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RESEARCH ARTICLE

Eurofins Covid-19 Sentinel™ Wastewater Test Provides Early Warning of COVID-19

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ABSTRACT
Estimates of viral content can be used as an indicator of the community infection rate, and as an indicator of resurgence of COVID-19 in well-defined sites such as production facilities, hospitals, nursing homes and wastewater treatment plants. The Eurofins Covid-19 Sentinel™ program was developed to monitor the evolution of the Covid-19 pandemic, and for early detection of corona disease outbreaks in local communities. This paper provides an extension of the Eurofins Sentinel program, and demonstrates further protocol optimization, method validation and real life application. The validation of the Eurofins Covid-19 Sentinel™ protocol include analysis of optimal storage conditions, sample stability, estimates of reproducibility, sensitivity, precision, and precision in viral recovery upon dilution of wastewater samples. The Eurofins Covid-19 Sentinel™ wastewater method was later implemented in the national wastewater surveillance program in Denmark, as it provides a robust, sensitive and semi-quantitative method to monitor the spread and occurrence of SARS-CoV-2 virus within local populations.

Keywords: COVID-19, SARS-CoV-2, Wastewater Surveillance, PCR

Introduction

The Eurofins Covid-19 Sentinel™ protocol has been developed to monitor the spread of SARS-CoV-2 in local communities, and thus provide an opportunity for early detection of localized increases in viral load, and potential disease outbreaks[1,2]. In Denmark, an extraordinary high frequency of human testing has been applied to track the spread of SARS-CoV-2 within the population. Clinical testing, however, suffer from a number of disadvantages and limitations, such as being a costly, individual-based, and a highly time consuming analysis. Furthermore, the clinical testing is particularly prone to privacy issues and is based in the willingness of the population to get tested. On the other hand, surveillance of wastewater from a defined area may be highly representative of the prevalence of SARS-CoV-2 in the entire community or in a single facility[3].

Wastewater monitoring is based on the fact that coronavirus is shed in human feces from infected patients[4–6]. Studies have shown that SARS-CoV-2 virus are shed via feces from infected individuals[7–9], even if the infected person does not show disease symptoms (i.e. is either asymptomatic or pre-symptomatic). In this way, analysis of wastewater containing fecal material will reveal whether infected individuals have contributed to the wastewater in a given catchment area. By accounting for the flow of wastewater, industrial outlet, and downpour, it is possible to infer on the level of infection in a given location[10–12].

Several protocols for detecting viruses in wastewater have been proposed [13–15], and the Eurofins Covid-19 Sentinel™ protocol serves as another promising, and highly cost-effective method. However, as of date, no consensus of protocol for testing wastewater samples for SARS-CoV-2 have been published. Some reports propose using the liquid phase[16] of the waste water for downstream analysis, others use the wastewater sludge phase[17], or both phases[18]. Early in the covid-19 pandemic, none of these methods were optimized for large-scale monitoring of disease outbreaks, the Eurofins Sentinel™ Program thus serves as one of the first "Proof of Concept" protocols for high throughput SARS-CoV-2 testing of wastewater. The Eurofins Covid-19 Sentinel™ protocol was implemented into the Danish National Monitoring Program, which was officially initiated on 1st July, 2021.

The objective of this study was to further strengthen the already published Eurofins Covid-19 Sentinel™ protocol[1], by demonstrating the properties of the method, including sensitivity, robustness and precision, and explore wastewater matrix effects and storage conditions on the analysis

Materials and Methods

Analysis and methodology

For successful detection of SARS-CoV-2 virus in wastewater, Eurofins Covid-19 Sentinel™ protocol includes the following steps; Collection of the samples, precipitation, RNA extraction from the liquid phase and Reverse Transcriptase (RT)-qPCR analysis.

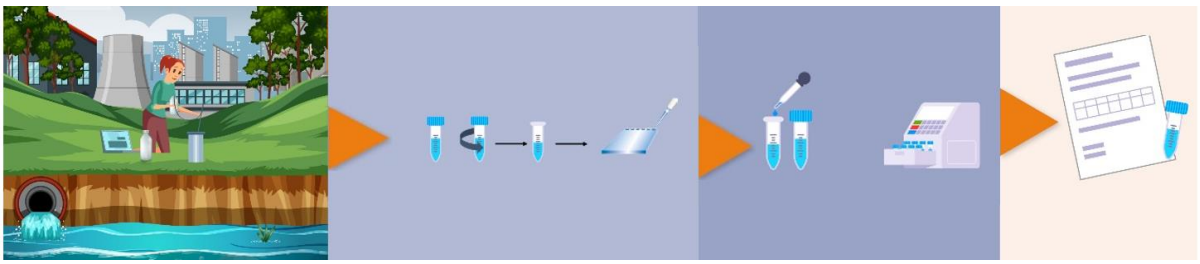

Figure 1. *Overview of the major analysis steps involved in the detection of SARS-CoV-2 from wastewater samples. 1) sample collection, 2) precipitation, 3) RNA extraction, and 4) PCR analysis.*

Sample collection

Wastewater samples used in this study were collected from a number of local wastewater treatment plants in Denmark. All samples were collected according to ISO 5667-10:2004 using a time-dependent composite sampler over a time period of 24-hours. Wastewater samples used in protocol validation were collected from May – December 2021. National monitoring was initiated July 2021. After sampling on site, all samples were transported directly (in a cooling box at 4 ± 2 °C) from the sampling location to Eurofins Environment Testing Denmark, Vejen, where all further analyses were performed. All samples were stored at 4 ± 2 °C until laboratory analysis. All analyses were performed in a class BSL-2 facility, and carried out by trained staff using appropriate safety measures.

Principle of precipitation

To optimize the detection of viral RNA (which may occur in relatively low concentration in an aqueous matrix such as wastewater), the viral content is firstly precipitated. This study protocol is based on the use of the flocculant, polyethylenglycol (PEG-8000), which is commonly applied for precipitation in a number of applications, such as protein concentration[19] and viral capture[20]. In the present protocol, wastewater samples were spiked with 50.000 copies of Murine norovirus (MNV) as a process control (GeneScan Technologies) before homogenization. Samples were subsequently centrifuged for 8 minutes, to separate the solid and liquid phases. Virus in the liquid phase was precipitated using a reagent of PEG-8000 and NaCl, which was added to each sample to a final concentration of5% PEG-8000 and 1,12% NaCl. The samples were then incubated for 30 minutes under constant shaking at 4 ± 2°C. Following the incubation samples were centrifuged for 30 minutes and the supernatant discarded. The aggregated viral pellet was used for RNA extraction. Samples were stored at 4 ± 2 °C until RNA extraction (if analysis were conducted within 4 hours) or frozen at -80°C for longer storage prior to RNA extraction.

Principle of nucleic acid extraction

Viral RNA (both SARS-CoV-2 and process control MNV) was extracted from the aggregated viral pellet. RNA was isolated by initial lysis of the cellular wall, followed by adhesion to silica coated magnetic beads and a number of wash steps. RNA extractions were performed according to manufactures protocol, using the VIRSeek RNA Extractor kit (Eurofins Technologies) kit on a semi-automated purification system (Auto-Pure 96, Allsheng). RNA extractions and downstream analysis were handled in an RNase free environment, and by use of RNase free consumables and reagents.

Principle of reverse transcription quantitative real-time polymerase chain reaction

Molecular detection of viral RNA is possible through the use of PCR techniques[21]. –SARS-CoV-2 RNA was detected using the VIRSe*e*k Ident kit (RdRP-gene) and the Mplex kit (N-gene) (Eurofins Technologies). Composition of the PCR kits and their application specifications are published elsewhere[2]. By including standard curves using IVT-RNA material (10-fold dilution series) a quantitative approach was applied for reporting the viral load in wastewater.

Using linear regression on log10 transformed concentrations of the IVT RNA, efficiency of the dilution series was determined; a slope of ~ -3,32 should appear between 10-fold dilutions[21]. After successful RT-qPCR analysis, all amplification curves were checked in the AriaMx software, and threshold settings were specified following manufacturers recommendation. Ct values were then converted to the number of copies in the initial volume of wastewater using amplification data from standard curves following the formula:

$$RNA\ Target\ \left[\frac{Copies}{mL}\right] = \frac{(b * m^{Ct}) * 20 * 1{,}3}{V}$$

where b and m are the starting point and growth factor of the standard curve, 20 is a factor used to calculate the number of RNA copies in the total RNA eluate (5 µl used in PCR/100 µl total eluate). 1,3 is a factor accounting for the loss of volume during RNA extraction, and V represent the volume of wastewater used for precipitation. Data for N and RdRP genes were compiled for each sample and organized into an excel sheet, in which Ct values were converted to copy numbers and multiple process controls were assessed. The quantified results (viral copies/volume) were interpreted elsewhere to be used in the national monitoring of SARS-CoV-2.

Protocol validation

Stability of wastewater samples

Stability of SARS-CoV-2 RNA in wastewater was tested in two separate trials by storing 10-L bulk wastewater samples from Danish wastewater treatment plants at 4 ± 2 °C. In part A, one bulk sample was stored for 14 days, and tested in multiple replicates at least every second day. Stability was then estimated by calculating the mean and 95% confidence interval for each date of testing. In part B, 67 different wastewater samples were tested on day 1, and re-tested after a varying number of days (between 8 and 35 days). Stability of viral RNA in wastewater over time, was then estimated by calculating the difference in Ct-values between the first and second analysis (for each SARS-CoV-2 gene target, N & RdRP-gene). Potential viral degradation and changes in viral load within wastewater samples were determined by fitting a linear regression with the difference between trial one and two as response variable, and the number of days

between first and second trial as explanatory variable.

Repeatability, precision and sensitivity

Repeatability was investigated by performing duplicate tests on 216 different wastewater samples, and calculating the difference between the Ct-values obtained in each replicate. Protocol precision and sensitivity can be evaluated by performing dilutions of wastewater samples. 10-fold dilution series were prepared on 5 different wastewater samples, and the viral load was then assessed in each dilution. The precision of dilutions and the overall sensitivity of the method, particularly in dilutions with low viral load, was thus evaluated by inspecting the slope of the best fitting linear regression between Ct-values and the dilution factor.

Process control murine norovirus

MNV was spiked into wastewater samples before precipitation (see "Principle of precipitation"). Presence of the process control in wastewater samples, which have undergone precipitation and RNA extraction, thus indicated successful completion of all steps in the analysis. Stability of MNV as a wastewater process control was evaluated by evaluating the presence of MNV in 1963 wastewater samples spiked with 50.000 copies of MNV.

Quantification – standard curve precision and repeatability

Repeatability and precision of the IVT RNA standard curves were evaluated by running 67 individual 10-fold serial dilutions and calculating the mean and standard deviation for each 10-fold dilution point. The best fitting linear regression slope and associated standard error was then calculated to determine the uncertainty contribution of the quantification method.

Results

In the following sections, results of validation of the Eurofins Covid-19 Sentinel™ protocol are presented.

Stability of wastewater samples

Trial A: Storage of a large bulk sample, tested in multiple replicates, showed no significant (CI 95%) effect of time on the recovery of SARS-CoV-2, and thus no evidence of systematic degradation in the individual wastewater sample after 14 days of storage at 4°C (Figure 2) was observed.

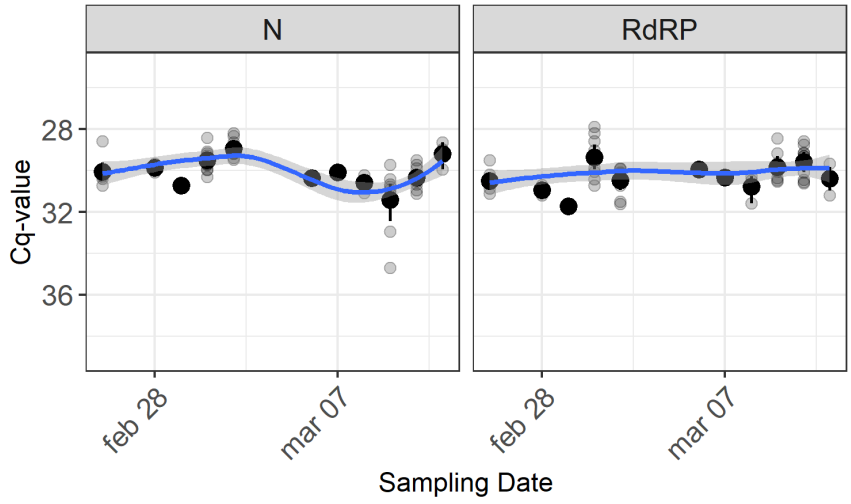

Figure 2. *Ct-values for one 10 L bulk sample tested in multiple replicates over time (14 days of storage).*

Trial B: Storage of 67 different wastewater samples for up to 35 days revealed a non-significant change in recovery of SARS-CoV-2 for the RdRP-gene (P = 0.139), whereas a slight (but non-significant) trend towards a degrading effect of time emerged for the recovery of the N-gene (P =0.295). The maximum observed difference between the first and second tests corresponded to approximately ± 2 Ct-values. Considering the magnitude of variation in waste water matrix along with slight variations in PCR-performance, the observed differences does not indicate any critical decay of SARS-CoV-2 in the present analysis for neither the N-gene nor the RdRP-gene (Figure 3).

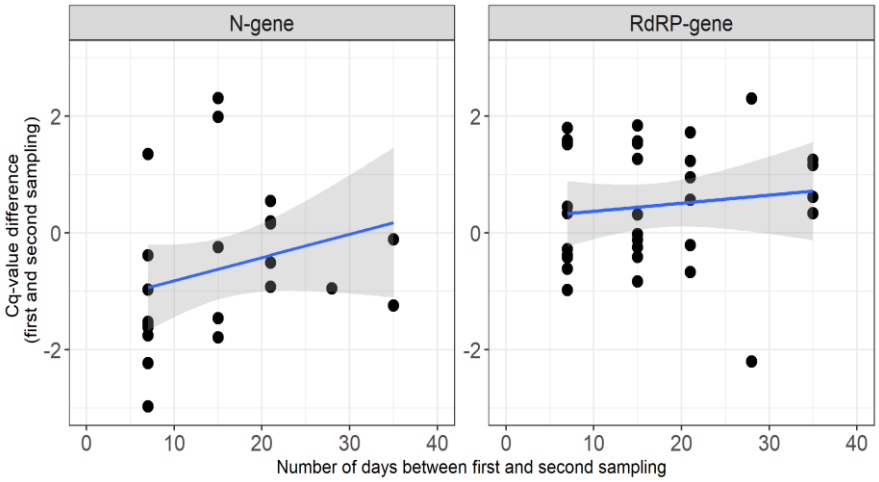

Figure 3. *The difference in Ct-values obtained between first and second sampling of 67 different wastewater matrices. Shown are raw difference estimates and the best fitting linear regression with difference as response and the number of days between first and second sampling as explanatory factors.*

Repeatability

Only minor variations between replicates of 216 different wastewater samples, were identified (Figure 4). The majority of the difference in replicates were within a range of ± 2 Ct-values, suggesting an adequate repeatability of the present -protocol.

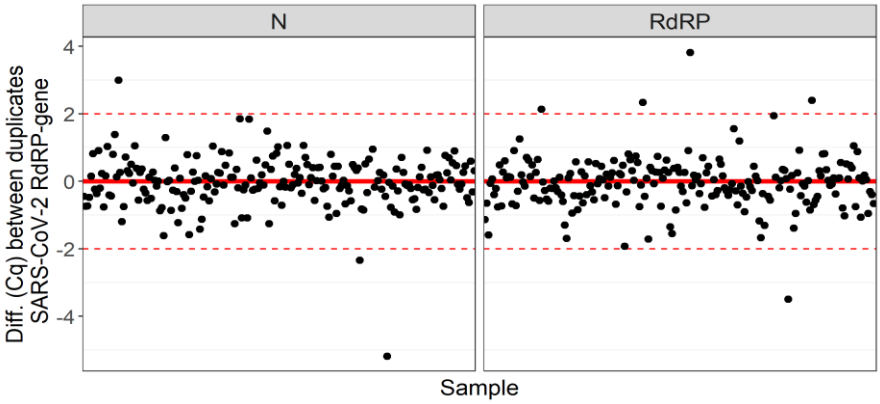

Figure 4. *The difference between duplicate runs of 216 different wastewater samples (where each black dot represents a different wastewater matrix). The majority of samples are within ±2 Ct-values (red dashed reference lines).*

Precision and sensitivity

Trial A: 10-fold dilution series of the spike-in process control (MNV) were performed on five different wastewater samples. The data showed a satisfying dilution series, and successful identification of MNV in low concentration (except for one sample). Similarly, for the two SARS-CoV-2 genes (RdRP and N-gene), serial dilutions also performed well, although the 100-fold dilution were undetected in 2-3 samples (Figure 5).

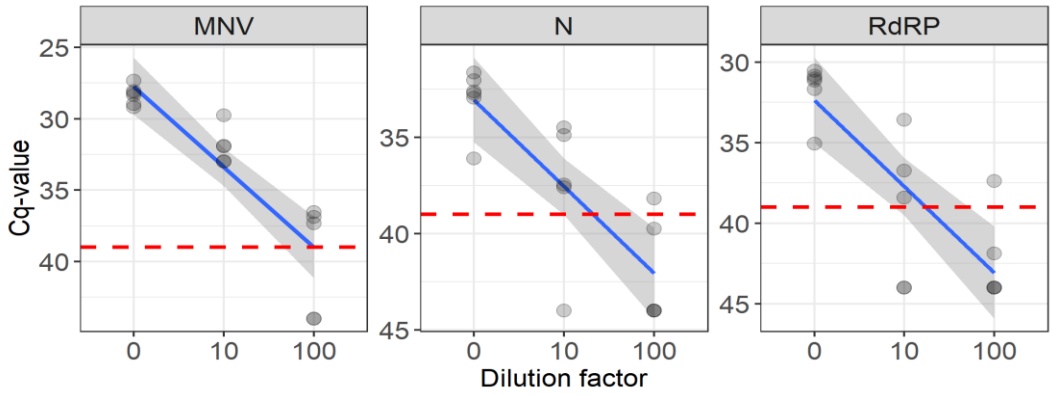

Figure 5. *10-fold dilution series of wastewater samples spiked with process control MNV. Shown here are Ct-values for each dilution series and the best fitting linear regression through the 5 samples in 10-fold dilutions. Note that only MNV should be comparable between samples.*

Process control murine norovirus

Among 1963 samples spiked with MNV, the majority of samples had Ct values of MNV between 26.5 and 28. Considering PCR technical variation, and slight variation in extraction efficiency, MNV is considered stable, robust and thus a suitable process control in wastewater analyses (Figure 6).

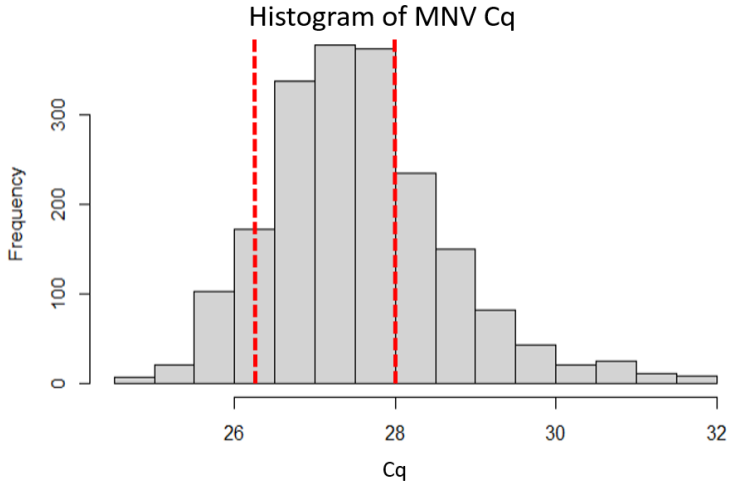

Figure 6. *Histogram of Ct-values obtained for the spike-in process control MNV in a total of 1963 wastewater samples.*

Quantification – standard curve precision and repeatability

A total of 67 replicate dilution series of IVT RNA (for both targets, N and RdRP) suggest high precision, efficiency, and repeatability in the preparation of the standard curves. By fitting a linear regression between Ct-values and log10-transformed IVT RNA concentrations (copies/reaction), slopes of -3.24 and -3.22 were obtained (corresponding to efficiencies of 103% and 104%) for N and RdRP genes, respectively (Figure 7).

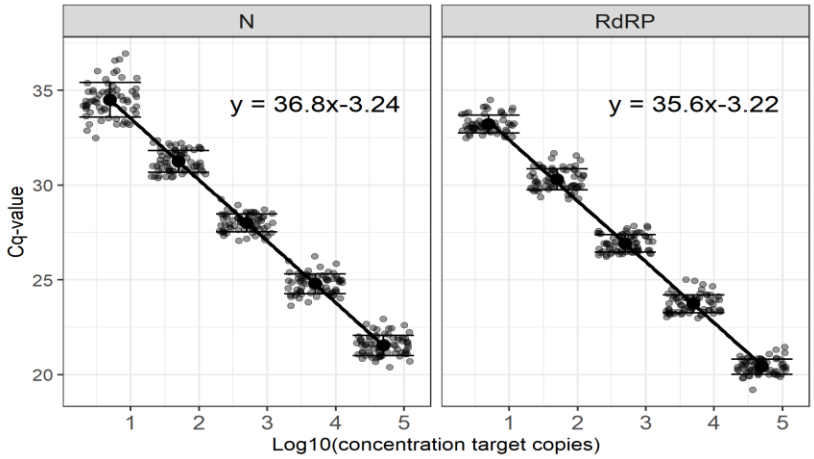

Figure 7. *Ct-values for a total of 67 individual 10-fold serial dilutions with N and RdRP IVT RNA. Shown are Ct-values, the mean for each concentration, and the associated 95% confidence interval. The best fitting linear regression (black solid line) and the corresponding regression formula for each fit is shown in the right-hand corner.*

Discussion

This paper provides an extensive validation of the Eurofins Covid-19 Sentinel™ SARS-CoV-2 wastewater testing protocol, including an evaluation of wastewater matrix properties and viral degradation. With this paper we hope to further strengthen the current knowledge of SARS-CoV-2 in wastewater, and to inspire other laboratory groups to streamline protocols. We provide a "Proof Of Concept" studies of method robustness, high throughput, and application in the Danish National SARS-CoV-2 Wastewater monitoring program[22].

Precipitation of viral matter using PEG-8000 has proven applicable in regards to consistent monitoring of SARS-CoV-2 given the stable performance of the precipitation method. However, the method of PEG-precipitation has disadvantaged as well, as the precipitation is not specific for viral matter. Inhibitors such as biological material, metals, and minerals are expected to be precipitated along with the viral matter. The inhibitors can affect the PCR-analysis in lesser or greater extent based on the efficiency of the RNA extraction and purification. In spite of the assumption of notable inhibition, PEG precipitation has proven highly favorable based on the stability of the method, the low cost, the quick process, and the nontoxicity of the reagents.

Robustness and repeatability of the Eurofins Covid-19 Sentinel™ protocol was investigated by analyzing 216 samples in duplicates, and comparing the obtained Ct-values. This study suggested that the method is highly repeatable, with a maximum of ±2 Ct-values between the replicates (Figure 4). Furthermore, results of dilution series revealed an acceptable level of recovery after 10-fold dilution, both for SARS-CoV-2 RNA, as well as the spike in process control (Figure 5). The efficiency of the dilution studies resembles that of the diluted IVT RNA used for the standard curves. Both dilution of wastewater samples and IVT RNA had an efficiency of approximately 100%. This consistency in efficiency supports the method of quantification in regards to preparing the standard curves using IVT RNA. In estimating the uncertainty of the standard curves we observed uncertainties within a ± 0,5 Ct-values. This result supports high repeatability in quantitative estimates of SARS-CoV-2 in wastewater samples. However, it should be noticed, that the uncertainty is not uniform through the range of quantification. Greater variations were observed at lower concentrations, which was expected due to the exponential amplification in RT-qPCR.

The MNV process control was carefully tested in a large number of samples, and found to be a highly reliable process control. If the concentration of MNV was out of scope for the individual samples a reanalysis was performed to insure no errors had occurred during the process of analysis. MNV is not fully recovered during precipitation, which is likely the case for SARS-CoV-2 as well. Though MNV is highly reliable as a process control, the level of recovery of MNV is not directly related to that of SARS-CoV-2. During the optimization processes of the additions of PEG, MNV was observed to behave differently than SARS-CoV-2 genes. Though stable within each alteration, MNV shoved a

sensitivity to the method of PEG-addition, whilst the Ct values of the SARS-CoV-2 genes were stable both within and between methods. The recovery percentage of SARS-CoV-2 during the precipitation is still unknown. The quantification of Ct-values should be used with caution, since the recovery of SARS-CoV-2 is unknown. The analysis provides a foundation for monitoring sampling sites over time and to compare different sites with similar matrix components. In spite of the assumption, that the quantification is subject to a negative bias, it allows for better monitoring and comparison than Ct-values, since the quantification eliminates the variations in RT-qPCR performance.

Whether viral RNA degrades over time (and how quickly) in wastewater, remains an unanswered question. Consensus however, seems to be that viral degradation is highly dependent on sampling location and matrix. Understanding the level of sample specific degradation is vital for retrospective analysis of stored wastewater samples[23]. Storage conditions and viral stability were tested in a large number of wastewater samples (from different locations), and no direct trend of degradation during 30 days of storage at 4°C (Figure 2 & 3) was observed. However, additional tests have shown that detection of viral RNA in wastewater from industries which release chemicals (e.g. waste cleaning services for industrial waste) or alter the pH-level of the wastewater, as well as wastewater from slaughter houses with high content of blood, is indeed impaired. On this basis, we recommend that sampling locations are carefully selected with respect to potentially interfering industries. We also highly recommend the use of flow-proportional- or time-dependent sampling, according to the guidelines provided in ISO 5667-10:2004[24].

By July, 2021, the Eurofins Covid-19 Sentinel™ protocol was implemented in the National SARS-CoV-2 Wastewater Monitoring Program in Denmark. Since its implementation, wastewater monitoring data has been compared to the extensive clinical sampling results, with particular focus on the changes in viral concentration in wastewater over time. Satisfactory correlation between wastewater titers and clinical estimates of infection rate was established early on in Denmark[22], as well as in many other countries[10,25]. The extensive wastewater dataset has furthermore paved the way for early warning of local increases in transmission and infection rate[26]. In such instances, local increases in the number of SARS-CoV-2 copies in the wastewater over a short period of time, has led to public recommendations for timely testing and for increasing the focus on social distancing, masks, and other hygienic measures.

In being inexpensive and fast the described method of precipitation and RT-qPCR has proven effective in regards to national monitoring of SARS-CoV-2 in waste water, since a great sample load can be executed in a short amount of time. Though the quantification is presumed biased, the bias is presumed stable for each individual sampling point. For future use, the recovery of SARS-CoV-2 during precipitation might be estimated with inactivated, intact SARS-CoV-2 copies. Furthermore, viruses with similar properties as SARS-CoV-2 could be used as substitutes in regards to determination of bias.

Conclusion

The essential protocol of this study shows a thorough validation of a robust, quantitative method for detecting the presence of SARS-CoV-2 in wastewater samples. The Eurofins Covid-19 Sentinel™ protocol has been broadly implemented, initially in the United Kingdoms as part of the national surveillance program in the United Kingdoms, and subsequently in the Danish National Monitoring Program. A highly repeatable - protocol with a broadly application potential for monitoring SARS-CoV-2 was demonstrated. The PEG precipitation protocol is easy to use, can sustain a high volume of samples, and is therefore very suitable for high throughput laboratories. The method furthermore includes a suitable, and robust process control, for the verification of successful completion of all analysis steps; precipitation of viral particles, RNA extraction, and RT-qPCR. The Eurofins Covid-19 Sentinel™ Program supports the strength of wastewater testing as an early warning tool, and as an indicator of local infection prevalence over time. Wastewater monitoring is however underlined with a number of uncertainties which should be considered throughout the process; from determination of sampling locations to the interpretation of analysis results. However, with increasing numbers of datasets and better understanding of the behavior of SARS-CoV-2 in wastewater and humans, laboratories continuously learn more about how to navigate the covid-19 pandemic, and how to best use the wastewater testing as the primary tool for surveillance in local communities.

Conflict of interest

The authors A. Jørgensen, J. Gamst, I.H. Rasmussen, and D.M. Ejegod are employed by Eurofins Environmental Testing Denmark, which designed

and provides the Eurofins Covid-19 Sentinel™ protocol.

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