | 5000 | 3411 | | | | | | | | | | | | | | | | x | x | x | x | x | | 5 |
| **Total** | **721000** | **437188** | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 5 | 5 | 6 | 8 | 10 | 8 | 11 | 8 | 7 | **92** |
| **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' | |

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).

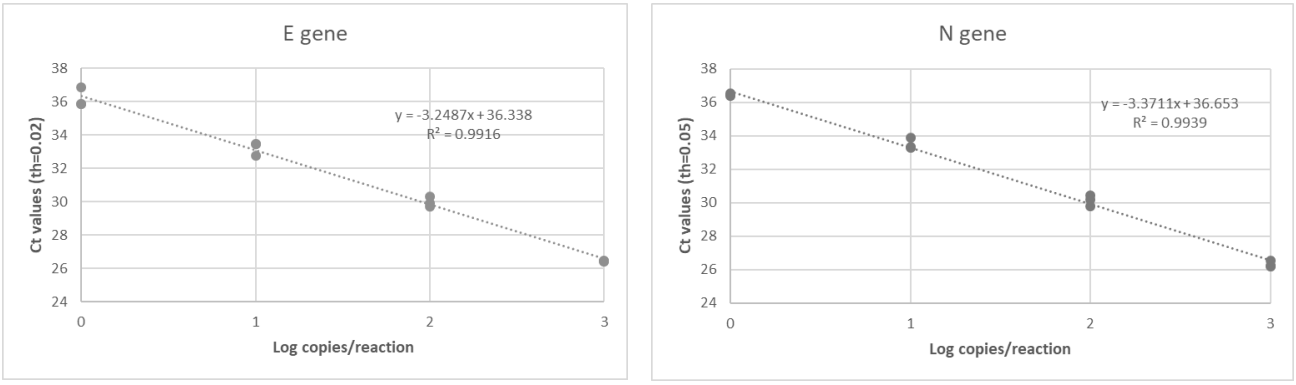


*Figure 1 – RT-qPCR standard curves established for both targeting genes (E gene and N gene) of SARS-CoV-2 using a commercially available standard (Biorad).*

**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, 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).

- 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.

- During the exit phase: Since approximatively May 10th, corresponding to the beginning of the exit phase, the viral RNA is no longer detectable in wastewater at most of the treatment plants tested, except for samples collected on the June 25th. **After a one-month period (May 10th – June 14th) with few or no detection of SARS-CoV-2 in wastewater, the viral genome is again detectable at significant concentrations (33.7 ≤ Ct values E gene ≤ 36.6) in the Luxembourg's major wastewater treatment plants (number of connected people superior to 30000). The SARS-CoV-2 concentrations were estimated to be $1.2x10^4$, $7.7x10^3$, $2.8x10^3$, $1.1x10^4$, $6.9x10^3$ and $1.4x10^4$ RNA copies/L for the wastewater treatment plants of Beggen, Bettembourg, Schifflange, Bleesbruck, Mersch and Petange, respectively.**

*Table 3 – Results of the screening of SARS-CoV-2 in 24h composite samples of incoming wastewater at different WWTP in Luxembourg. Red: samples positive for SARS-CoV-2, Yellow: presumptive samples for SARS-CoV-2, Green: negative samples for SARS-CoV-2*

| | | **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 | Tested samples | Positive samples | **Positive rate (%)** |
| Beggen | 139731 | | | | | | | | | 0 | 0 | | + | + | + | + | + | + | 6 | 6 | **100** | + | ± | - | - | - | - | + | 7 | 3 | **43** |
| Bettembourg | 53606 | | | | | | | | | 0 | 0 | | | | | | | | 0 | 0 | | - | - | - | - | - | ± | + | 7 | 2 | **29** |
| Schifflange | 68143 | - | - | - | - | - | - | ± | + | 8 | 2 | **25** | + | + | + | + | + | + | 6 | 6 | **100** | - | ± | - | - | - | - | + | 7 | 2 | **29** |
| Bleesbrück | 30930 | | | | | | | | | 0 | 0 | | | | | | | | 0 | 0 | | | - | - | - | - | - | + | 6 | 1 | **17** |
| Mersch | 30473 | | | | | | | | | 0 | 0 | | | | | | + | + | 2 | 2 | **100** | - | + | - | - | - | - | + | 7 | 2 | **29** |
| Pétange | 59481 | - | - | - | - | - | - | - | + | 8 | 1 | **13** | | | | | + | + | 2 | 2 | **100** | - | - | - | + | - | + | + | 7 | 3 | **43** |
| Hesperange | 15479 | | | | | | | | | 0 | 0 | | | | | | ± | + | 2 | 2 | **100** | ± | - | - | - | - | - | - | 7 | 1 | **14** |
| Echternach | 7499 | | | | | | | | | 0 | 0 | | | | | | | | 0 | 0 | | | | | | - | | | 1 | 0 | **0** |
| Uebersyren | 18600 | | | | | | | | | 0 | 0 | | | | | | | | 0 | 0 | | | | - | | - | | | 2 | 0 | **0** |
| Grevenmacher | 9835 | | | | | | | | | 0 | 0 | | | | | | | | 0 | 0 | | | | - | | - | | | 2 | 0 | **0** |
| Trois Vierges | 3411 | | | | | | | | | 0 | 0 | | | | | | | | 0 | 0 | | | - | - | - | - | - | | 5 | 0 | **0** |
| **Total** | **437188** | | | | | | | | | **16** | **3** | **19** | | | | | | | **18** | **18** | **100** | | | | | | | | **58** | **14** | **24** |

**Quantitative Results –** Quantitative results are shown in Figure 2 and 3 for the WWTP of Beggen and Schifflange, respectively; both treatment plants were sampled weekly starting in mid-March.

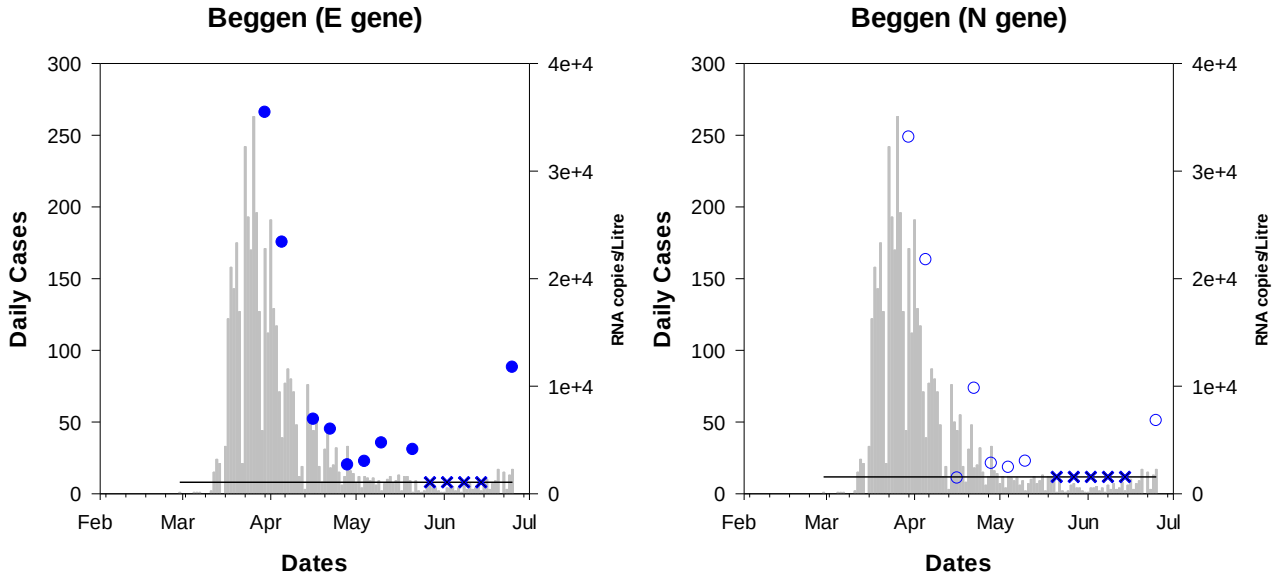


*Figure 2 - RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E and N genes) in wastewater samples from Beggen wastewater treatment plant (from March 31st to June 25th). Grey squares: daily-confirmed cases[1], blue dots: positive samples, blue cross: tested but negative samples, black line: limit of quantification (LOQ).*

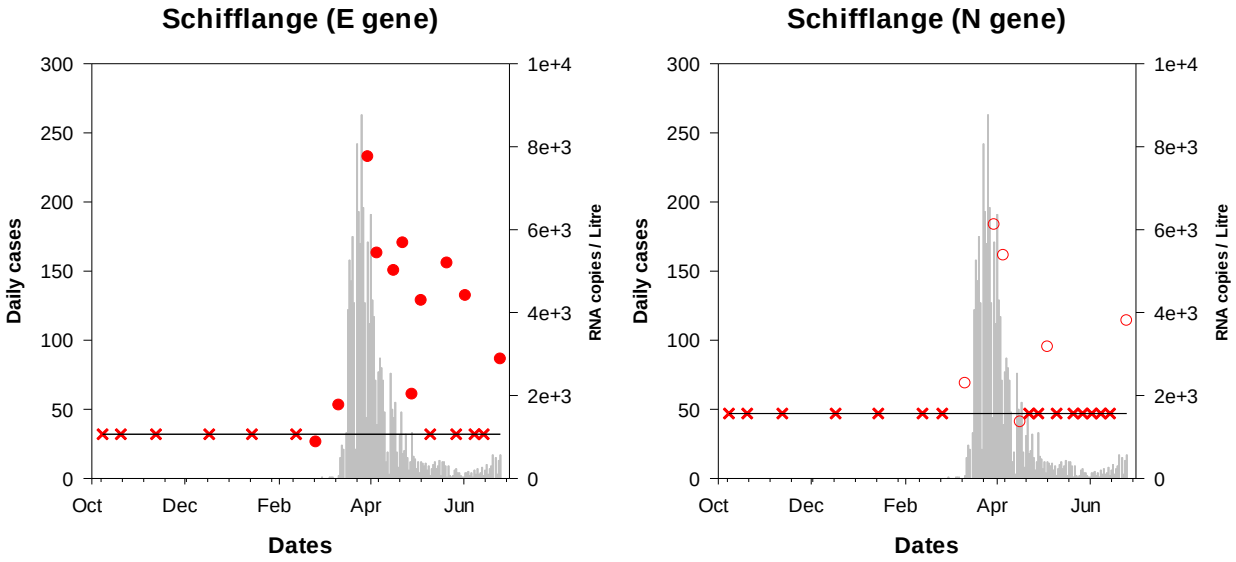


*Figure 3 – RT-qPCR quantification time-course monitoring of SARS-CoV-2 (E gene) in wastewater samples from Schifflange wastewater treatment plant (from Oct 8th to June 25th). Grey squares: daily-confirmed cases[2], red dots: positive samples, red cross: tested but negative samples, black line: limit of quantification (LOQ).*

We compared the average level of SARS-CoV-2 genome in wastewater samples over time with the number of confirmed COVID-19 cases in Luxembourg. As assumed, we confirmed that the increase and the subsequent

[2] *https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6*

decrease of the viral RNA copies in wastewater influents accurately followed the increase and the decrease of the COVID-19 cases observed at the national level (Figures 2 and 3). This is even truer and even more visible for the Beggen WWTP, which is the largest in the country. **The increase in SARS-CoV-2 RNA concentration observed last week is suspicious and needs further attention. Results for the coming weeks (daily cases as well as concentrations in wastewater) should support this observation**.

In conclusion, the work undergoes 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. Indeed, our results showed 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.

## References


1. Xu, Y., et al., *Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding.* Nature Medicine, 2020.
2. Xiao, F., et al., *Evidence for gastrointestinal infection of SARS-CoV-2.* Gastroenterology, 2020.
3. Wu, Y., et al., *Prolonged presence of SARS-CoV-2 viral RNA in faecal samples.* The Lancet Gastroenterology & Hepatology, 2020. **5**(5): p. 434-435.
4. Lescure, F.-X., et al., *Clinical and virological data of the first cases of COVID-19 in Europe: a case series.* The Lancet Infectious Diseases, 2020.
5. Holshue, M.L., et al., *First Case of 2019 Novel Coronavirus in the United States.* New England Journal of Medicine, 2020. **382**(10): p. 929-936.
6. Young, B.E., et al., *Epidemiologic Features and Clinical Course of Patients Infected With SARS-CoV-2 in Singapore.* JAMA, 2020.
7. Wurtzer, S., et al., *Time course quantitative detection of SARS-CoV-2 in Parisian wastewaters correlates with COVID-19 confirmed cases.* medRxiv, 2020: p. 2020.04.12.20062679.
8. Medema, G., et al., *Presence of SARS-Coronavirus-2 in sewage.* medRxiv, 2020: p. 2020.03.29.20045880.
9. Ahmed, W., et al., *First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community.* Science of The Total Environment, 2020: p. 138764.
10. Rimoldi, S.G., et al., *Presence and vitality of SARS-CoV-2 virus in wastewaters and rivers.* medRxiv, 2020: p. 2020.05.01.20086009.
11. Kitajima, M., et al., *SARS-CoV-2 in wastewater: State of the knowledge and research needs.* Science of The Total Environment, 2020: p. 139076.
12. Randazzo, W., et al., *SARS-CoV-2 RNA titers in wastewater anticipated COVID-19 occurrence in a low prevalence area.* medRxiv, 2020: p. 2020.04.22.20075200.
13. Skraber, S., et al., *Concentration and diversity of noroviruses detected in Luxembourg wastewaters in 2008-2009.* Applied and Environmental Microbiology, 2011. **77**(15): p. 5566-5568.
14. Kremer, J.R., et al., *Genetic diversity of noroviruses from outbreaks, sporadic cases and wastewater in Luxembourg 2008-2009.* Clinical Microbiology and Infection, 2011. **17**(8): p. 1173-1176.
15. Skraber, S., et al., *Occurrence and persistence of enteroviruses, noroviruses and F-specific RNA phages in natural wastewater biofilms.* Water Research, 2009. **43**(19): p. 4780-4789.
16. Ogorzaly, L., et al., *Human adenovirus diversity in water samples using a next-generation amplicon sequencing approach.* Food and Environmental Virology, 2015. **7**(2): p. 112-121.
17. Corman, V.M., et al., *Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR.* Eurosurveillance, 2020. **25**(3): p. 2000045.