Methods of the SARS-CoV-2 wastewater surveillance data in Denmark

1. How is SARS-CoV-2 monitored in wastewater?

For the national wastewater surveillance of SARS-CoV-2, 29 wastewater samples are currently taken from 28 treatment plants across Denmark – two samples are taken from two inlets of one treatment plant (Lynetten).

1.1 Coverage of wastewater surveillance

- Since 04.02.2023 and onward, 29 samples are taken from 28 treatment plants twice a week.
- In the period from 09.07.2022 to 03.02.2023, 87 samples were taken from 83 treatment plants twice a week. In addition, two weekly samples from seven decentralized sampling sites were included.
- In the period from 01.01.2022 to 08.07.2022, 202 samples were taken from 198 treatment plants and 26 samples from decentralized sampling sites three times a week.

1.2 Laboratory analysis

- Until 31.03.2023, the commercial sub-contracted laboratory Eurofins Environment Testing Denmark, Eurofins Miljø A/S performed laboratory analysis.
- Since 01.04.2023, TestCenter Danmark (TCDK) performs laboratory analysis at Statens Serum Institut (SSI).

2. How is SARS-CoV-2 measured in wastewater?

- The SARS-CoV-2 virus is excreted with the faeces of approximately half of infected individuals, and it can be therefore measured in wastewater.
- Wastewater samples are taken using an automated sampler that collects small amounts of wastewater over approximately 24 hours. Wastewater samples are then transported refrigerated to SSI where laboratory analysis is performed by TCDK. In the laboratory, the virus is concentrated and purified from wastewater and then analysed with a PCR-test (RT-qPCR) to measure to number of RNA copies of selected SARS-CoV-2 genes per litre of wastewater and for another harmless and naturally occurring virus, the Pepper mild mottle virus (PMMoV), which is also excreted with faeces. PMMoV can be used as an indirect measure of the amount of faeces in wastewater. By normalising the measured SARS-CoV-2 concentration with PMMoV, results can take into account both the dilution of wastewater (for example due to rain) and the number of users of the wastewater system in a specific catchment area.
- The laboratory results of the wastewater analyses are reported to the Department of Infection Disease Epidemiology and Prevention at SSI, where they are validated and processed in statistical analyses. The results are presented on the SSI's website as the National surveillance of SARS-CoV-2 in wastewater.

3. How is the normalised concentration of SARS-CoV-2 in wastewater calculated?

For each wastewater sample (purified sample) a total of four analysis are made divided between two PCR analysis kits:

- Kit 1; SARS-CoV-2 genes N1 and E,
- Kit 2; SARS-CoV-2 gene N2 and the faecal indicator PMMoV.

The PCR analyses are performed as technical triplicates, i.e. each gene is analysed by three independent PCR tests from the same purification. This is done to increase the precision and robustness of the test results. Genes N1, N2 and PMMoV are included in the following analysis. Results from the E gene are not used as they are associated with great uncertainty.

The result of the PCR tests is calculated as the number of SARS-CoV-2 copies or the number of PMMoV copies per well, i.e. per the amount of purified material that has been PCR tested. The PCR test has a signal if RNA is detected in the PCR well. A missing signal in one or more of the three PCR tests is assigned the value of zero copies per well. Since the analysis is based on triplicates, it is estimated that both the detection and quantification limits for the average of the triplicates is 3 copies per well. See background in Annex 1.

For the SARS-CoV-2 genes, the average number of copies measured in the three PCR tests is calculated. In the event that results are available for fewer than three of the triplicates, a decision is made together

with the laboratory as to whether the result for the gene in question can be used. For approved samples, the average number of copies is determined as follows:

- With a signal in at least two of the three triplicates and an average number of copies ≥3, the average is directly used in the subsequent calculations.
- With a signal in at least two of the three triplicates and an average number of copies <3, the average number of copies is determined to be 1.5.
- With a signal in just one of the three PCR tests, the average number of copies is determined to be 0.5.
- With no signal in all the three PCT tests, the average number of copies is determined to be 0.25[1].

The number of RNA copies per well is then converted to the number of copies per litre of wastewater using the expression

$$RNA_{copies/litre} = RNA_{copies/well} * 20 * 100$$

The factor 20 corrects for the fact that 0.005 mL out of 0.1 purified mL is used in the PCR reaction. The factor 100 corrects for using 0.1 mL from 10 purified mL of wastewater.

The number of RNA copies per litre of wastewater is $\log_{10}$ transformed. Then the following calculations and analyses are carried out.

For each sample, the target is the faecal-normalized concentration of SARS-CoV-2 in the wastewater. The concentration is calculated as an average of SARS-CoV-2 RNA copies per litre of wastewater, measured with the two SARS-CoV-2 genes. Then, the SARS-CoV-2 average is normalized with the faecal indicator PMMoV. This gives that

$$\log_{10}(RNA_{faeces\ mean\ normalised}) = \frac{\log_{10}(N1) + \log_{10}(N2)}{2} - \log_{10}(PMMoV).$$

Before the average normalized values are presented in graphs, the values are back-transformed from $\log_{10}$ scale to normal scale. The back transformation also uses the scaling factor 10^7, which was introduced to make the results easier to read and understand. This gives that

$$\widehat{RNA}_{faeces\ mean\ normalised} = 10^{\log_{10}(RNA_{faeces\ mean\ normalised})} * 10^7 = RNA_{faeces\ mean\ normalised} * 10^7$$

[1] As the values in the subsequent calculations are logarithm-transformed, the average number of copies in case of missing a signal in all the tests is set to 0.25 instead of 0.

4. How are the final wastewater results calculated?

The wastewater results are presented aggregated for the whole country, for the five regions and for each sampling location. All calculations related to the wastewater measurements are made on the basis of logarithm-transformed values (log_{10}). There are two general categories of the wastewater results, which are described below.

4.1. Weekly weighted average

The virus concentration of SARS-CoV-2 in the wastewater is calculated as the average weekly number of SARS-CoV-2 RNA copies, in relation to the aforementioned faecal indicator PMMoV (see sections 2 and 3). In the overall graphs (national and regional), the results from each treatment plant are weighted according to the number of inhabitants in the catchment area (log_{10} inhabitants).

4.2. Trends (growth rate)

At the national level, a growth rate is calculated to describe the trend in the SARS-CoV-2 concentration based on the last three weeks of data. The growth rate is thus the average weekly change in concentration over the past three weeks. The growth rate is calculated by using a mixed-effects model with sewage treatment plant as a random effect.

The estimate from the model is converted to an average weekly percentage change as

$$average\ weekly\ growth = (10^{growth\ rate * 7} - 1) * 100\%.$$

4.2.1 Categorization and visualization of the growth rate

The growth rate is classified as one of the seven categories below.

Very strong increase	≥50%	
Strong increase	25% to 49%	
Increase	10% to 24%	
Stable	-9% to 9%	
Decrease	-24% to -10%	
Strong decrease	-49% to -25%	
Very strong decrease	≤-50%	

The growth rate is shown in the figure of the normalized concentrations of SARS-CoV-2 in the wastewater. The most recent three weeks, that the estimate for the growth rate is based on, are colored according to the category that the growth rate falls within, and is shown along with the actual numerical estimate of the growth rate. This can be seen in the weekly publication of wastewater results on the website for the national monitoring of SARS-CoV-2 in wastewater.

4.3 Level assessment

Based on the highest and lowest observed values of the weighted weekly average of SARS-CoV-2 concentrations in the period from 29.06.23 to 07.12.23, five levels have been established. The boundaries between these levels are evenly distributed on a logarithmic scale and calculated so that the lowest and highest concentrations fall into the categories 'Very low level' and 'Very high level', respectively. The established levels can be seen in Table 2.

The levels are marked as horizontal bands in the graph of the normalized concentrations of SARS-CoV-2 in the wastewater, which is updated weekly on the website.

Table 2: Levels for the normalized SARS-CoV-2 concentration in wastewater.

Level	**Normalized SARS-CoV-2 concentration (copies / L)**	
Very high level	≥ 12800	
High level	3200 to 12800	
Medium level	800 to 3200	
Low level	200 to 800	
Very low level	< 200	

Annex

5. Laboratory determination of detection and quantification limits

To determine the detection and quantification limits, three independent dilution series are made for each SARS-CoV-2 gene. For each dilution, 24 technical replicates are made, so that there is a total of 72 data points per concentration for each gene.
The detection limit is set so that detection is expected in at least 95% of the wells and the quantification limit is set so that the coefficient of variation is below 35%.
In an experiment, dilutions were made where 1, 2, 4, 8, 16, 32, 64 and 128 copies per well were expected. On this basis, the detection and quantification limits have been chosen as the lowest concentrations that meet the criteria.

Table 1 shows the limits found for the two SARS-CoV-2 genes that are included in the analysis.

Table 1: Detection and quantification limits for the two SARS-CoV-2 genes in single wells

	Detection limit (copies per well)	Quantification limit (copies per well)
N1 gene	4	8
N2 gene	2	16

6. Data processing

Based on the above laboratory determination of the detection and quantification limits for individual wells, here we describe the background for the selected limits based on the data analysis of the technical triplicates.

6.1 Detection Limit

The starting point for choosing a detection limit is that one wants to be sure of obtaining a signal in 95% of the tested samples. The number of copies in a well follows a Poisson distribution. Given the Poisson variation, the theoretical limit is that on average there must be at least 3 copies per well to obtain a signal in 95% of the analysed samples. If one looks at technical triplicates, instead of looking at the individual well, then, with a 95% probability of detection in each well, one will have >99% probability of detection in at least two wells. In case there is a signal in less than two wells, the estimated number of copies per well is based on the number of wells with a signal.

6.2 Quantification limit

As described above, the quantification limit is chosen so that the coefficient of variation (CoV) must be below 35% - the reason is again because of a Poisson variation. By using the average of the three triplicates, this variation is reduced. Figure 1 shows the result of a simulation experiment, which was made to investigate this. For a number of concentrations, expressed as the expected number of copies per well, 100 simulations of the calculation of the CoV were made using single measurements and triplicates, respectively. The CoV was determined as the standard deviation divided by the mean of 30 determinations.

Figure 1 shows boxplots for the 100 simulations for each concentration and number of replicates. The blue boxes correspond to the above experiments. In the experiment with single wells, the CoV was determined to be 8 or 16 for the three genes. No tests were carried out with intermediate concentrations. Figure 1 shows that the median at 8 copies per well is close to a CoV < 35%. If the experiment is repeated, CoV will therefore be determined to be 8 in half of the cases and 16 otherwise, since intermediate concentrations were not investigated. The experiment is therefore in full accordance with the theoretical expectations. If the calculations of the CoV are instead based on the average of triplicates (red boxes), the quantification limit becomes 3, as the median here is below 35%.

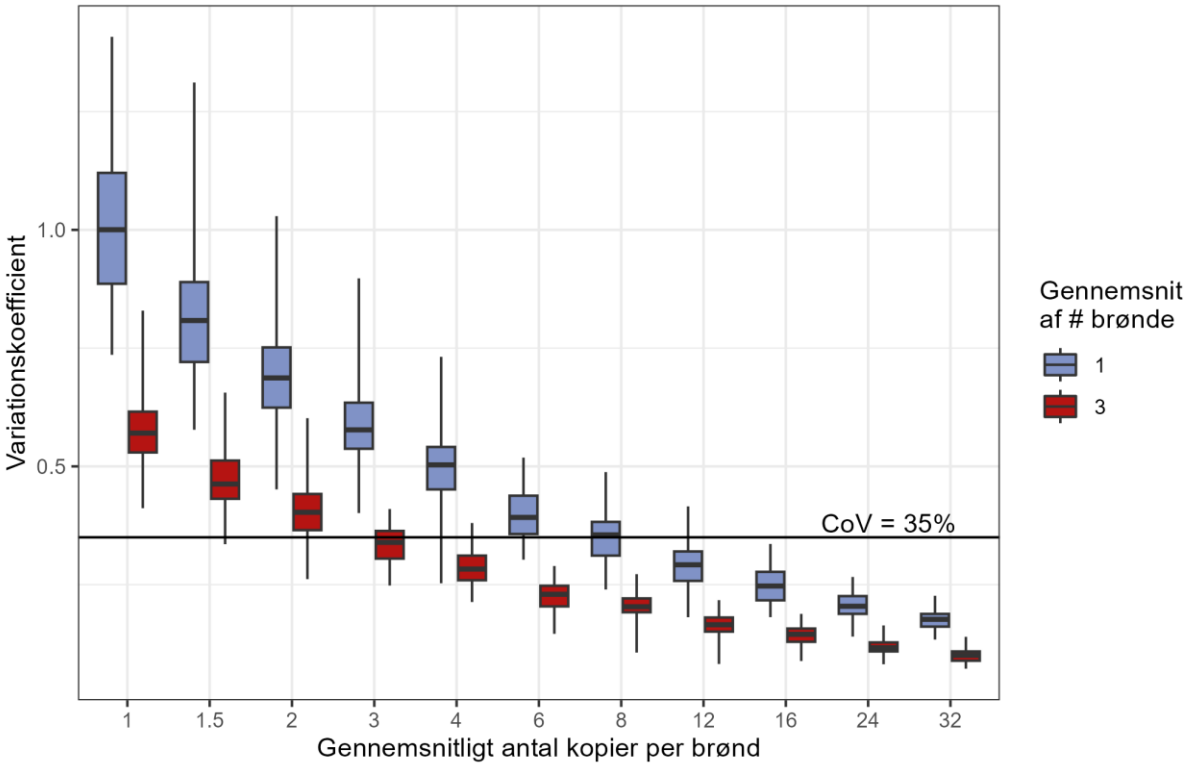

Figure 1: Simulated coefficients of variation (CoV) for different concentrations and for single wells and average of three technical triplicates.

Based on the above, it has been chosen to accept levels of quantification when the average of the triplicates is above 3. In practice, there can be several reasons why detection does not occur in a well. Therefore, the average is not used if there is only detection in one of the triplicates, nor if the average is over 6, when the probability of observing zero copies in a well is very small. In these cases, the observation is omitted until a discussion occurs with the laboratory about what is best to do.