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SARS-CoV-2 detection in wastewater using multiplex quantitative PCR

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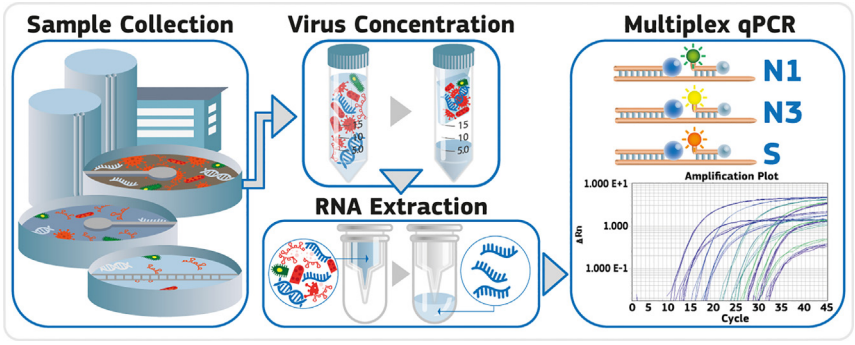
HIGHLIGHTS

- Set up of multiplex RT-qPCR for detection of three target SARS-CoV-2 RNA genes
- Method tested on four untreated local wastewater samples showing the robustness
- Multiplex advantages are decreased sample number, cost and time for the analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

A multiplex reverse transcription quantitative PCR (RT-qPCR)-based method was designed for the simultaneous detection of different SARS-CoV-2 genes. In this study, we used three target genes encoding for the nucleocapsid 1 and 3 (N1, N3), and the spike (S) proteins, all commonly used in the detection of SARS-CoV-2 in human and environmental samples. The performance of the multiplex assay, compared to the single assay was assessed for the standard calibration curve, required for absolute quantification, and then, for the real environmental samples to detect SARS-CoV-2. For this latter, four environmental samples were collected at a local wastewater treatment plant (WWTP). The results showed that the cycle threshold (Ct) values of the multiplex were comparable to the values obtained by the singleplex PCR. The amplification of the three target genes indicated the presence of SARS-CoV-2 in the four water samples with an increasing trend in February and these results were confirmed in the multiplex approach, showing the robustness of this method and its applicability for the relative abundance analysis among the samples. Overall, both the laboratory and field work results demonstrated that the multiplex PCR assay developed in this study could provide a method for SARS-CoV-2 detection as robust as the single qPCR, but faster and cost-effective, reducing by three times the number of reactions, and consequently the handling time and reagents.

1. Introduction

In December 2019, a novel coronavirus pneumonia (COVID-19) outbreak arose in Wuhan, China, caused by a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 has been a public health emergency of international concern, with cases reported in 223

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countries and territories (WHO, 2021a). The main route of transmission for this new virus is by person-to-person contact (contact transmission), respiratory excretions generated by sneezing, coughing, and aerosols in intensive care units (Allen and Marr, 2020; CDC, 2020; Tang et al., 2020).

Although SARS-CoV-2, like other coronaviruses, shows low stability in the environment (La Rosa et al., 2020a), the presence of viral genetic material in the stool of positive subjects could reflect the abundance of SARS-CoV-2 RNA content in untreated wastewaters (Foladori et al., 2020; Wurtzer et al., 2020a) and surface waters to which wastewaters are directly discharged (Guerrero-Latorre et al., 2020). The monitoring of enteroviruses and bacterial pathogens in water is a recognized epidemiological practice within public health surveillance strategy (Asghar et al., 2014; Moazeni et al., 2017). On this basis, several studies showed that water-based surveillance of SARS-CoV-2 could be used as a complementary method to clinical diagnostic and early warning system for COVID-19 local re-emergence (Aguiar-Oliveira et al., 2020; La Rosa et al., 2021; Murakami et al., 2020; Randazzo et al., 2020; Saguti et al., 2021; Wu et al., 2020; Wurtzer et al., 2020b). Although the correlation between viral RNA quantification and epidemiological cases has not yet been established, the monitoring in waters could help to draw a dynamic trend of the virus in the population and to assess the efficacy of actions taken to contain the outbreak. The wastewater surveillance becomes critical particularly in areas with limitations in clinical testing, and can be used to obtain spatial and temporal distribution map of the viral infection in a community (Hamouda et al., 2021). Indeed, in low-income countries, WWTP monitoring can be an important tool for the fight against COVID-19 spread. However, the sampling should be planned according to the country's needs, as in many cases a significant portion of produced sewage does not reach the WWTP. In this respect, public schools and community toilets could represent an easier track of the community health (Calabria de Araujo et al., 2020).

Thus far, technical limitations are among the main challenges in achieving fast and reliable outcomes (Ahmed et al., 2020; Medema et al., 2020a; Patel et al., 2021). Polymerase chain reaction (PCR) based methods currently employed for accurate and sensitive detection of SARS-CoV-2 target genes in environmental samples mainly rely on quantitative PCR (qPCR) and reverse transcription qPCR (RT-qPCR). However, dissimilar rates of detection have been reported for various SARS-CoV-2 genes when using different assays, primers and probes, reason for which inclusion of multiple targets in the PCR testing was recommended (Patel et al., 2021). In turn, major volumes of samples and reagents are required along with increased time for an overall analysis, especially when screening for SARS-CoV-2 variants.

Multiplex PCR-based approach allows a simultaneous detection and/or quantification of multiple gene targets in one sample with the same sensitivity as detected in singleplex. The use of a multiplex, in a monitoring analysis, permits to obtain more information using less sample volume. It is time-saving, more cost-effective as the amount of reagents is reduced, and it helps to minimize the variability due to the pipetting. However, given the complexity of the multiplex PCR assay, the assay often requires optimization to avoid the interaction between primers/probe sets or the competition for common reagents within the reaction targeting different genes. Several multiplex approaches have been developed for providing a quick molecular diagnosis of COVID-19 (Attwood et al., 2020; Visseaux et al., 2020; Waggoner et al., 2020). Some multiplex protocols were developed for a dual detection of SARS-CoV-2 and influenza virus (Chung et al., 2021; Mancini et al., 2020; Nörz et al., 2021), for the detection of multiple respiratory pathogens (Hirotsu et al., 2020; Panning et al., 2020; Williams et al., 2020), and for the detection of variants (Islam et al., 2021). Other multiplex approaches were aimed at library preparation for genome sequencing (Paden et al., 2020; Tyson et al., 2020; Xiao et al., 2020) and respiratory viral infection trend analysis (Marriott et al., 2020). Simultaneous detection of viral targets by droplet digital PCR (ddPCR) was developed using SARS-CoV-2 genome obtained from cultured in Vero E6 cells with spiked control (Nyaruaba et al., 2021), nasopharyngeal/throat swabs and saliva samples (Cassinari et al., 2021, de Kock et al., 2021, Deiana et al., 2020, Williams et al., 2020). However, few studies reporting the use of multiplex RT-PCR for environmental testing, have been published. One of them involved the detection of microbiological contamination on cloth masks during COVID-19 pandemic (MacIntyre et al., 2020).

This study presents the development of a multiplex method for a simultaneous detection of three target genes (N1, N3 and spike (S)), which are commonly used for the detection of SARS-CoV-2 (Ahmed et al., 2020; La Rosa et al., 2020b; Medema et al., 2020b; Sherchan et al., 2020). The gene N1 is the most frequently used as an indicator for SARS-CoV-2 detection in wastewater samples followed by N2 gene, and N3. This latter showed to be more sensitive than N2 (Hamouda et al., 2021; Medema et al., 2020b) and for this reason, it was included in the present study. The S gene was selected in order to investigate whether, later on, it could be used to distinguish among the variants since the key mutations occur in this gene. We demonstrated that the multiplex has the same sensitivity as the single step in a real wastewater sample. Moreover, differences in sensitivity between the target genes and the gene copy number variations over a three-month period (December 2020–February 2021) have been determined and confirmed also by the multiplex approach. These results showed that the multiplex method provides a robust, rapid and reliable approach applicable to monitoring SARS-CoV-2 in wastewater samples.

2. Materials and methods

2.1. Reference material for controls and quantification

The Directorate for "Health, Consumers and Reference Materials" of the European Commission's Joint Research Centre (JRC) kindly provided the reference material (RM)-EURM-019 as positive control for the SARS-CoV-2 detection (Corbisier et al., 2020). The RM is a synthetic RNA sequence of 880 nucleotides (nt) which contains the target regions of eight different genes widely used in the SARS-CoV-2 testing, such as N1, N2 and N3 genes (CDC, 2020), RdRp and E genes (Corman et al., 2020), the NIID 2019-nCOV_N (Nao et al., 2020), the Thailand N gene (WHO, 2020) and the S gene (Corbisier et al., 2020). Five microlitres of a 1000× dilution of RM was used as control to verify the correct transcription and amplification during the SARS-CoV-2 detection by RT-qPCR in single and multiplex assays.

Plasmid pCoV2 was used to generate a calibration standard curve to quantify SARS-CoV-2 RNA amplified by using the RT-qPCR primers/probe sets either in single or multiplex assays. pCoV2 is a standard pUC57 vector containing a synthetic cDNA sequence of RM EURM-019 (Twinhelix, Italy) as shown in Fig. 1. The plasmid was transformed in *E. coli*, and positive colonies were picked either for growing in liquid media for DNA extraction or stored at −80 °C in glycerol stock.

To generate the standard curves, the concentration of the pCoV2 DNA was determined by Nanodrop (Spectrophotometer 8000) and a stock solution of 1×10^8 genomic copy (gc) per mL was prepared. This stock solution was then 10-fold serial diluted down to 1×10^3 gc/mL, and aliquots were stored at −20 °C. In the qPCR, a final volume of 5 μL of each dilution, for concentrations ranging from 500,000 to 5 viral copies number, was used to perform the amplification as described in Section 2.4. The standard curve was generated for each run using the primers either in a single or in a multiplex assay. Three technical replicates were run each time to generate the standard curves. The run data was analyzed with SDS software v2.4 (Applied Biosystems, Thermo Fisher Scientific) and the cycle threshold (Ct) was calculated as the intersection between an amplification curve and the baseline of the background signal. The Ct values were plotted against the Log viral copy number (5.70 to 0.70) to generate the standard curve for each target gene. Linear regression was performed for each curve and the trend line equation ($Ct = slope \times (\text{Log (viral copy number)}) + intercept$)

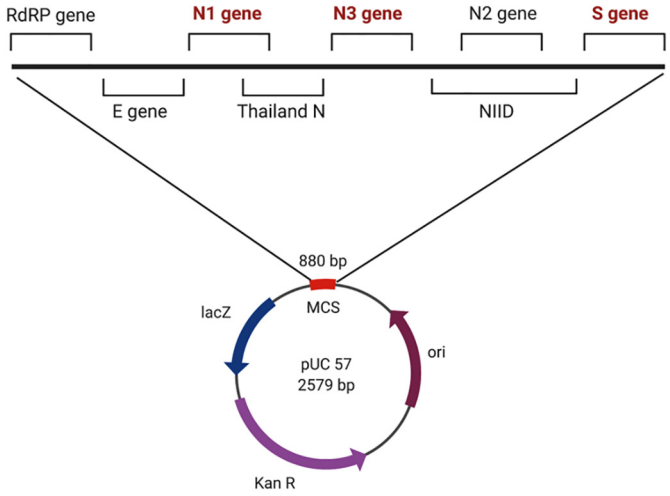

Fig. 1. Schematic organization of plasmid pCoV2. The DNA plasmid was used to generate a standard curve for the quantification of the SARS-CoV-2 N1, N3 and S target genes. The different blocks represent the target genes and their location in the synthetic DNA sequence of 880 nt length. In red the three target genes used in this study. Figure was created with BioRender (https://biorender.com).

used to calculate the copy number for each sample. The slope (m) and intercept (b) values were obtained from the linear regression of the standard curves ($y = mx + b$), plotting the 10-fold serial dilution (log copy number) vs the Ct values obtained from each dilution. The LOD was 5 copies, which corresponds to the lowest number of copies that could be amplified in triplicates.

The amplification efficiency (E) was calculated for all the standard curves by, *i*) first plotting the Ct values versus the Log of the input gc to derive the slope of the linear curve fit and then, *ii*) by using the following equation:

$$E = \left(10^{\left(-\frac{1}{Slope}\right)} - 1\right) \times 100$$

The coefficient of correlation (R^2), obtained for the linear regression, is a measure of how well the data fit the standard curve. It is used to evaluate the performance of primer sets and its value ideally should be >0.9.

2.2. Wastewater sample collection and processing

A total of four untreated wastewater samples were collected from the influent of a WWTP located in Lombardy area (North Italy) between December 2020 and February 2021. The WWTP is a local treatment plant which treats domestic and hospital wastewater, and represents a population of 1.1×10^5 habitants. The wastewater samples (500 mL) were sub-sampled in sterilized plastic bottles from a 24 h composite sampler on December 11th 2020, January 11th and 18th 2021 and February 1st 2021, transported at 4 °C, aliquoted in 100 mL volumes and processed on the same day.

Briefly, following an initial centrifugation at 4500 ×*g* for 30 min without break at 4 °C to precipitate large particles, the supernatant was collected and concentrated with 10 kDa Amicon®Ultra 15 centrifugal filters (Merck, Germany) (Gonçalves et al., 2021). The centrifugal device had a sample volume of 15 mL, therefore all the volume (100 mL) was centrifuged six times at 4000 xg for 45 min without break. The concentrated wastewater sample was collected from the reservoir with a pipette and transferred to a clean microtube (~500 μL), and stored at −80 °C until further analysis.

Two 50 mL aliquots of influent wastewater samples were artificially inoculated with ~10^6 gc/L of a heat-inactivated SARS-CoV-2 virus kindly provided by the National Public Health Centre from Hungary. The virus was isolated from an anonymous patient (isolate SZ-16) and heat-inactivated of the National Safety Laboratory in the National Public Health Centre. The aliquots were shipped frozen to the JRC and were defrosted at 4 °C before the concentration step using 10 kDa Amicon®-Ultra 15 centrifugal filters (Merck, Germany). The inoculated samples were used to evaluate the concentration and extraction method of viral RNA from wastewater samples. The samples were tested in singleplex reaction using N3 gene by RT-qPCR in triplicates. The recovery efficiency, which takes into account the known amount of virus added to the water sample and the final quantification of viral RNA measured by qPCR, was calculated using the following formula:

$$Recovery\ efficiency\ (\%) = \frac{Total\ viral\ RNA\ gene\ copies\ recovered}{Total\ viral\ RNA\ gene\ copies\ seeded} \times 100$$

2.3. RNA extraction and reverse transcription to cDNA

A 250 μL aliquot of concentrated wastewater sample was transferred to a 2 mL bead lysis tube and RNA was extracted using Quick-RNA™ Fecal/Soil Microbe Microprep kit (Zymo Research Europe GmbH, Germany) according to the manufacturer's instructions with slightly modifications. Briefly, a volume of 800 μL of S/F RNA lysis buffer supernatant was taken instead of 400 μL, and the RNA was eluted in 50 μL. A blank sample was included during the RNA extraction to monitor any possible cross contamination during sample processing. Fifteen microlitres of the extracted sample was retro-transcribed to cDNA using SuperScript™ IV (Invitrogen, Thermo Fisher Scientific). The cDNA was aliquoted and stored at −20 °C.

2.4. SARS-CoV-2 analysis by single and multiplex RT-qPCR

To minimize cross contamination, good laboratory practices were observed i.e. using different laboratory locations for sample processing, RNA extraction, reverse-transcription of samples and qPCR.

All RT-qPCR were performed on Applied Biosystems 7900HT Real Time PCR Systems with ABI 7900 software SD2.4. The genes N1, N3 and S were used for the detection of SARS-CoV-2 in environmental samples using the TaqMan chemistry system in either singleplex or multiplex assays. The master mix was prepared using 12.5 μL of Environmental Master Mix 2.0 (Thermo Fisher Scientific), primers and probes as listed in Table 1, and RNAse/DNAse free water to a final volume of 25 μL per reaction. The cycle conditions for the qPCR were 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, and 58 °C for 30 s. All the RT-qPCR reactions were run in triplicates. To detect any RT-qPCR inhibition, the RNA samples were tested using different volumes (10 μL, 5 μL, and 2.5 μL).

For the detection of target genes, three different 5′ fluorescent reporter dyes were used for the probe, 6-Carboxyfluorescein (6-FAM, ~517 nm) to detect N1 gene, 2′-chloro-7′phenyl-1,4-dichloro-6-carboxyfluorescein (VIC, ~551 nm) for N3 gene, and 2′-chloro-5′-fluoro-7′,8′-benzo-1,4-dichloro-6-carboxyfluorescein (NED, ~575 nm) for S gene. The quenchers at the 3′ end of the probe were QSY (Thermo Fisher Scientific) coupled to 6FAM and VIC dyes, while minor groove binder non-fluorescent quencher (MGBNFQ) was coupled to NED dye (Table 1).

2.5. Statistical analysis

Ct and gc/L values for single and multiplex PCR were compared using RStudio Software (R version 3.6.1) and Prism version 9.0.2 (GraphPad Software, California USA). Shapiro-Wilk test was used to evaluate the normality of the data. The mean range and the standard error of Ct

Table 1
List of primers and probes used in the single and multiplex assays. The table lists the concentrations used from each primer and probe in the final qPCR reaction, and the expected amplified fragment size. N, nucleocapsid; S, spike; Fw, forward; Re, reverse; P, probe. In bold, the 5′ fluorescent reporter dyes of the TaqMan probes, FAM, VIC and, NED and the 3′ quenchers, QSY and MGBNFQ are listed.

Target gene	Primer or probe	Sequence (5′-3′)	Concentration (nM)	Product size	Ref
	N1_Fw	GACCCCAAAATCAGCGAAAT	200		
N1 gene	N1_Re	TCTGGTTACTGCCAGTTGAATCTG	200	72	(CDC, 2020, Medema et al., 2020b)
	N1_P	**FAM**-ACCCCGCATTACGTTTGGTGGACC-**QSY**	200		
	N3_Fw	GGGAGCCTTGAATACACCAAAA	200		
N3 gene	N3_Re	TGTAGCACGATTGCAGCATTG	200	72	(CDC, 2020, Medema et al., 2020b)
	N3_P[a]	**VIC**- AYCACATTGGCACCCGCAATCCTG-**QSY**	200		
	S_Fw	GACATACCCATTGGTGCAGG	900		
S gene	S_Re	TGACTAGCTACACTACGTGCC	900	83	(Corbisier et al., 2020)
	S_P	**NED**- AGACTCAGACTAATTCTCCTCGGCG-**MGBNFQ**	450		

[a] International Union of Pure and Applied Chemistry (IUPAC) codes: Y = C or T.

and gc/L values were calculated, the data were compared using a paired *t*-test analysis or a two-way ANOVA test, followed by a Tukey's post hoc test. The slopes of the standard curves were compared using an analysis of covariance (ANCOVA), followed by a Tukey's post hoc test. The *p* values <0.05 were considered statistically significant.

Since an RNA sample can yield RT-qPCR positive signal above the LOD, a cut-off of <45 Ct was used to determine positive samples as suggested in the paper by Ahmed and his team (Ahmed et al., 2021). In order to consider a sample as positive for SARS-CoV-2, two of the three replicates should give an amplification with a Ct value below 45. If only one of the replicates shows amplification, the sample is considered inconclusive and listed as negative for SARS-CoV-2.

3. Results and discussion

3.1. Generation of calibration/standard curve and limit of detection in multiplex assay

To develop the multiplex, two different concentrations of primers and probes, two annealing temperatures and three different master mixes were tested in order to obtain the same sensitivity as in the singleplex approach. During the setup of the multiplex, we observed competition for reagents, reason for which one of the three genes could not amplify. This technical problem was overcome by changing the mastermix (Fig. S1, Table S1). We also accounted for the amplification efficiency at very low cDNA amount for the standard curve, therefore different temperatures and master mixes were tested to determine the best conditions, as reported in Material and Methods.

To evaluate the LOD for the total viral RNA's copies and to quantify N1, N3 and S genes, the plasmid pCoV2, containing those target genes, was used to generate a 10-fold dilution series (5×10^5 to 5 genomic copies). The Ct values for each concentration were determined in triplicates by performing either a single or multiplex assay in order to compare the efficiency of amplification of both methods for each target gene (Fig. 2). As shown in Table 2, the Ct values obtained with the multiplex assay were similar to the singleplex RT-qPCR, and no statistical differences were found between standard curves (paired *t*-test, $p > 0.05$), or by comparing the slope of standard curves (ANCOVA, $p > 0.05$). The R^2 ranged between 0.997 and 0.999, and the amplification efficiency was above 95% for all primers/probe sets (Fig. 2 and Table 2). The assay was sensitive enough to detect 5 gene copies both in single and multiplex reactions. These data show that our multiplex RT-qPCR assay has a similar performance for the detection of SARS-CoV-2 as using a single gene assay approach.

Robustness and reliability, similar to the qPCR of each single target gene, are provided by the specificity and sensitivity of the primers/probe set and the method set up. Linked to this latter, the reliability has been determined by the constant amplification efficiency resulting in all reactions higher than 95%. In addition, multiplex is faster since it reduces by three fold the number of replicates, assuming that for each target gene the analysis of three replicates is performed. Consequently, handling time as well as the cost of reagents become lower, particularly convenient for a high number of samples.

3.2. Viral recovery and RT-qPCR performance for WWTP samples

To determine the viral recovery, two wastewater samples were spiked with 2.77×10^6 copies/L of a heat-inactivated SARS-CoV-2. The extracted RNA was reverse transcribed and used to amplify the target N3 gene in triplicates (see Materials and methods, Section 2.4). N3 amplification generated a Ct average value of 35.52 ± 0.27 for sample 1 and 35.96 ± 1.11 for sample 2, corresponding to a concentration of genomic copy numbers of 5.14×10^4 and 4.54×10^4 gc/L for, respectively, derived from the absolute quantification (see Fig. S2). The recovery

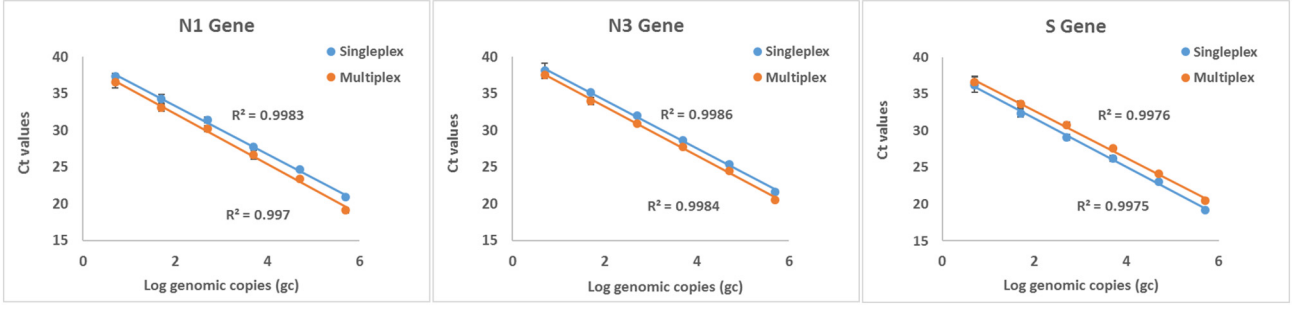

Fig. 2. Comparison of single and multiplex assay performance for the three SARS-CoV-2 target genes (N1, N3 and S). The graphs show the standard curves obtained for each gene using either the single or the multiplex approaches. Ct values were based on the 10-fold serial dilution from 500,000 to 5 viral genomic copies using the plasmid pCoV2. The genomic copies are presented as logarithm (Log). All qPCR assays were run in triplicates. The vertical bars represent the standard errors. R^2 values are also shown in the graphs for each assay, top for singleplex and bottom for multiplex. No significant differences were observed between assays (*t*-test $p > 0.05$). Ct, Cycle threshold.

Table 2
Ct values in a 10-fold dilution series of pCoV2 plasmid to determine the limit of detection. The R^2, and efficiency of amplification are also listed. The RT-qPCR was run in triplicates. Ct, cycle threshold; SD, standard deviation.

Genomic copies/reaction	Average Ct values ± SD					
	N1		N3		S	
	Singleplex	Multiplex	Singleplex	Multiplex	Singleplex	Multiplex
500,000	20.91 ± 0.02	19.12 ± 0.36	21.64 ± 0.29	20.48 ± 0.16	19.23 ± 0.06	20.46 ± 0.27
50,000	24.64 ± 0.17	23.37 ± 0.07	25.40 ± 0.25	24.48 ± 0.03	23.09 ± 0,09	24.14 ± 0.21
5000	27.77 ± 0.19	26.63 ± 0.58	28.66 ± 0.20	27.76 ± 0.12	26.23 ± 0.41	27.59 ± 0.21
500	31.40 ± 0.38	30.25 ± 0.38	31.97 ± 0.08	30.92 ± 0.07	29.05 ± 0.39	30.78 ± 0.34
50	34.29 ± 0.57	33.07 ± 0.45	35.12 ± 0.09	33.99 ± 0.48	32.38 ± 0.56	33.60 ± 0.46
5	37.32 ± 0.44	36.54 ± 0.82	38.08 ± 1.05	37.57 ± 0.42	36.25 ± 1.05	36.60 ± 0.78
R^2	0.998	0.997	0.999	0.998	0.998	0.998
Efficiency (E)	102.01	95.93	101.89	99.00	105.03	100.57

efficiency in our laboratory was 1.85% and 1.64% for the respective samples (Table S2).

Four WWTP samples were tested to detect the SARS-CoV-2 RNA. A 100 mL aliquot of each influent wastewater sample was concentrated immediately after the collection and afterwards processed for the viral RNA extraction as described in Materials and methods (Sections 2.2 and 2.3). The RT-qPCR was performed either for each single target gene or together in a multiplex mode to evaluate the performance and sensitivity of the primers/probe set. In all assays, the positive sample was the RM (EURM-019) to ensure the correct transcription and amplification. The Ct values generated by the multiplex qPCR were similar to those obtained by the singleplex assay as shown in the Fig. 3 and Table 3. The N1 and N3 genes were detected in all water samples analyzed either in single or multiplex mode with a Ct ranging from 33.77 to 40.82 (Table 3). Differently, the S gene was detected, with both assays, only in the water samples collected on January 11th and February 1st 2021, while in December 2020, it was observed only in samples analyzed using the single assay. To assess the repeatability and reproducibility of the system, two wastewater samples (11.12.20 and 18.01.21) were tested again on a different day, giving similar results as the ones obtained in the first analysis (Table S3).

In our assay, the S gene was always detected at very high Ct values, close to the LOD, except for the sample collected in February. A possible explanation could be the low viral recovery we observed, compared to other authors (Ahmed et al., 2020, Gonçalves et al., 2021, Michael-Kordatou et al., 2020).

However, the S gene seems less sensitive for the detection of SARS-CoV-2 in environmental samples compared with the N1 and N3 genes. A possible explanation could be that *i*) N transcripts are more abundant than the S ones, as recently reported by Kim et al. (2020) in their study on SARS-Co-V-2 transcriptomics. Indeed, in the reverse transcription, either genomic RNA or the transcripts are all reverse transcribed due to poly(A) tail targeted by the poly(dT) primers. *ii*) Different stability of the sub-genomic RNAs, carrying the RNA genomic transcripts and possibly the one containing the S transcript could be more prone to the degradation in the environmental sample; or *iii*) a combination of both hypotheses. Differences in performance among SARS-CoV-2 detection assays have also been reported in other studies, such as the lower sensitivity of detection in clinical samples for the RdRp gene due a mismatch in the reverse primer (Reijns et al., 2020; Vogels et al., 2020). However, for S gene, to our knowledge, no studies have been described so far. Further investigations are therefore needed to better understand such differences.

Other multiplex approaches have also been developed for the detection of SARS-CoV-2 in clinical samples which involved quantification of up to four SARS-CoV-2 target genes (open reading frame 1ab (ORF1ab), nucleocapsid (N1, N2, N3), RNA dependent RNA polymerase (RdRp) and/or envelope (E) genes), with the majority of studies employing two genes (N1 and N2) in addition to a non-target control (Hirotsu et al., 2020, Kudo et al., 2020, Perchetti et al., 2020, Petrillo et al., 2020). Similar to our approach, results obtained with the multiplex in those studies were comparable to the singleplex (Ishige et al., 2020; Reijns et al., 2020) showing that a faster, sensitive and robust RT-qPCR for SARS-CoV-2 detection can be available at a fraction of the cost which makes the monitoring of viral RNA more achievable. Moreover, the sensitivity of 5 gene copies per reaction reached in the present approach was in the range of the lowest values reported (Petrillo et al., 2020, Reijns et al., 2020). Studies on environmental matrices such as

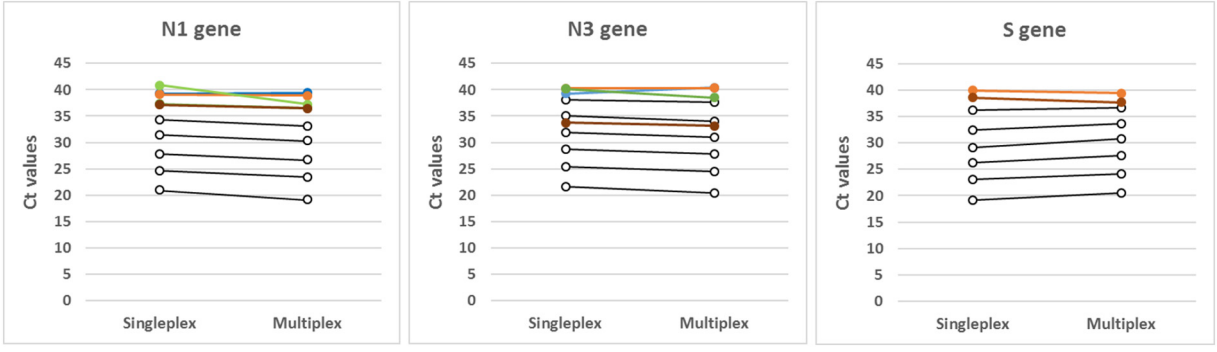

Fig. 3. Ct values comparison between single and multiplex assay. Ct values from the standard curve are in black lines and open circle, and wastewater samples in colored lines for the three SARS-CoV-2 target genes (N1, N3 and S). Similarities between single and multiplex are visualized by parallel lines connecting Ct values for each gene. Sampling dates are represented by the following colors: blue, December 11th 2020; orange, January 11th; green, January 18th and brown, February 1st 2021. No significant differences were observed between assays (*t*-test $p > 0.05$).

Table 3
The average of Ct detected in the wastewater samples. N1, N3 and S genes were amplified either in single or in multiplex assays, and run in triplicates. The table shows the mean of cycle thresholds (Ct) and the standard error ($n = 3$). The reference material (RM, EURM-019) was used as a positive control to evaluate the correct transcription and amplification. UND, undetermined.

Sample	Ct					
	N1		N3		S	
	Singleplex	Multiplex	Singleplex	Multiplex	Singleplex	Multiplex
RM	21.34 ± 0.08	18.10 ± 0.12	23.90 ± 0.04	22.55 ± 0.20	20.53 ± 0.30	21.56 ± 0.32
11.12.20	39.23 ± 0.21	39.51 ± 0.71	39.28 ± 0.28	40.36 ± 0.10	41.46 ± 0.49	UND
11.01.21	39.1 ± 0.64	38.90 ± 1.09	40.23 ± 1.02	40.32 ± 0.60	39.91 ± 0.38	39.43 ± 1.07
18.01.21	40.82 ± 1.49	37.22 ± 0.06	40.20 ± 1.05	38.48 ± 1.26	UND	UND
01.02.21	37.18 ± 0.55	36.45 ± 1.39	33.77 ± 0.08	33.17 ± 0.18	37.70 ± 1.51	38.59 ± 0.05

face masks during COVID-19 pandemic, showed the advantage of using multiplex RT-PCR for a simultaneous detection of various viruses rather than its specificity due to employment of multiple SARS-CoV genes (MacIntyre et al., 2020).

3.3. Quantification of SARS-CoV-2 RNA in WWTP samples

Viral genomic RNA was quantified to evaluate differences between sampling campaigns. The water samples showed an average of 3.46×10^2 to 9.10×10^3 gc/L in the N1 multiplex assay and 2.92×10^2 to 2.90×10^4 gc/L in the N3 multiplex assay (Fig. 4).

No significant differences were observed between the results obtained by single or multiplex assay (Two-way ANOVA, Tukey's post hoc test $p > 0.05$), while significant differences were observed among the sampling dates (Two-way ANOVA, Tukey's post hoc test $p < 0.05$). Indeed, N1 and N3 genes were significantly higher in the sample collected on February 1st compared with the other three samples (December 11th, January 11th and 18th). These results confirmed that the multiplex assay is reliable as the single assay and it can be used for quantification and relative comparison among the sample collections. As described previously, S target gene was amplified with lower Ct in the sample showing higher genomic copy number. In further studies, we will investigate the low detection rate of the S gene and its sensitivity in the detection of SARS-CoV-2 RNA in natural samples. Additionally, we will assess whether the S gene detection is correlated with high copy numbers of viral RNA in a given sample and consequently with the increased of COVID-19 cases in the population. To this aim, we will start a large screening of wastewaters by collecting the samples on weekly basis following analysis with the multiplex approach. As our sampling scheme was set at weekly basis, the epidemiological data will be converted as a 7-days cumulative in order to find correlations between the epidemiological data and our results. Furthermore, during this phase, the viral recovery will be improved by optimising the concentration and extraction step to achieve a better viral RNA yield.

4. Conclusions

In the present study, we demonstrated that the multiplex assay, covering the simultaneous amplification of three SARS-CoV-2 target genes, is a good methodology for monitoring the concentrations of viral genetic material in wastewaters. The advantages are *i*) reduction of possible variability among samples since the qPCR occurs in one reaction, and *ii*) decreased number of samples, therefore cost and time necessary for the analysis are minimized. More importantly nowadays, the multiplex could also be used to target genes in order to discriminate, where possible, between the variants and their relative abundances. Indeed, the design of primers/probe system for a specific region of the gene, such as the nucleotide deletions present in some variants (e.g. B.1.1.7) (Vogels et al., 2021), could specifically target the mutants discriminating them from the Wuhan type in only one reaction. This approach would allow tracing the presence and modulation of mutants within the population. Our current studies are focused on protocols for the detection of SARS-CoV-2 variants in wastewater.

In conclusion, the multiplex assay could contribute to the SARS-CoV-2 surveillance in wastewater, an initiative advocated at European and global level, and recommended by the European Commission and the World Health Organization (EC, 2021; WHO, 2021b).

Disclaimer

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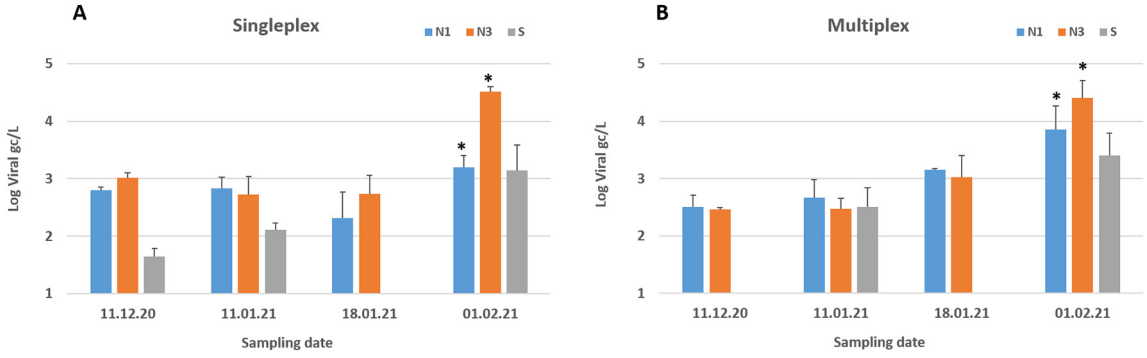

Fig. 4. Quantification of viral RNA in samples from influents of the wastewater treatment plant (WWTP). The genomic copies (gc) were calculated per litre and are expressed as logarithmic value for three SARS-CoV-2 target genes (N1, N3 and S). The vertical bars represent the standard errors. No statistical differences were found between single (A) or multiplex (B) assays. Asterisks denote statistical significance among sampling dates for each target gene (ANOVA, Tukey test $p < 0.05$).

CRediT authorship contribution statement

Anna Navarro: Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Livia Gómez:** Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Isabella Sanseverino:** Writing – original draft, Writing – review & editing. **Magdalena Niegowska:** Visualization, Writing – original draft, Writing – review & editing. **Eszter Roka:** Investigation, Formal analysis, Writing – review & editing. **Rosalba Pedraccini:** Investigation, Formal analysis. **Marta Vargha:** Writing – review & editing. **Teresa Lettieri:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148890.

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