Supporting information

Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands.

Environmental Science and Technology Letters

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Methods

Sample processing
The samples were transported to the laboratory on melting ice and RNA was isolated within 24h of sampling. Larger particles (debris, bacteria) were removed from the samples by pelleting using centrifugation of the sample in 50 or 250 ml conical centrifuge tubes at 4654xg for 30 mins without brake. A volume of 100-200 ml supernatant was filtered through Centricon® Plus-70 centrifugal ultrafilters with a cut-off of 100 kDa (Millipore, Amsterdam, The Netherlands) by centrifugation at 1500xg for 15 minutes. The Centricon concentrate was 0.44 – 1.79 g.

RNA extraction
Two RNA extraction procedures were used to extract RNA from the concentrated sewage samples. The samples of February 6 and March 5 were processed using the RNeasy PowerMicrobiome Kit (Qiagen, Hilden, Germany) according to the manufacturers protocol. For practical reasons it was decided for all the samples from March 15 onwards to use the magnetic extraction reagents of the Biomerieux Nuclisens kit (Biomerieux, Amersfoort, the Netherlands) in combination with the semi-automated KingFisher mL (Thermo Scientific, Bleiswijk, The Netherlands) purification system to extract RNA from Centricon concentrates as previously described. Elution of RNA was done in 100 µl elution buffer for both RNA extraction methods. In the first sampling round, the equivalent of 100-150 ml of sewage sample was used for RNA extraction. In subsequent sampling rounds, this was approx. 50 ml of sewage. Exact volumes per sample are given in Table S2.

Real-time RT-PCR
Primers/probe sets that were published by US CDC³ and a European study⁴ were used in this study. Four primer sets were selected (Table S4): the N1-N3 set from CDC that each target a different region of the nucleocapsid (N) gene and the set targeting the envelope protein (E) gene from Corman et al.⁴, to include targets against two separate SARS-CoV-2 genes. The specificity of these primer/probe sets against other (respiratory) viruses, including human coronaviruses, had been confirmed by others.⁴,⁵ Each individual reaction contained 5 µl of the total volume of 100 µl eluted RNA template (meaning that 5% of each sewage sample is analysed with each qRT-PCR), 4 µl of 5x Taqman Fast Virus 1-Step Master Mix (Applied Biosystems, Fisher Scientific, Landsmeer, The Netherlands), different concentrations of primers and probes (Table S4), 2 µl of 4 mg/ml BSA (Bovine Serum Albumin, Roche Diagnostics, Almere, The Netherlands) and the reaction volume was adjusted to a final volume of 20 µl with ultrapure DNase/RNase free distilled water (Invitrogen, Fisher Scientific, Landsmeer, The Netherlands). Thermal cycling reactions were carried out at 50 °C for 5 minutes, followed by 45 cycles of 95 °C for 10 and 60 °C for 30 seconds on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands). Reactions were considered positive if the cycle threshold was below 40 cycles. Quantification of N1, N2 and N3 specific assays was performed using four 10-fold dilutions (ranging from 1.0E+02 to 1.0E+05 copies per reaction) of the 2019-nCoV_N_Positive Control plasmid DNA from IDT (Leuven, Belgium) with specified concentrations as calibration suspension.
Virus concentration control
In a subset of 16 samples, the concentration of F-specific RNA phages was measured by the Double Agar Layer plaque assay method according to ISO 10705, before and after the centrifugation and ultrafiltration step, to determine the virus recovery of these steps.

RT-PCR controls
To check the recovery efficiency RNA extraction for the qRT-PCR, a previously described non-target RNA fragment was added to the lysed sewage concentrates as an internal control (IC). This IC-RNA consists of an in vitro transcribed RNA fragment with a length of 412 bases derived from dengue virus type 2 and was synthesized as previously described. qRT-PCR analyses using primers IC-F (5‘-ATGACAGCCACTCCTCCG-3’), IC-R (5‘-GGAACGAACACAGTCTTC-3’) and probe IC-P (5‘-TexasRed-AGCAGAGACCATTCCCTGAGC-BHQ-3’) were used to amplify and detect part of (length: 149 bp) this dengue virus fragment. Quantification of IC-RNA was done by performing qRT-PCR’s on serial 10-fold dilutions of quantified synthetic double stranded DNA (gBlock, IDT, Leuven, Belgium). The concentration of IC-RNA in the extracted sample-RNA was compared with the concentration in the added IC-RNA suspension to determine the recovery efficiency (%) of the IC-RNA by the RNA-extraction procedure and the possible presence of qRT-PCR inhibitors. High-resolution automatic electrophoresis was performed on an Agilent 2100 Bioanalyzer using Agilent 1000 DNA Kit (Agilent, Santa Clara, USA) to confirm the length of the three N-gene and the E-gene specific SARS-CoV-2 RT-PCR-products. Negative controls were included by performing qRT-PCR reactions on RNA isolated from 1 ml of ultrapure DNase/RNase free distilled water (negative extraction control) and by performing qRT-PCR reactions directly on 5 µl ultrapure DNase/RNase free distilled water (negative qRT-PCR control).
Tables

Table S1. Sampling dates and cumulative number of COVID-19 cases in the Netherlands, as reported through the health surveillance system.6

| Sampling round | Dates  | Cumulative number of reported COVID-19 cases (national total) | Cumulative number of COVID-19 cases per 100,000 people (national total) |
|----------------|--------|-------------------------------------------------------------|---------------------------------------------------------------------|
| 1              | February 5 | 0                                                           | 0.0                                                                 |
|                | February 6 | 0                                                           | 0.0                                                                 |
|                | February 7 | 0                                                           | 0.0                                                                 |
| 2              | March 4    | 38                                                          | 0.2                                                                 |
|                | March 5    | 82                                                          | 0.5                                                                 |
| 3              | March 15   | 1135                                                        | 67                                                                  |
|                | March 16   | 1413                                                        | 8.3                                                                 |
| 4              | March 25   | 6412                                                        | 37.7                                                                |

Table S2. Wastewater treatment plants and population served.

| Wastewater treatment plant | Design capacity (inhabitant equivalents) | Inhabitants in sewershed (estimate\(^a\)) |
|---------------------------|------------------------------------------|-------------------------------------------|
| Amsterdam                 | 1014000                                  | 709800                                   |
| Den Haag                  | 1400000                                  | 980000                                   |
| Utrecht                   | 530000                                   | 371000                                   |
| Apeldoorn                 | 350000                                   | 245000                                   |
| Amersfoort                | 335000                                   | 234500                                   |
| Schiphol                  | 54000                                    | Not determined                           |
| Tilburg                   | 375000                                   | 262500                                   |

\(^a\) The number of inhabitants in the catchment of the WWTP was estimated from the design capacity (in inhabitant equivalents) of each of the WWTP and the average difference between design capacity and number of inhabitants in the Netherlands (1:0.7).
Table S3. Volumes processed in RNA extraction per site and date.

| WWTP   | Date     | Sewage volume for RNA extraction (ml) |
|--------|----------|----------------------------------------|
| Amsterdam | 7-2-2020 | 123                                    |
|         | 5-3-2020 | 45                                     |
|         | 15-3-2020 | 56                                     |
|         | 25-3-2020 | 50                                     |
| Den Haag  | 6-2-2020 | 120                                    |
|         | 4-3-2020 | 46                                     |
|         | 5-3-2020 | 44                                     |
|         | 15-3-2020 | 55                                     |
|         | 16-3-2020 | 51                                     |
| Utrecht  | 5-2-2020 | 150                                    |
|         | 5-3-2020 | 45                                     |
|         | 15-3-2020 | 44                                     |
|         | 25-3-2020 | 50                                     |
| Apeldoorn | 6-2-2020 | 136                                    |
|         | 5-3-2020 | 55                                     |
|         | 15-3-2020 | 61                                     |
|         | 25-3-2020 | 50                                     |
| Amersfoort | 6-2-2020 | 129                                    |
|         | 5-3-2020 | 41                                     |
|         | 15-3-2020 | 56                                     |
|         | 25-3-2020 | 49                                     |
| Tilburg   | 15-3-2020 | 42                                     |
|         | 16-3-2020 | 49                                     |
|         | 25-3-2020 | 52                                     |
| Schiphol  | 7-2-2020 | 121                                    |
|         | 5-3-2020 | 36                                     |
|         | 15-3-2020 | 48                                     |
|         | 25-3-2020 | 48                                     |
Table S4. Primer-probe sets

| Assay | Target gene | Primer/Probe | Concentration | Sequence<sup>a</sup> | Reference |
|-------|-------------|--------------|---------------|----------------------|-----------|
| N1    | Nucleocapsid (N) | 2019-nCoV_N1-F | 200 nM | 5′-GACCCCAAAATCAGCGAAAT-3′ | 3 |
|       |              | 2019-nCoV_N1-R | 200 nM | 5′-TCTGGTTACTGCCAGTGGATCTG-3′ | 3 |
|       |              | 2019-nCoV_N1-P | 200 nM | 5′-FAM-ACCCGCATTACGTGTTGGTAGACC-ZEN/iowa Black-3′ | 3 |
| N2    | Nucleocapsid (N) | 2019-nCoV_N2-F | 200 nM | 5′-TTCAAAACATTGGCCGCAAA-3′ | 3 |
|       |              | 2019-nCoV_N2-R | 200 nM | 5′-GCGGACATTCCGAAGA-3′ | 3 |
|       |              | 2019-nCoV_N2-P | 200 nM | 5′-FAM-ACAATTTCAGCAGCTTAG-ZEN/iowa Black-3′ | 3 |
| N3    | Nucleocapsid (N) | 2019-nCoV_N3-F | 200 nM | 5′-GGGAGCCTTGAATACACCAAA-3′ | 3 |
|       |              | 2019-nCoV_N3-R | 200 nM | 5′-TGTACGAGAACGAGCAT-3′ | 3 |
|       |              | 2019-nCoV_N3-P | 200 nM | 5′-FAM-AYCAATTGGCCAGCGCAATCTG-ZEN/iowa Black-3′ | 3 |
| E     | Envelope (E)  | E_Sarbeco_F | 400 nM | 5′-ACAGGTACGTTAATATAGGACGC-3′ | 4 |
|       |              | E_Sarbeco_R | 400 nM | 5′-ATATGTCAGCAGTCATGCAACA-3′ | 4 |
|       |              | E_Sarbeco_P1 | 200 nM | 5′-FAM-ACACTAGCCATCCTACTGCGCTCG-ZEN/iowa Black-3′ | 4 |

<sup>a</sup> Y=C/T. FAM: 6-carboxyfluorescein; ZEN/iowa Black: internal ZEN and iowa Black double-quenched probe
References

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