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SARS-COV-2 VIRUS IN FINNISH WASTEWATER

Optimization of RT-ddPCR method and comparison with the RT-qPCR method in virus detection

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ABSTRACT

Erja Janhonen: SARS-CoV-2 virus in Finnish wastewater
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Background and aims: SARS-CoV-2 is a single-stranded envelope RNA virus that causes currently occurring COVID-19 pandemic. Those infected with the virus excrete it in wastewater through urine, feces, saliva and various body secretions, and thus the virus is transmitted to the sewer system. Asymptomatic can also secrete the virus. At the population level, the presence of the virus and its concentration in wastewater can be studied from the samples collected from the wastewater treatment plants. The advantage of wastewater monitoring versus clinical testing take in healthcare is greater coverage, and also it detects patients who have not applied for the test. The aim of this work was to study the detection of the virus by the droplet digital PCR (ddPCR) and to compare the method to the more commonly used quantitative PCR (qPCR) method. The results of these methods were compared to the regional COVID-19 statistics of the Infectious Disease Registry of Finland. **Materials and methods:** In the method optimization part of the work the most sensitive way to detection of SARS-CoV-2 RNA in the wastewater samples by ddPCR is searched. SARS-CoV-2 RNA isolated from patient samples as well as wastewater samples were used for the method optimization and detection of PCR inhibition. The optimized methods were compared to the methods used in the international laboratories using QCMD test panel. The thesis work compares the differences between PCR primers recognizing different gene regions of SARS-CoV-2. Wastewater samples from the wastewater treatment plants were concentrated by a Centricon Plus -70 filter and RNA was isolated from the samples using the Qiagen Viral Mini RNA Kit and an isolation robot with Chemagic Viral RNA/DNA Kit. The presence of SARS-CoV-2 in the samples and its concentrations were studied by ddPCR and qPCR, and sensitivity and specificity of these methods were compared. The epidemiological part of the work compares the obtained results with the regional COVID-19 situation. **The results:** N1 and E PCR primers showed the best performance in ddPCR, of which N1 was slightly more sensitive. As a virus isolation method in ddPCR, the Qiagen Viral Kit performed somewhat better than the Chemagic Viral Kit. N2 has been the most functional PCR primer in qPCR, although it proved to be insensitive in ddPCR. In qPCR slightly better accuracy was obtained using the Chemagic Viral Kit than the Qiagen Viral Kit. In the test performed, no inhibition of wastewater was observed with ddPCR. ddPCR detected low viral concentrations, 1:80 000 diluted RNA at the lowest and it was as sensitive as the detection limit values given in the literature. The lowest detected amount of viral RNA was 1:5 000 clinical sample by qPCR so ddPCR was more sensitive than qPCR. Both methods proved to be specific in detecting all SARS-CoV-2 positive samples in the test and both gave negative results to samples containing other coronaviruses. SARS-CoV-2 was present in 16/20 of the samples which had been collected on February and March 2021 from the wastewater treatment plants (WWTPs). These WWTPs were Oulu, Helsinki, Espoo, Tampere, Turku, Kuopio, Lappeenranta and Rovaniemi. According to the Infectious Disease Registry of Finland, COVID-19 cases occurred in the areas of these treatment plants. No virus was detected in Seinäjoki and Pietarsaari and according to the Infectious Disease Registry, there were no cases in Pietarsaari in either week or in Seinäjoki in March. In Seinäjoki there were 7 cases in February and result of the sample was negative. **Conclusion:** ddPCR is a sensitive and a specific method in detection of SARS-CoV-2. The highest accuracy on this work was obtained using N1 primer and the Qiagen Viral Kit. The method detects the virus and its concentration in the wastewater as well an in the clinical samples. In addition to sensitivity and specificity, the method has the advantage of good tolerance to PCR inhibitors. The challenges of the method compared to qPCR are multi-step, higher cost, and longer analysis time. The method detected the virus in 8 WWTPs. The prevalence of SARS-CoV-2 in wastewater correlates with diagnosed COVID-19 cases and virus monitoring is a promising method to monitor the development of a population-level SARS-CoV-2 pandemic.

Keywords: SARS-CoV-2, coronavirus, wastewater, wastewater treatment plant, ddPCR, qPCR.
The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

TIIVISTELMÄ

Erja Janhonen: SARS-CoV-2 viruksen esiintyvyys suomalaisissa jätevesissä
Pro gradu, Tampereen yliopisto, Molekyylibiologian maisteriohjelma
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Sivut: 50

Työn tausta ja tavoitteet: SARS-CoV-2 on meneillään olevan COVID-19-pandemian aiheuttanut yksijuosteinen vaipallinen RNA-virus. Virustartunnan saaneet henkilöt erittävät virusta jätevesiin virtsan, ulosteen, syljen sekä muiden ruumiineritteiden välityksellä, ja siten virusta kulkeutuu viemäriverkostoon. Myös oireettomat voivat erittää virusta. Väestötasolla viruksen esiintymistä ja sen pitoisuuksia jätevesissä voidaan tutkia vedenpuhdistamoilta kerätyistä näytteistä. Etuna terveydenhuollossa otettuihin nenänielunäytteisiin nähden jätevesien tutkimisella on suurempi kattavuus, ja sillä saadaan mukaan myös ne sairastuneet, jotka eivät hakeudu testeihin. Työn tavoitteena on tutkia viruksen havaitsemista droplet digital PCR (ddPCR) -menetelmällä ja verrata saatuja tuloksia yleisemmin käytössä olevalla qPCR-menetelmällä saatuihin tuloksiin sekä tartuntatautirekisterin alueellisiin COVID-19-tilastoihin. **Materiaalit ja menetelmät:** Työn menetelmäoptimointiosuudessa etsitään sensitiivisintä tapaa havaita SARS-CoV-2-viruksen RNA:ta jätevesinäytteistä ddPCR-menetelmän avulla. Optimoinnissa ja PCR-inhibition havaitsemisessa käytetään potilasnäytteistä eristettyä SARS-CoV-2 RNA:ta sekä jätevesinäytteitä. Kehitettyjä menetelmiä verrattiin kansainvälisiin laboratorioiden käyttämiin menetelmiin QCMD kontrollipaneeliin avulla. Työssä vertaillaan eri geenialueita tunnistavien PCR-alukkeiden välisiä eroja. Jätevesipuhdistamoilta kerätyt jätevesinäytteet konsentroidaan Centricon Plus 70 -filtterillä, näytteistä eristetään RNA:ta Qiagen Viral Mini -kitin avulla sekä eristysrobotilla Perkin Elmer Chemagic Viral RNA/DNA-kittiä käyttäen, ja näitä eristysmenetelmiä vertaillaan keskenään. SARS-CoV-2 esiintymistä näytteissä ja viruksen pitoisuuksia tutkitaan ddPCR- ja qPCR-menetelmien avulla ja näiden menetelmien herkkyyksiä vertaillaan. Työn epidemiologisessa osuudessa verrataan jätevesinäytteiden tuloksia alueellisiin COVID-19-tartuntatilastoihin. **Tulokset:** N1- ja E- PCR-primerit toimivat parhaiten ddPCR:ssa, ja N1 oli hieman herkempi. Viruseristystapana ddPCR:ssa Qiagen Viral Mini -Kit toimi jonkin verran paremmin kuin Perkin Elmer Chemagic Viral RNA/DNA-Kit. Vastaavasti N2 on ollut toimivin PCR-aluke qPCR:ssa, vaikka se osoittautui ddPCR:ssa epäsensitiiviseksi, ja qPCR:ssa Chemagic Viral RNA/DNA-kittia käyttäen saatiin hieman parempi tarkkuus, kun Qiagen Viral Mini -Kitillä. Tehdyissä testeissä jätevedestä aiheutuvaa inhibitiota ei huomattu ddPCR:n kanssa. ddPCR havaitsi matalia viruspitoisuuksia, alimmillaan 1:80000 laimennetun RNA:n, joka sijoittuu kirjallisuudessa esiintyvien detektioraja-arvojen väliin. qPCR havaitsi alimmillaan 1:5000 RNA-laimennoksen, joten ddPCR oli sensitiivisempi kuin qPCR. SARS-CoV-2-RNA:ta havaittiin 16/20 helmikuussa ja maaliskuussa 2021 puhdistamoilta kerätyistä jätevesinäytteistä. Nämä puhdistamot olivat Oulu, Helsinki, Espoo, Tampere, Turku, Kuopio, Lappeenranta ja Rovaniemi. Tartuntatautirekisterin mukaan kyseisten puhdistamoiden alueella esiintyi COVID-19-tartuntoja mittausajankohtina. Virusta ei havaittu Seinäjoella eikä Pietarsaaressa mittausajankohtina, eikä tartuntatautirekisterin mukaan tapauksia esiintynyt Pietarsaaressa kumpanakaan mittausajankohtana eikä Seinäjoella viikolla 12. Viikolla 8 Seinäjoelle kirjattiin 7 tartuntatapausta, mutta kyseinen jätevesinäyte oli testauksessa negatiivinen. **Johtopäätökset:** ddPCR on sensitiivinen ja spesifinen menetelmä SARS-CoV-2-detektiossa. Suurin tarkkuus tässä työssä saatiin käyttämällä N1-primeriä ja Qiagen Viral Mini -kittiä Bio Rad One-Step ddPCR for Probes -kitin kanssa. Menetelmä havaitsee viruksen sekä sen pitoisuuden vaihtelut jätevedestä sekä kliinisistä näytteistä. Sensitiivisyyden ja spesifisyyden lisäksi menetelmän etuna on sen hyvä PCR-inhibiittoreiden sieto. Menetelmän haasteita qPCR:ään verrattuna ovat monivaiheisuus, korkeampi hinta sekä menetelmän suurempi ajallinen kesto. Menetelmällä havaittiin virusta kahdeksan suomalaisen jätevedenpuhdistamon näytteistä. SARS-CoV-2 viruksen pitoisuus jätevedessä korreloi diagnosoituihin COVID-19 tapauksiin ja viruksen monitorointi on lupaava menetelmä populaatiotason SARS-CoV-2 pandemian kehittymisen seurantaan.

Avainsanat: SARS-CoV-2, koronavirus, jätevesi, jätevedenpuhdistamo, ddPCR, qPCR
Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck-ohjelmalla.

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LIST OF SYMBOLS AND ABBREVIATIONS

aa	amino acid
ACE2	angiotensin converting-enzyme 2
ddPCR	droplet digital polymerase chain reaction
cDNA	complementary DNA
COVID-19	Corona Virus Disease 2019
CoVs	coronaviruses
E	envelope
FNR	false negative result
FPR	false positive result
F-primer	forward primer
LoD	limit of detection
MERS	Middle East Respiratory Syndrome
N	nucleocapsid
NTC	no template control
qPCR	quantitative polymerase chain reaction
R_0	basic reproduction number
RBD	receptor binging domain
RBM	receptor binding motif
R-primer	reverse primer
RT	reverse transcriptase (enzyme)
S	spike
SARS	Severe Acute Respiratory Syndrome
ssRNA	single stranded RNA
THL	Terveyden ja hyvinvoinnin laitos, Finnish institute for health and welfare
WHO	World Health Organization
WWTP	wastewater treatment plant
µl	10^{-6} liter

1. INTRODUCTION

Ongoing COVID-19 (Coronavirus disease) pandemic caused by the positive-sense single stranded RNA virus SARS-CoV-2 or 2019-nCoV (Severe Acute Respiratory Syndrome Coronavirus) (Wang et al. 2020). COVID-19 epidemic started in Wuhan in China in December 2019. As the epidemic accelerated WHO declared the epidemics to global pandemic 11.3.2020. In Finland, the first COVID-19 case was detected end of January 2020 from a clinical sample (Haveri et al. 2020). At the time of writing, there have been 84 287 COVID-19 cases and 923 deaths in Finland (THL 19.4.2021).

Primarily the virus transmits by droplet infection, but it transfers by through direct contact also (Junejo et al. 2020; THL Infektiotaudit ja rokotukset). SARS-CoV-2 causes respiratory symptoms and some individuals with the infection are asymptomatic. Common symptoms of COVID-19 are fever, myalgia, fatigue, dry cough and dyspnea and in 2-10% of COVID-19 patients has gastrointestinal symptoms, as diarrhea (Kitajima et al. 2020). The morality rate has ranged from 1.7-2.4% globally (Terveyskirjasto Uusi koronavirus (COVID-19)). The mortality is lower among young and healthy people and high age raises to the risk of death (Ioannidis 2021). Fatality rate of SARS-CoV-2 is lower than other beta coronaviruses, SARS up to 50% and 34.4-69.2% for MERS (Kitajima et al. 2020). Reproduction number (R_0) of SARS-CoV-2 is 1.4-6.5 which means that each infected individual could transmit the virus to another 1.4-6.5 cases (Kitajima et al. 2020).

The virus ends up in wastewater through the feces and the other secretions of the infected individuals. In Finland, 73% of the population are users of the sewage network and monitoring wastewater provides information on viruses and illicit drug use in the general population (Suomen ympäristökeskus, Vesihuollon tietojärjestelmä, Julkiraportti; Suomen virallinen tilasto (SVT): Väestörakenne 2019; THL Koronaviruksen jätevesiseuranta; Kankaanpää et al. 2014; Delogu et al. 2018). Hence, wastewater is a potential source of epidemiological data; a change of amount of the virus in the effluent indicate a regional change in infection situation (Kitajima et al. 2020; THL Koronaviruksen jätevesiseuranta). In the case of COVID-19, correlation between viral concentration of wastewater and confirmed COVID-19 cases exists ja wastewater surveillance can act as an early warning system for an epidemic (Ahmed et al. 2020; WHO 2020).

Many studies have been done about detection and quantification of SARS-CoV-2 from patient samples by RT-qPCR and RT-qPCR is the golden standard of detection of the virus from clinical samples (Dang et al. 2020; Falzone et al. 2020). In the use of ddPCR in the detection of SARS-COV-2 has been less studied than qPCR, and only few articles have been published on ddPCR use with wastewater samples. In the case of wastewater monitoring, lack of an optimized and standardized protocol of SARS-CoV-2 detection and quantification from wastewater samples is a challenge (Kitajima et al. 2020; Cervantes-Avilés et al. 2021). Suo et al. (2020) stated that there it is an urgent need for more accurate and sensitive detection method than RT-qPCR.

Wastewater samples must be concentrated before RNA extraction because secretions are diluted when mixed with wastewater and amount of RNA per unit volume is small. Centricon Plus-70 filter can concentrate most 70 mL solutions down to 350 µl (Millipore, Centricon Plus-70 manual). There is no need to concentrate clinical samples, so they are not diluted. RNA must be isolated from both wastewater sample and clinical samples prior to detection by ddPCR or qPCR. Qiagen QIAmp Viral Mini Kit can be used for isolation of viral RNA from a many type of viruses. The kit based on silica-based membrane with the speed of micro spin technology. In the protocol, sample is lysed, and RNases are inactivated first. Next, a viral RNA binds to the membrane and contaminants washed away. Last, RNA is eluted to the buffer and it is ready for detection (QIAmp Viral Mini Kit Handbook). Chemagic RNA/DNA Kit based on the binding of RNA to magnetic beads and the retrieval of the beads from the sample by magnets. Finally, the RNA is eluted in buffer (Perkin Elmer Chemagic purification protocol).

1.1 Detection methods: droplet digital PCR (ddPCR) and real-time quantitative PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) is method used for the detection and quantitation of gene expression in real time (Hawkins and Guest 2016). It is fast, accurate and specific tool for diagnostic (Hamed 2020). RT-qPCR (reverse transcriptase qPCR) is the golden standard method for the diagnosis of COVID-19 from upper respiratory samples (Dang et al. 2020; Falzone et al. 2020; Gonzalez et al. 2020). RT-qPCR is method used globally in healthcare to detect COVID-19 infection from a patient sample and WHO approved qPCR tests developed many different laboratories are used to detect SARS-CoV-2 (Sule and Oluwayelu 2020). In qPCR, virus amplification is measured as

a quantification cycle that is commonly reported as a cycle threshold (Ct) (Sule and Oluwayelu 2020).

In the reverse transcriptase (RT) reaction, the synthesis from RNA to cDNA occurs by action of a reverse transcriptase enzyme prior to DNA amplification (Bustin et al. 2020). Since SARS-CoV-2 is an RNA virus and the detection is based on the identification of the DNA, RNA must be synthesized into cDNA either before detection by qPCR or ddPCR. The DNA- polymerase enzyme needs primers to amplification, one for the forward strand and one for the reverse strand. These primers are complementary to the template region of the viral genome. The most used target genes for SARS-CoV-2 detection by qPCR are Orf1ab gene (Open reading frame), the gene N that encoding the nucleocapsid and the gene S that encoding the spike protein (Falzone et al. 2020). In addition, probes are also often used for detection to confirm the specific amplification of the target gene. Probes are fluorescently labelled oligonucleotides which give fluorescent signal that is recognized by qPCR or ddPCR.

The amplification of the target gene is done similarly in ddPCR as in qPCR presented earlier. However, in ddPCR, the sample is divided into a maximum 20 000 droplets, each which is nanoliters in size (Li et al. 2018). The droplets consist of the sample with ddPCR oil, and every droplet is individual reaction (Li et al. 2018). If the droplet contains at least one copy of target DNA, the droplet digital reader detects the fluorescent signal and interprets the sample as positive. If there are not copies of target DNA, no fluorescent signal is generated, and the reader interprets the sample as negative (Li et al. 2018).

Workflows of both methods, RT-qPCR and RT-ddPCR are show in the *Figure 1.* The figure visualizes that ddPCR is a more multi-stage detection method than qPCR, because ddPCR based on the water-emulsion droplet technology and the preparation of these droplets brings to extra work step to the protocol. In qPCR, the samples must be added to the 96-well plate with master mix, the plate must be sealed with a film and vortexed before PCR. In ddPCR, the samples should be added to the tubes with master mix and these must be pipetted to the cartridge with oil for formation of the droplets. The droplets have to transferred to the 96-plate by a multi-channel pipette and the plate is should be sealed with alum foil before PCR reaction. The results of ddPCR are read on separate device, the droplet digital reader.

Figure 1. *Workflows of RT-qPCR and RT-ddPCR.*

RT-qPCR is a widely used method for identifying the SARS-CoV-2 RNA from clinical samples, but its challenge is false-negative results with low viral load, and it is sensitive to PCR inhibitors (Dinge et al. 2013; Falzone et al. 2020). The studies with clinical samples show that ddPCR is a more sensitivity method for SARS-CoV-2 detection than qPCR and the limit of detection of RT-ddPCR is lower than that of RT-qPCR (Dang et al. 2020; Falzone et al. 2020; Suo et al. 2020). Dang et.al. (2020) detected patient samples in their study; 28 of the 30 samples were positive by qPCR and all 30 were positive by ddPCR. In another study, 63 patients in the two hospitals were double-tested by qPCR and ddPCR; 21 were positive by qPCR and 49 were positive by ddPCR, 41 were negative by qPCR and 10 were negative by ddPCR and 4 were suspected SARS-CoV-2 infections (Suo et al. 2020).

Droplet digital PCR is used for SARS-CoV-2 detection from clinical samples, but it has been used rarely to study wastewater samples. Studies using clinical samples show a higher sensitivity of ddPCR detection compared to that of RT-qPCR (Dang et al. 2020; Falzone et al 2020). Falzone et al. (2020) tested limit of detection in ddPCR and RT-qPCR with housekeeping gene human ß-actin and the detection limit of qPCR was 0.0187-1.87 ng and 0.00374 ng in ddPCR. Detection limit of ddPCR was 2.10 copies/reaction (0.105 copies/µl) with ORF1ab gene and 1.80 copies/reaction (0.09 copies/µl) with N gene but limit of detection (LoD) of qPCR was 1 039 copies/reaction (51.95 copies/µl) with ORF1ab and 873.2 copies/reaction (43.66 copies/µl) with N gene (Suo et al. 2020). Gonzalez et al. (2020) reported that the LoD of qPCR are 14.6 copies/reaction for N1 gene, 2.00 copies/reaction for N2 gene and 2.18 copies/reaction for N3 gene.

The ddPCR method also has some weakness compared to the ddPCR method. The ddPCR analysis is more time consuming and slightly more expensive than qPCR method (Table 1). Generating of the droplets and reading of the plate take more time in ddPCR. The 8-well cartridge, the droplet generator gasket and the droplet generator oil are materials that are needed in ddPCR but not in qPCR. *Figure 1* shows that ddPCR has more steps than qPCR. Hence, ddPCR take approximately 15% more time and 5-10% more cost than RT-qPCR (Falzone et al. 2020; Gonzalez et al. 2020). However, reaction inhibition causes less interference to ddPCR than to RT-qPCR (Dinge et al. 2013; Rački et al. 2014; Falzone et al. 2020). Rački et al (2014) study to PCR inhibition in ddPCR and qPCR from different matrices, one of which was wastewater. They noticed it ddPCR showed higher resilience to inhibitors than qPCR. Liu et al. (2020) tested different primers and probe sets by qPCR and ddPCR in their study, and their comparison between qPCR and ddPCR showed that there were less false negative results (FNRs) with ddPCR than with qPCR (Gonzalez et al. 2020).

Table 1. *Comparison of ddPCR and qPCR in based on literature.*

character	**ddPCR**	**qPCR**
limit of detection	1.8-21 copies/reaction (Suo et al. 2020) 0.00374 ng (Falzone et al. 2020)	873.2-1 039 copies/reaction (Suo et al. 2020) 0.0187-1.87 ng (Falzone et al. 2020)
false negative results	less than with qPCR (Gonzalez et al. 2020)	with low viral load (Falzone et al. 2020)
time	approximately 2 h more working time than in the RT-qPCR (Falzone et al. 2020)	faster than ddPCR
costs	5-10% more expensive than the RT-qPCR (Falzone et al. 2020)	
inhibition	not sensitive (Dinge et al. 2013, Rački et al 2014)	sensitive (Dinge et al. 2013, Rački et al 2014)
replicates	only one reaction per sample	parallel reactions are needed to confirm the result

2. A NOVEL CORONAVIRUS SARS-COV-2

2.1 Structure and genome

SARS-CoV-2 is a single stranded enveloped RNA virus (ssRNA). SARS-CoV-2 is retained 99.8-99.9% homology of nucleotide sequence with beta bat CoV and 50% similarity with MERS-CoV and 75-80 % similarity with SARS-CoV (Junejo et al. 2020; Kitajima et al. 2020). SARS-CoV-2 binds to the receptor named angiotensin-converting enzyme 2 (ACE2) via its receptor binding motif (RBM) in the receptor binging domain (RBD) (Kitajima et al. 2020; Shang et al. 2020). RBD located in the spike protein (S) in the outer surface of the virus and the spike has key role in ACE2-receptor recognition and the fusion to the host cell (Huang et al. 2020).

Size of human CoVs is 80-160 nm in diameter and they have the largest genome among the RNA viruses (Wang et al. 2020). Length of the genome of SARS-CoV-2 is ~29 kb and it contains 11 coding genes (Khailany et al. 2020; Nakagawa et al. 2020). The SARS-CoV-2 consist of 5´ untranslated region (UTR) that is followed by Orf1ab, S, Orf3a, E, M, Orf6a, Orf7a, Orf7b, Orf8, N and Orf10 genes. The virus genome has also 3´UTR region (Khailany et al. 2020). *Figure 2* shows the genes of the SARS-CoV-2. The common structural proteins of SARS-CoV-2 (Figure 3) are an envelope protein (E) 74 aa, a membrane protein (M) 222 aa, a nucleocapsid protein (N) 419 aa and a spike protein (S) 1273 aa (Khailany et al. 2020; Nakagawa et al. 2020). The N protein forms a capsid around to the genome of the virus and M, E and S proteins form envelope surrounding this (Wang et al. 2020). The whole genome of the virus contains 29 881 base pairs and 9860 aa (GenBank no. MN908947; Huang et al. 2020).

Figure 2. *Genes of SARS-CoV-2*

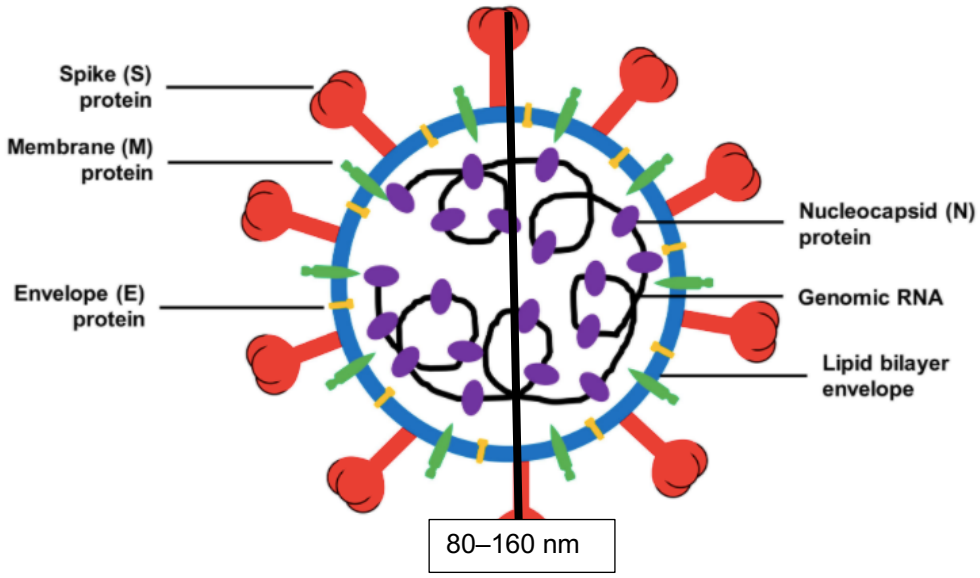


Figure 3. *The structure of SARS-CoV-2 (modified figure from Wang et al. 2020). The size of the virion is about 80-160nm.*

2.2 Origin and classification

Seven coronaviruses have been isolated from human and three of these are emerging pathogenic coronaviruses; SARS-CoV, MERS-CoV and the new coronavirus SARS-CoV-2 (Wang et al. 2020). *Figure 4* shows classification of SARS-CoV-2. SARS-CoV-2 is a member of a genera Beta coronaviruses of a genus Coronavirinae of a subfamily Okariviruses of a family Coronaviridae of an order Nidovirales. In addition to SARS-CoV-2, other beta coronaviruses are SARS-CoV and MERS-CoV (Junejo et al. 2020).

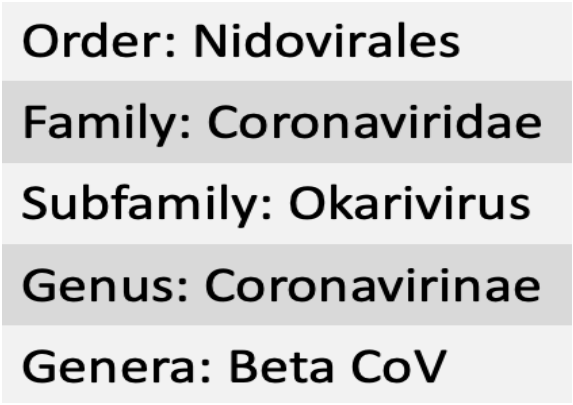


Figure 4. *Classification of SARS-CoV-2*

SARS-CoV-2 was observed first time in December 2019 in Wuhan, in China (Junejo et al. 2020). The natural reservoir of coronaviruses are bat and rat, which the virus has spread to intermediate host such as cow or pig. The intermediate host of SARS-CoV-2 is thought to be a pangolin, as the critical functional regions of the spike protein of the virus are nearly the same than the virus of pangolin, but specific pathway to human is unclear (Junejo et al. 2020). It is also possible, that there is no intermediate host, and the virus could have been transmitted directly from natural reservoir to the human (Shang et al. 2020).

2.3 COVID-19

SARS-CoV-2 is an RNA virus that causes COVID-19 (Corona Virus Disease 2019). The virus spreads mainly through droplets of human airway but also through direct contact (Junejo et al. 2020; THL Infektiotaudit ja rokotukset). In Finland, the first COVID-19 case was confirmed 29 January 2020 from patient´s nasopharyngeal sample (Haveri et al. 2020).

Symptoms of COVID-19 are fever, myalgia, fatigue, dry cough, dyspnea and diarrhea and the morality rate of COVID-19 is 1.7-2.4 % (Kitajima et al. 2020; Terveyskirjasto Uusi koronavirus (COVID-19)). It was reported that around 60% of infections are asymptomatic so asymptomatic individuals can spread to the virus unknowingly (Suo et al. 2020, Bai et al. 2020). COVID-19 causes more mortality among the elderly and debilitated people. The risk of mortality increases with age (Ioannidis 2021).

3. WASTEWATER MONITORING

Almost 60% of the Finnish population is subject to wastewater monitoring (THL: Väestötason huumeiden käyttö). Population-based illicit drug use has been studied through wastewater monitoring and this has provided information on what drugs are used at the population level, what regional differences exist and whether new drugs are emerging (Kankaanpää et al. 2014). Also, the presence of various polioviruses in population has been detected by wastewater surveillance (Delogu et al. 2018). Wastewater surveillance is a part of the WHO global polio eradication program and it is a powerful tool for surveillance of enteroviruses in population (Delogu et al. 2018). In addition to poliovirus, the changes of antimicrobial resistance (AMR) are also monitored in wastewater (WHO, Scientific brief 2020). In Finland, polio wastewater monitoring is carried out with the aim of detecting disease-causing polioviruses if they occur in sewer network (THL: Polion jätevesiseuranta).

The challenge of wastewater monitoring is the low viral load caused by dilution of secretions. Therefore, wastewater samples must be concentrated before RNA isolation. Reliability of the measurement is increased by standardizing the measurement conditions. The sample collection takes 24 hours at the time and it is scheduled for the same day of the week. Seasonal variation such as temperature variation must be taken into account, as an increase in temperature accelerates the breakdown of the virus.

Wastewater has inhibitors to which PCR-based methods are sensitive. These inhibitors, such polysaccharides, metal ions, and Rnases, may cause a decrease in the sensitivity of the method or false negative results. The composition of wastewater varies within the treatment plant and especially between wastewater treatment plants and hence the number and quality of inhibitors also vary (Ahmed et al. 2020).

3.1 SARS-CoV-2 in wastewaters

SARS-CoV-2 RNA has been detected in wastewaters throughout the world during COVID-19 pandemic (Ahmed et al. 2020; WHO: Status of environmental surveillance for SARS-CoV-2 virus; Gonzalez et al. 2020). Infected individuals secrete the SARS-CoV-2

to urea, feces, saliva, respiratory secretions and through these secretions, the virus enters wastewater (Kitajima et al. 2020; THL: SARS-CoV-2 jätevedenpuhdistamoilla). Significantly, SARS-CoV-2 has also been isolated from feces of asymptomatic individuals, indicating that asymptomatic individuals can also secrete the virus (Kitajima et al. 2020).

SARS-CoV-2 inactivate rapidly under increased temperature and by major disinfectants such as bleach, ethanol, benzylalkonium chloride, povidone-iodine and chloroxylenol (Kitajima et al. 2020). In wastewater, the inactivation time of the virus could be between 2.3-3.5 days at 23°C. The virus is sensitive by heat. (Gundy et al. 2009, Hamouda et al. 2020). Hamouda et al. (2020) wrote that temperature is most significant factor affecting the survival of viruses and when the temperature increases, the virus inactivation also increases. SARS-CoV-2 inactivates in 5 minutes in 70°C (Chin et al. 2020)

The presence of microorganism in the wastewater affects to the survival of the virus by accelerating their degradation (Hamouda et al. 2020). Some ions in wastewater can change the structure of the membrane of SARS-CoV-2 and they affect its selectivity and its hydrophobicity (Cervantes-Avilés et al. 2021). The pH of the wastewater also has effect on the survival of viruses, but SARS-CoV-2 appears to survive over a wide pH range (3-10) in room temperature (Chin et al. 2020). Suspend solids of wastewater could adsorbed to the coronaviruses and at the same time protect viruses from inactivation. The charge of the surface of the virus affects to the ability of the virus to adhere to charged surfaces and charged particles (Hamouda et al. 2020).

Wastewater-based COVID-19 surveillance is cost-effective and correlation between SARS-CoV-2 concentration and clinical cases of COVID-19 has been assigned (Ahmed et al. 2020; WHO: Status of environmental surveillance for SARS-CoV-2 virus). Wastewater-based surveillance is cheaper method than the clinical monitoring (Gonzalez et al. 2020). It can identify symptomatic but also asymptomatic individuals and thus it can provide a more comprehensive picture of the disease situation than the clinical COVID-19 trials (Gonzalez et al. 2020). Also, all types of human secretions end up in the wastewater, but the swab samples are local samples. Zhang et al. (2020) examined throat and stool samples from three children all of whom had COVID-19 infection and found stool samples to be SARS-CoV-2 positive for another 10 day after the throat samples were negative. Cheung et al. (2020) detected the virus from stool samples, in stool was collected after respiratory samples had negative test results. In addition, wastewater-based surveillance is time saving compared to testing people individually

(Hamouda et al. 2020). Kitajima et al. (2020) wrote that surveillance strategy can be useful as an “early warning” system.

4. OBJECTIVES

The specific aims of this thesis work were:

I. Optimize the ddPCR method for SARS-CoV-2 detection
II. Test the functionality of the ddPCR method in wastewater samples
III. To correlate copy number of SARS-CoV-2 with wastewater treatment plant specific prevalence of COVID-19 by location.

5. MATERIALS AND METHODS

Workflow with wastewater samples

Wastewater samples were concentrated using Centricon filters. The samples were then subjected to RNA isolation by QIAmp Viral RNA Mini Kit or Chemagic Viral RNA/DNA Kit. The presence and amount of SARS-CoV-2 copy number has been detected using the ddPCR and qPCR methods. The *Figure 5* outlines the main steps of this thesis work with wastewater samples. The work steps are described in more detail below.

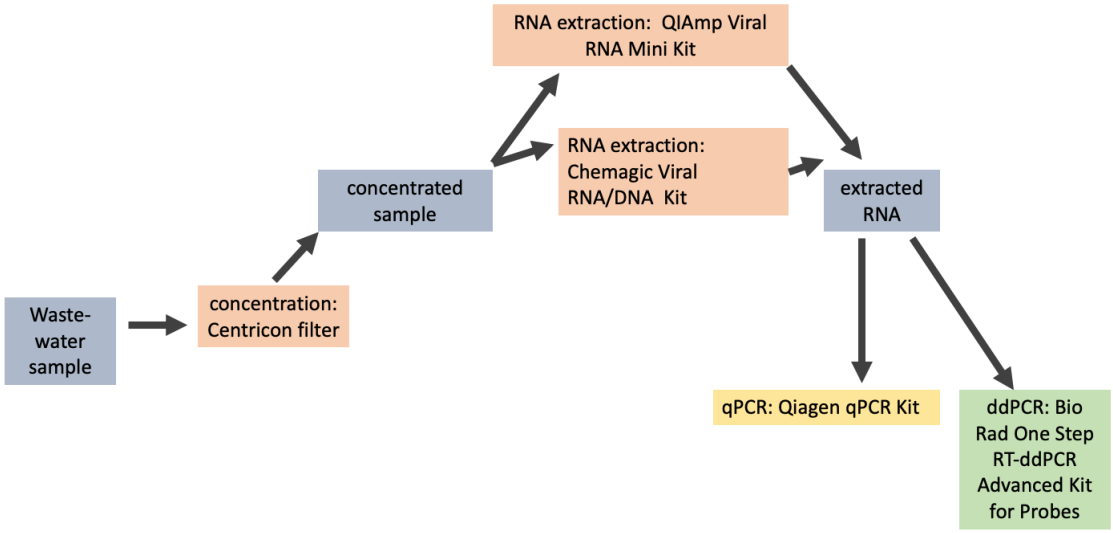


Figure 5. *Workflow with wastewater samples.*

5.1 Biosafety

WHO (World Health Organization) has published to *Laboratory biosafety guidance related to coronavirus disease (COVID-19)* for safe working with SARS-CoV-2. It says that in non-propagative laboratory work such as in nuclei acid amplification test, the procedures according to Biosafety Level 2 (BSL-2) should be used. The guidance has been followed in this thesis work. Also, double gloves and the hand protections were used when treating wastewater.

5.2 Samples

5.2.1 Clinical patient samples

Throat swab samples positive for SARS-CoV-2 were inactivated incubating sample at 60°C for 30 minutes before RNA extraction. The sample tubes were not opened before inactivation.

5.2.2 QCMD (Quality Control for Molecular Diagnostics) samples

Quality Control for Molecular Diagnostics (QCMD) samples are quality control samples for laboratories. QCMD is an independent International External Quality Assessment (EQA) / Proficiency Testing (PT) organization. QCMD provides a wide-ranging quality assessment service primarily focused on molecular infectious diseases to over 2 000 participants in over 100 countries (Quality Control for Molecular Diagnostics). The samples were diluted into transport medium. *Table 2* shows 10 QCMD samples that were included in this thesis work.

Table 2. *QCMD samples analyzed in the study.*

Table shows the sample content and copy number of the virus in milliliters. Sample relationship indicates the duplicate samples in the test panel.

sample content	copy number (Log10 Copies/ml)	sample relationship
SARS-CoV-2	3.16	1
coronavirus 229E	3.93	
SARS-CoV-2	2.48	2
SARS-CoV-2	4.29	
SARS-CoV-2	3.27	3
SARS-CoV-2	2.48	2
SARS-CoV-2	3.27	3
SARS-CoV-2	3.16	1
negative	negative	
coronavirus OC43	4.0	

5.2.3 Wastewater samples

Wastewater samples were collected from ten Finnish wastewater treatment plants (*Table 17*). The samples were collected within 24 hours with an automatic collection device and were sent express cold delivery to the laboratory. The shipments lasted overnight and arrived at the laboratory the next morning. The samples were collected from ten Finnish treatment plants: Seinäjoki, Oulu, Helsinki, Rovaniemi, Turku, Kuopio, Espoo, Lappeenranta, Pietarsaari and Tampere.

5.2.4 PCR inhibition test samples

The inhibition test samples contained Finnish wastewater to which had been added isolated SARS-CoV-2 RNA from positive patient samples. The wastewater samples were

collected in 2017 and 2018, time before the first documented COVID-19 case, so they were negative for SARS-CoV-2 RNA. All wastewater samples were spiked with SARS-CoV-2 using same final copy number. Shortly, 1:2 500 diluted SARS-CoV-2 RNA were added to wastewater samples in a 1:1 ratio and thus 1:5 000 diluted samples were obtained *(Table 3).* "No inhibitor control" sample was SARS-CoV-2 RNA diluted to 1:5 000 with sterile water and since this control sample did not contain wastewater, it did not show wastewater-induced PCR inhibition.

Table 3. *PCR inhibition test samples analyzed in the study.*

sample
no inhibition control
WWTP1 28.9.2017
WWTP2 12.12.2017
WWTP1 12.12.2017
WWTP1 7.2.2018
WWTP1 27.4.2018
WWTP2 12.6.2018

5.3 Wastewater concentration

Wastewater samples were concentrated prior to RNA extraction because secretions are diluted in the wastewater network and virus concentrations are therefore low. The wastewater samples were concentrated using Centricon Plus -70 100 kDA filters. About 70 mL of wastewater sample was concentrated to a volume of 350 µl.

The filter was washed with milli q water before concentration. First 70 mL of the sample added to the sample filter cup with filtrate collection cup and sealed with the cap. It was centrifuged at 3 500 x g for 30 min. in centrifuge bucket by Thermo Scientific Sorvall Lynx 4000 centrifuge. After this, the sample filter cup was removed and turned upside down and it was placed on top of the concentrate collection cup. This was centrifuged at 1 000 x g for 2 min. Then the concentrate cup was removed, and the sample was pipetted to the tube.

5.4 RNA extraction

The RNAs are extracted from concentrated wastewater samples by Qiagen QIAmp Viral RNA Mini Kit and Perkin Elmer Chemagic Viral DNA/RNA 300 Kit by Chemagic isolation robot.

5.4.1 RNA extraction from clinical samples

The clinical samples were inactivated before RNA extraction. After inactivation, QIAmp Viral RNA Mini Kit protocol was used for RNA extraction: a 140 µl sample was pipetted to Eppendorf-tube, 560 µl of AVL-carrier was added to the tube and after this followed a 10 min. incubation time in room temperature. Next, 560 µl of ethanol was added to the tube, this was vortexed and then a 630 µl sample was pipetted to spin column. Then the tube was centrifuged at 8 000 rpm for 1 min and it was moved from the spin column to a new tube. Next, 630 µl of the sample was pipetted again to the spin column and these were centrifuged. 500 µl of AW1 buffer was added with the sample and these were centrifuged at 8 000 rpm for 1 min. After this, the spin column was moved to the new tube. Next, 500 µl of AW2 buffer was added to the tube and the tube was centrifuged at 14 000 rpm for 3 min. After this, the spin column was moved to the new tube. 60 µl of AVE-buffer was pipetted to the tube and the sample was incubated for 1 min. in room temperature and it was centrifuged at 8 000 rpm for 1 min.

5.4.2 RNA extraction from wastewater samples

RNA was extracted from concentrated wastewater samples using QIAmp Viral Mini Kit as described in *Chapter 5.4.1*. The samples were extracted using Perkin Elmer Chemagic Viral RNA/DNA Kit with the Chemagic isolation robot using manufacturer's protocol. Before starting, 440 µl of the Poly(A) RNA Buffer was added to the Poly(A) RNA tube and mixed thoroughly. Dissolved lyophilized Proteinase K was added in H_2O.

Prior the extraction the robotic station was filled as follows:

position 1: Rack with Disposable Tips

position 2: low-well-plate prefilled with 150 µl Magnetic Beads

position 3: deep-well-plate containing 300 µl sample, 4 µl Poly(A) RNA, 10 µl Proteinase K, 300 µl Lysis Buffer 1, binding Buffer 2

position 4: empty deep-well-plate

position 5: empty deep-well-plate

position 6: empty deep-well-plate

position 7: deep-well-plate prefilled with 50 - 100 µl Elution Buffer 6.

Elution Buffer 6 was prefilled, and the thoroughly resuspended magnetic beads accorded to the sample positions. 4 µl Poly(A) RNA, 10 µl Proteinase K and then 300 µl Lysis Buffer 1 were added to the wells containing sample.

5.5 Primers and probes for SARS-CoV-2 detection

In this study four different SARS-CoV-2 gene regions were tested for detection of SARS-CoV-2. The primers and the probes target the envelope gene (E) and N1, N2 and N3 regions of the nucleocapsid gene. CoV2019_N1, CoV2019_N2, CoV2019_N3 and CoV2019_E primers and probes were used (Table 4).

Table 4. *Primers and probes used in this study.*

gene	primers	probe
N1	CoV2019_N1-F **GACCCCAAAATCAGCGAAAT**	CoV2019_N1-P 6-FAM / BHQ-1 **ACCCCGCATTACGTTTGGTG-GACC**
	CoV2019_N1-R **TCTGGTTACTGCCAG-TTGAATCTG**	
N2	CoV2019_N2-F **TTACAAACATT-GGCCGCAAA**	CoV2019_N2-P HEX / BHQ-1 **ACAATTT-GCCCCCAGCGCTTCAG**
	CoV2019_N2-R **GCGCGACATTCCGAA-GAA**	
N3	CoV2019_N3-F **GGGAGCCTT-GAATACACCAAAA**	CoV2019_N3-P 6-FAM / BHQ-1 **AYCACATTGGCACCCG-CAATCCTG**
	CoV2019_N3-R **TGTAG-CACGATTGCAG-CATTG**	
E	CoV2019_E_F **ACAGGTACGTTAA-TAGTTAATAGCGT**	CoV2019_E_P 6-FAM / BHQ-1 **ACAC-TAGCCATCCTTACTGCGCTTCG**
	CoV2019_E_R **ATATTGCAGCAGTAC-GCACACA**	

5.6 Droplet digital PCR workflow

The ddPCR reaction was done using a mixture of pre-mixed primer and probe needed for the reaction. The oligo mixture was vortexed well and it was stored in ice before the usage.

A mixture of primers and probe was prepared (for 10 samples):
2.7 µl 100 nM F-primer
2.7 µl 100 nM R-primer
3 µl 25 nM probe
6.6 µl H_2O

Further a mastermix was done in a separate tube using following reagents and volumes. A master mix was made (for 10 samples):
81 µl H_2O
55 µl supermix (Bio Rad One-Step RT-ddPCR Advanced Kit for Probes)
11 µl 300 mM dithiothreitol (DDT)
11 µl the primers-probe-mixture
22 µl reverse transcriptase enzyme

All components were vortexed before pipetting. 18 µl of the master mix was pipetted into each 0.5 ml Eppendorf-tube and then 4 µl of sample was added into the tube. The mixture was then vortexed. Total volume of each tube was 22 µl.

20 µl of the reaction mix was transferred from the tubes to middle wells of the DG8 droplet generator cartridge. 70 µl of droplet generator oil for probes was pipetted into the bottom wells of the cartridge. The gasket was hooked to the cartridge and after this the cartridge was moved into QX200 droplet generator.

40 µl of the contents of the top wells of cartridge was pipetted with a 8-channel pipette into a 96-well PCR plate. The plate was setting on top of the PX1 support block. The plate was covered with an aluminum foil and the foil was closed to plate in PX1 PCR plate sealer. *Table 5* shows thermal cycling conditions of PCR.

There was one NTC sample for each of the seven samples without replicate samples. Positive control was neither used.

RT-PCR

Bio Rad T100 Thermal cycler was used for RT-PCR under follow protocol in *Table 5*.

Table 5. *Thermal cycling conditions of RT-ddPCR.*

Step	Temperature (C°)	Time	Number of cycles
hold	25	3 min	1
reverse transcription	50	60 min	1
enzyme activation	95	10 min	1
denaturation	95	30 sec	40
annealing/extension	55	1 min	40
enzyme deactivation	98	10 min	1
hold	4	infinite	1

Droplet reader

Droplets were analyzed in Bio Rad QX200 Droplet reader and QuantaSoft Software was used. FAM/HEX channel was selected. For the analysis of the results a threshold value was set manually.

5.7 qPCR workflow

Qiagen Quantitect Probe RT-PCR Kit was used with qPCR.

Reaction mix was made (per reaction):

Quantitect RT Master mix 25 µl
F-primer final concentration 0.4 µM
R-primer final concentration 0.4 µM
probe final concentration 0.1-0.2 µM
Quantitect RT mix 0.5 µl
template RNA 1 pg-500 ng
Nuclease free water

Total volume of reaction was 50 µl.

Master mix was made into the tube and it was distributed on 96-well plate, the samples were pipetted into the 96-well PCR plate. The plate was vortexed and iScience 7900HT Fast Real-Time PCR System was used for RT-PCR run using protocol *(Table 6).*

Table 6. *Thermal cycling conditions of RT-qPCR.*

Step	**Temperature (ºC)**	**Time**	**Number of cycles**
reverse transcription	50	30 min.	1
PCR initial activation	95	15 min.	1
denaturation	94	15 sec	40-45
annealing/extension	60	60 sec	40-45

6. RESULTS AND DISCUSSION

In this work, PCR primer sets and RNA extraction methods were optimized for ddPCR to detect SARS-CoV-2 for wastewater samples. Two different RNA extraction methods: Qiagen Viral RNA Mini Kit and Perkin Elmer Chemagic RNA/DNA Kit with an isolation robot were compared. According to the literature ddPCR is less sensitive for PCR inhibitors than qPCR, so its tolerance for PCR inhibitors was tested with wastewater samples (Dinge et al. 2013, Rački et al 2014). Also, the effect of freezing and thawing for RNA preservation was tested. Some studies show that ddPCR is more sensitive method than qPCR, so sensitivity and specificity of ddPCR and qPCR were compared (Dang et al. 2020; Falzone et al. 2020; Suo et al. 2020). Correlation of COVID-19 cases of WWTP areas and RNA copy number of SARS-CoV-2 of wastewater samples was examined. In this thesis work results of ddPCR have been given in form copies/µl but in some studies results has been given form copies/reaction. The reaction volume of ddPCR reaction is 20 µl, thus a result can be changed to different format by multiplying or dividing by 20.

Of the ddPCR results, those with a droplet count >8 000 have been accepted and below this value have been rejected. The thresholds used with ddPCR are shown in the ddPCR amplitude figures.

In this work viral RNA was extracted by Qiagen Viral RNA Mini Kit before primer optimization and PCR inhibition test. The results are shown in tables and also original ddPCR analysis results are shown. *Figure 6* and *Figure 7* show instructions for interpreting the ddPCR image.

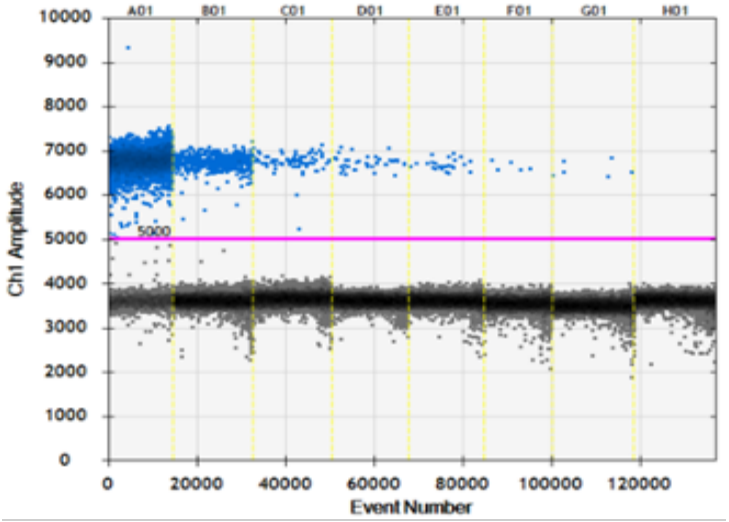


Figure 6. *Instructions for interpreting a ddPCR amplitude-event number image.*

A01-H01 are names of the wells of PCR plate. Ch1 Amplitude is fluorescence intensity of channel 1 (FAM). Blue populations are positive droplets and grey populations are negative droplets. Black area means a lot of grey populations. Pink line is a threshold value. Yellow line is the portion of droplets of reaction in the total droplets.

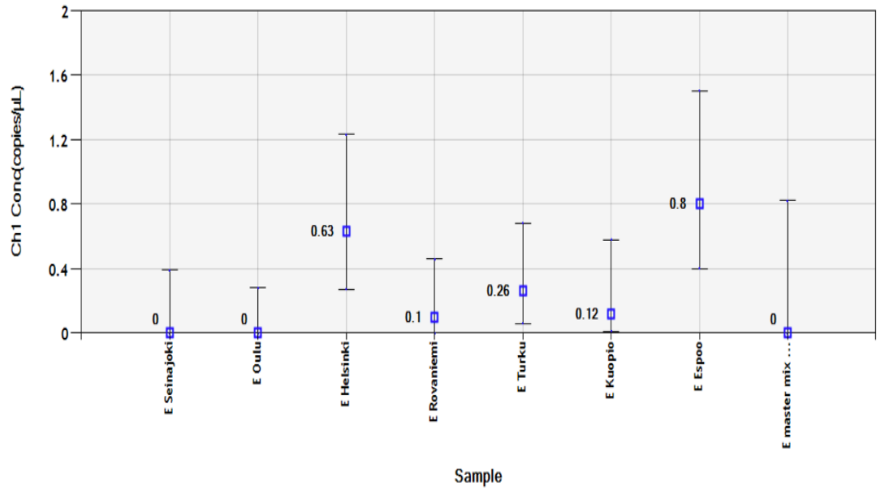


Figure 7. *Instructions for interpreting a ddPCR concentration image.*

The figure shows concentrations of the samples. Sample names are in the x-axel and concentration of the samples (copies/µl) is in the y-axel.

6.1 Primer optimization for ddPCR

WHO approved primers was used in SARS-CoV-2 detection by qPCR: E, N1, N2 and N3 (*Table 4*). These primers were tested to obtain the most sensitive methods for the wastewater analysis. It is important to use as sensitive detection method as possible for

SARS-CoV-2 analysis to be able to detect also low virus positivity in wastewater samples. The PCR amplification of N2 method proved to be poorly in ddPCR, so it was omitted from testing. The N3 method was less sensitive than N1 and E, so N1 and E were chosen to continue (Table 7).

Table 7. *Primer sets comparison between N1, N2, N3 and E primer sets. The results were analyzed by ddPCR.*

primer set	sample	
	1:10 SARS-CoV-2 RNA (copies/µl)	**1:100 SARS-CoV-2 RNA (copies/µl)**
N1	227	20.5
N2	negative	negative
N3	157	negative
E	66.2	9.8

The sensitivity of the two selected primer sets was tested using dilution serie of SARS-CoV-2 sample. For the comparison test RNA were extracted from positive SARS-CoV-2 clinical sample and it was diluted 1:10, 1:500, 1:2500, 1:5 000 and 1:10 000. N1 and E primers were tested with this dilution serie (Table 8, Figure 8). The copy number of the tested samples was higher by N1 primer set than with E primer set. The comparison of the primers shows that N1 was most sensitive primer set for SARS-CoV-2 detection by ddPCR. Better sensitivity of N1 also appears in wastewater samples (*Table 9)*.

Table 8. *Method comparison between N1 and E primer sets for ddPCR.*

sample	N1 primer (copies/µl)	E primer (copies/µl)
SARS-CoV-2 RNA 1:10	**1 779**	729
SARS-CoV-2 RNA 1:500	**31.6**	15.2
SARS-CoV-2 RNA 1:2500	**7.1**	2.6
SARS-CoV-2 RNA 1:5000	**3**	1.4
SARS-CoV-2 RNA 1:10000	**2.7**	0.65

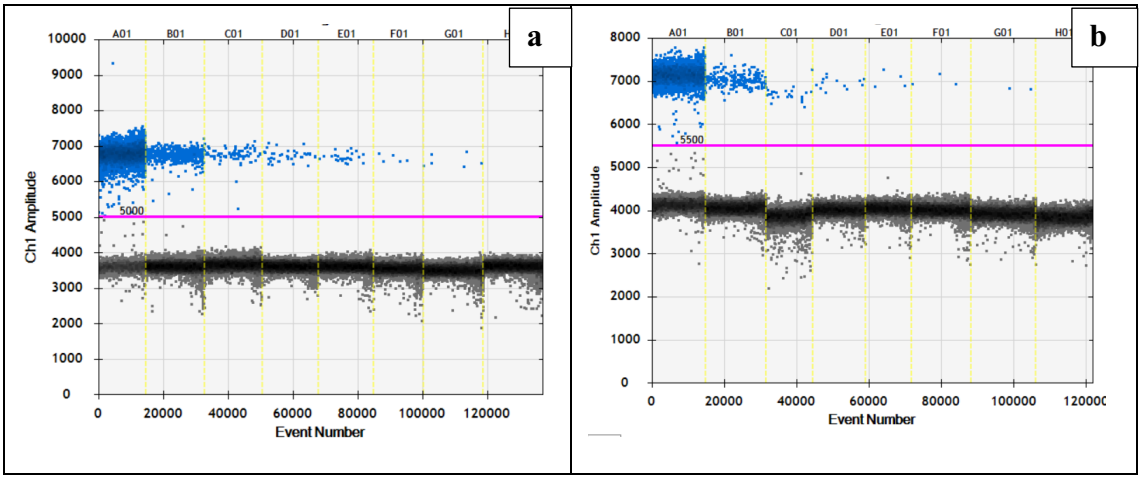


Figure 8. *Primer test in ddPCR.*

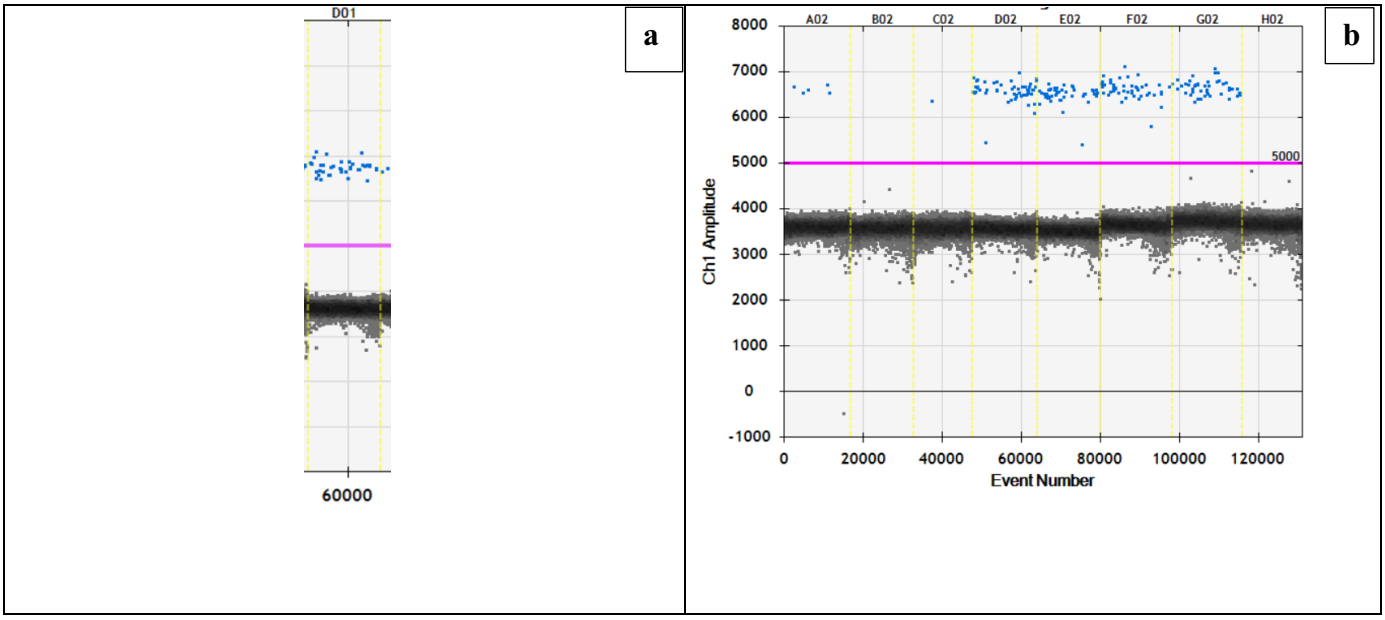
a) N1 primer A01 1:10 diluted RNA, B01 1:500 diluted RNA, C01 1:2 500 diluted RNA, D01 1:5 00 0diluted RNA, E01 1:10 000 diluted RNA.
(b) E primer A01 1:10 diluted RNA, B01 1:500 diluted RNA, C01 1:2 500 diluted RNA, D01 1:5 000 diluted RNA, E01 1:10 000 diluted RNA.

6.2 PCR inhibition in wastewater samples in ddPCR

PCR inhibition test was performed using wastewater samples that were collected before the SARS-CoV-2 pandemic started. Tested concentration of “no inhibition control” was 2.6 copies/µl (N1) and 1 copies/µl (E) by ddPCR. SARS-CoV-2 samples were diluted to the same concentration (1:5 000) with the wastewater samples which were collected at different time and place. The viral concentrations were 2.9-3.9 copies/µl (N1) and 1.2-2.3 copies/µl (E) by ddPCR (Table 9). The ddPCR amplitudes are present in *Figure 9*. The threshold was set at 5 000 with N1 primer and at 5 500 with E primer. A slightly higher number of copies of SARS-CoV-2 was detected in all wastewater samples than in “no inhibitor control”. Hence, no inhibition was observed in ddPCR of the wastewater samples in this test. Inhibition would be observed if the concentration in samples containing wastewater were lower than in samples diluted with pure water.

Table 9. *ddPCR PCR inhibition test using wastewater samples for both N1 and E primers.*

sample	N1 primer (copies/µl)	E primer (copies/µl)
no inhibition control	2.6	1
WWTP1 28.9.2017	3.8	1.2
WWTP2 12.12.2017	3.6	1.6
WWTP1 12.12.2017	2.9	2.3
WWTP1 7.2.2018	3	1.9
WWTP1 27.4.2018	2.9	1.4
WWTP2 12.6.2018	3.9	1.4



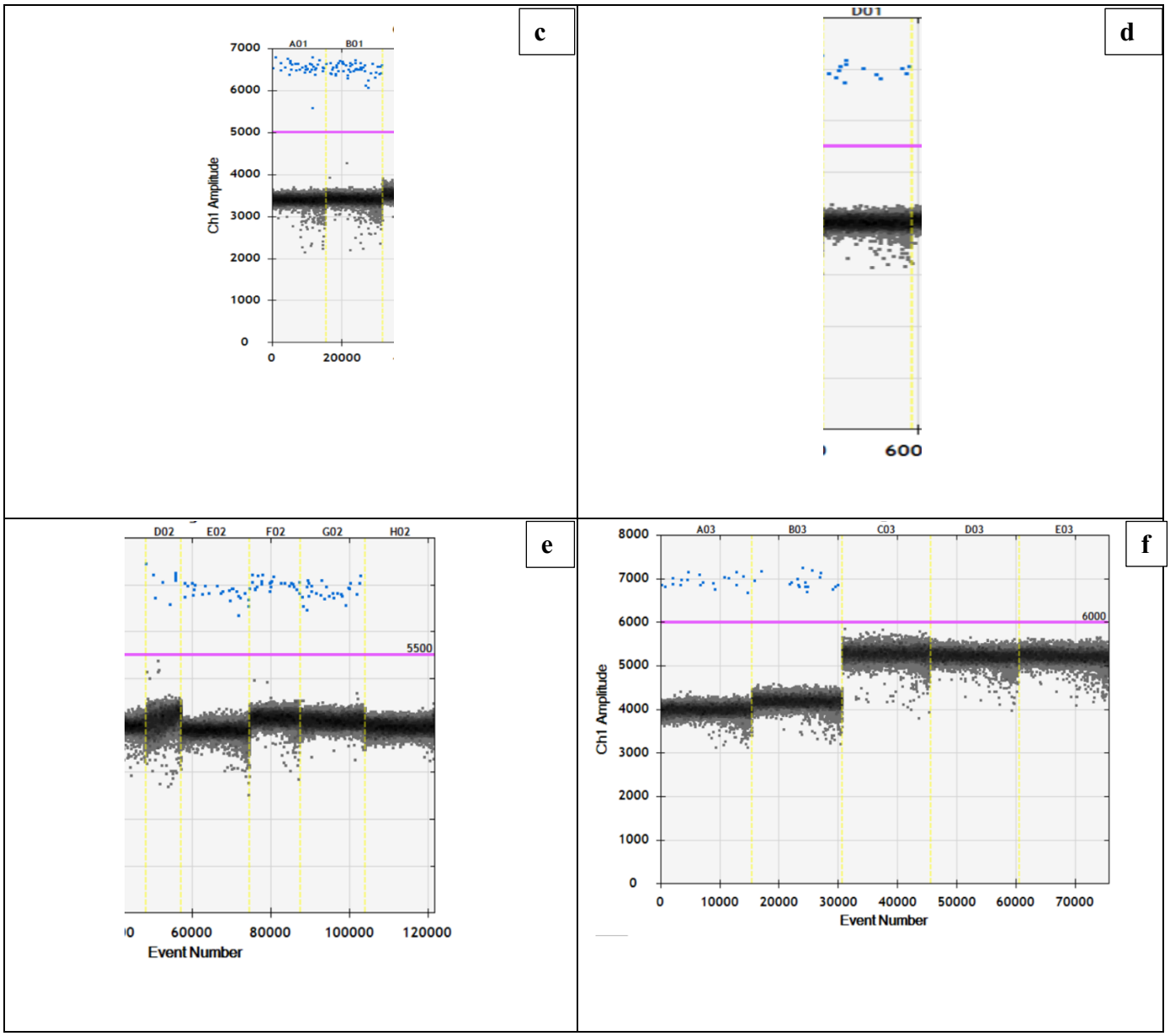


Figure 9. *PCR inhibition tested by ddPCR.*

(a) ddPCR amplitudes with N1 primer. Threshold was set as 5000. D01 no inhibition control. (b) ddPCR amplitudes with N1 primer. Threshold was set as 5000. D02 WWTP1 27.4.2018, E02 WWTP2 12.6. 2018, F02 WWTP1 12.12.2017, G02 WWTP1 7.2.2018, H02 NTC. (c) ddPCR amplitudes with N1 primer. Threshold was set as 5000. A01 WWTP1 27.4.2018, B01 WWTP2 12.6.2018. (d) ddPCR amplitudes with E primer. Threshold was set as 5500. D01 no inhibition control. (e) ddPCR amplitudes with E primer. Threshold was set as 5500. D02 WWTP1 28.9.2017, E02 WWTP2 12.12.2017, F02 WWTP1 12.12.2017, G02 WWTP1 7.2.2018, H02 NTC. (f) ddPCR amplitudes with E primer. A03 WWTP1 27.4.2018, B03 WWTP2 12.6.2018, C03 and D03 are blanks.

6.3 RNA extraction method optimization

The optimal RNA extraction method is important to obtain as sensitive detection method as possible. SARS-CoV-2 RNA from wastewater samples were extracted by the two methods: Qiagen RNA Viral Mini Kit and Perkin Elmer Viral RNA/DNA Chemagic 360 Kit. RT-qPCR and RT-ddPCR were made for the extracted RNA samples of both methods. Qiagen Quantitech Probe PCR Kit was used with RT-qPCR and Bio Rad One-Step RT-ddPCR Advanced Kit for Probes was used with RT-ddPCR. qPCR results of QCMD samples are listed in *Table 10* and RT-ddPCR in *Table 11.*

Comparison results of RT-ddPCR between QIAmp Viral Mini Kit and Chemagic Viral DNA/RNA Kit show that extraction of the RNA worked well with both kits and no significant differences were detected. The effect of primer sets seems to be stronger than for the sensitivity to detect the virus than effect of extraction method (Table 11). In qPCR, the detection of the virus was slightly more sensitive with Chemagic Viral DNA/RNA Kit than with the Viral RNA Kit (Table 10).

Table 10. *Comparison between RNA extraction methods in qPCR.*

The lowest Ct-values of each sample are bolded indicating the most sensitive detection of the virus. Ct-values are averages from the three parallel qPCR reactions

	extraction method					
	Viral Kit			**Chemagic**		
sample content	N1	N2	E	N1	N2	E
SARS-CoV-2	**33.11**	37.94	33.95	34.32	37.92	36.44
coronavirus 229E	negative	negative	negative	negative	negative	negative
SARS-CoV-2	36.95	negative	negative	**36.65**	negative	37.55
SARS-CoV-2	**29.37**	34.24	31.89	29.39	33.05	30.79
SARS-CoV-2	**32.48**	37.82	35.94	32.69	36.45	33.68
SARS-CoV-2	37.62	negative	37.61	**35.54**	37.97	36.53
SARS-CoV-2	32.55	37.25	35.05	**32.51**	36.91	34.25
SARS-CoV-2	33.8	37.78	36.47	**33.38**	38.44	35.39
negative	negative	negative	negative	negative	negative	negative
coronavirus OC43	negative	negative	negative	negative	negative	negative

Table 11. *Comparison between extraction methods in ddPCR, values copies/µl. The highest concentration of each sample is bolded. "no result" means that too few droplets have formed in the sample i.e., the number of the droplets was less than 8 000, and the result has been discarded*

	extraction method			
	Viral Kit		**Chemagic**	
sample content	N1	E	N1	E
SARS-CoV-2	**2.2**	1.4	0.17	1.4
coronavirus 229E	negative	negative	negative	negative
SARS-CoV-2	0.36	negative	**0.9**	0.4
SARS-CoV-2	**37.7**	24.7	36.6	24.9
SARS-CoV-2	**4.3**	2.5	3.7	3.5
SARS-CoV-2	negative	**1.4**	negative	0.7
SARS-CoV-2	4.4	3.5	**7.7**	2.8
SARS-CoV-2	**2.7**	1.8	2.6	2.5
negative	negative	negative	no result	negative
coronavirus OC43	negative	negative	no result	negative

6.4 The effect of freezing and thawing on SARS-CoV-2 RNA preservation

The effect of freezing and thawing may have an effect for the preservation of SARS-CoV-2 RNA. The processing of the wastewater sample has several steps including shipment, concentration, RNA extraction and ddPCR analysis. This process is time consuming and for practical reasons, the samples are sometimes freezed during the analysis. Therefore, the effect of freezing and thawing on SARS-CoV-2 RNA preservation was tested. SARS-CoV-2 RNA from wastewater samples that were collected between 21st and 22st of February 2021 were detected by ddPCR after RNA isolation and after one freeze-thaw-cycle.

The results are shown in *Table 12*. The ddPCR amplitudes and concentrations of non-frozen RNAs is shown in *Figure 10* and ddPCR amplitudes and concentration of frozen and thawed samples are shown in *Figure 14*. The SARS-CoV-2 copy number were similar from fresh and after on freeze-thaw-cycle. Interestingly even the samples that showed a low copy number of SARS-CoV-2 genome from un-freezed samples were positive after freezing-thawing cycle. Based on this comparison, one freeze and thaw cycle have no significant effect on the copy number of SARS-CoV-2 RNA in the samples. This is an important result for the analysis process of SARS-CoV-2 in wastewater samples. It indicates that this difficult and time-consuming process can be interrupted, if necessary, by freezing the sample before the ddPCR or qPCR analysis.

Table 12. *Effect of freezing and thawing on SARS-CoV-2 RNA preservation analyzed by ddPCR.*

WWTP	**no freezing or thawing of RNA**		**once frozen and thawed RNA**	
	N1 primer	**E primer**	**N1 primer**	**E primer**
Seinäjoki	negative	negative	negative	negative
Oulu	negative	negative	negative	0.17
Helsinki	1.6	0.63	1	0.9
Rovaniemi	0.24	0.1	0.22	0.09
Turku	0.38	0.26	1	0.9
Kuopio	0.13	0.12	0.08	0
Espoo	1.7	0.8	1.5	1.2
Pietarsaari	negative	negative	negative	negative
Lappeenranta	0.19	negative	0.29	0.15
Tampere	0.09	0.21	0.1	0.07

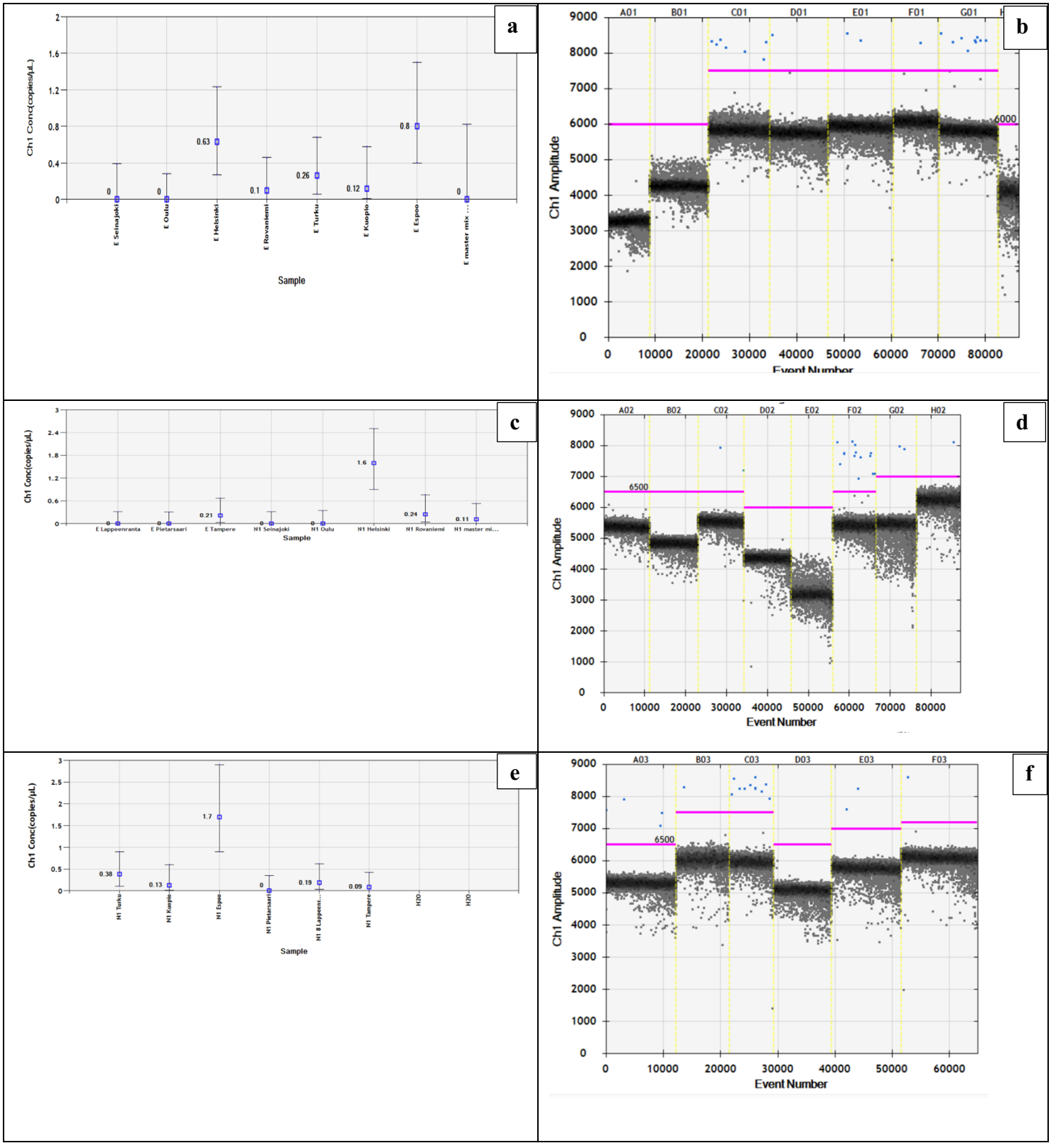

a
Ch1 Conc(copies/µL)
2
1.6
1.2
0.8
0.4
0
0
0
0.63
0.1
0.26
0.12
0.8
0
E Seinajoki
E Oulu
E Helsinki
E Rovaniemi
E Turku
E Kuopio
E Espoo
E master mix ...
Sample
b
A01
B01
C01
D01
E01
F01
G01
Ch1 Amplitude
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
6000
0
10000
20000
30000
40000
50000
60000
70000
80000
Event Number
c
Ch1 Conc(copies/µL)
3
2.4
1.8
1.2
0.6
0
0
0
0.21
0
0
1.6
0.24
0.11
E Lappeenranta
E Pietarsaari
E Tampere
N1 Seinajoki
N1 Oulu
N1 Helsinki
N1 Rovaniemi
N1 master mi...
Sample
d
A02
B02
C02
D02
E02
F02
G02
H02
Ch1 Amplitude
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
6500
0
10000
20000
30000
40000
50000
60000
70000
80000
Event Number
e
Ch1 Conc(copies/µL)
3
2.5
2
1.5
1
0.5
0
0.38
0.13
1.7
0
0.19
0.09
N1 Turku
N1 Kuopio
N1 Espoo
N1 Pietarsaari
N1 Lappeen...
N1 Tampere
H2O
H2O
Sample
f
A03
B03
C03
D03
E03
F03
Ch1 Amplitude
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
6500
0
10000
20000
30000
40000
50000
60000
Event Number

Figure 10. *On 21-22.3.2021 collected wastewater samples without freezing and thawing the RNA of the samples analyzed by ddPCR.*

Concentrations and amplitudes: (a&b): E primer Seinäjoki, Oulu, Helsinki, Rovaniemi, Turku, Kuopio, Espoo and NTC. (c&d) E primer and Lappeenrata, Pietarsaari and Tampere. N1 primer and Seinäjoki, Oulu, Helsinki, Rovaniemi and NTC. (e&f) N1 primer and Turku, Kuopio, Espoo, Pietarsaari, Lappeenranta, Tampere and 2 x blank.

6.5 Repeatability, sensitivity and specificity of the methods

It is important to detect low copy number of the virus from wastewater if only few individuals are infected in the population. In ddPCR, the lowest detected amount was 1:80 000 diluted RNA at 0.56 copies/µl with N1 primer and 1:40 000 diluted RNA at 0.59 copies/µl with E primer. These results were obtained by examining clinical samples. In qPCR, the lowest detected dilution was 1:5 000 at Ct-value 38.91 with E primer and 1:2 500 at Ct-value 39.75 with N1 primer. This result was in line with the previous reports that showed that ddPCR was more sensitive than qPCR (Dang et al. 2020; Falzone et al. 2020; Suo et al. 2020).

The optimized methods were compared also using SARS-CoV-2 QCMD panel. This panel in the QCMD program was analyzed in international laboratories using various methods including also commercial methods. The results of 497 datasets were reported in this QCMD panel round. Both tested qPCR and ddPCR methods detect all positive samples of QCMD panel and the methods did not detect the other coronaviruses, 229E or OC43, indicating that they were specific for SARS-CoV-2 (Quality Control for Molecular Diagnostic. Individual Report). False negative results were not observed. The comparison of the results to other participating laboratories confirmed that the methods optimized in this thesis are sensitive and specific: out of the 497 datasets only about 84% analyzed all samples correctly. The comparison of the results between different methods is often difficult or even impossible. The optimized methods were compared to test if these methods correlate. The amplification of both qPCR and ddPCR methods correlated to the copy numbers of the virus in the panel (*Table 13*). *Figure 11* shows there is a linear correlation between viral copies of sample and the ddPCR result.

Table 13. *Detection of the QCMD samples by qPCR and ddPCR.*

QCMD samples		detection method	
sample content	(copies/µl)	qPCR (Ct)	ddPCR (copies/µl)
SARS-CoV-2	1.45	34.3	2.2
coronavirus 229E	8.51	negative	negative
SARS-CoV-2	0.30	36.7	0.36
SARS-CoV-2	19.5	29.4	37.7
SARS-CoV-2	1.86	32.7	4.3
SARS-CoV-2	0.30	35.5	1.4
SARS-CoV-2	1.86	32.5	4.4
SARS-CoV-2	1.45	33.4	2.7
negative	0.00	negative	negative
coronavirus OC43	10.0	negative	negative

The QCMD sample serie included duplicate sample pairs. The informed copy number of the sample pairs were 0.3, 1.45 and 1.86 copies of SARS-CoV-2 genome copies in µl. The variability between sample pairs was low especially in higher concentrations using ddPCR (0.36 vs. 1.4; 2.2 vs. 2.7 and 4.3 vs. 4.4 respectively). The results indicated that the ddPCR quantifies the samples accurately. The qPCR results (35.5 vs, 36.7; 33.4 vs. 34.3 and 32.5 vs. 32.7 respectively) showed 2.3; 1.9- and 1.1-fold difference between the replicates, indicated that the accuracy was similar compared to ddPCR (Table 13).

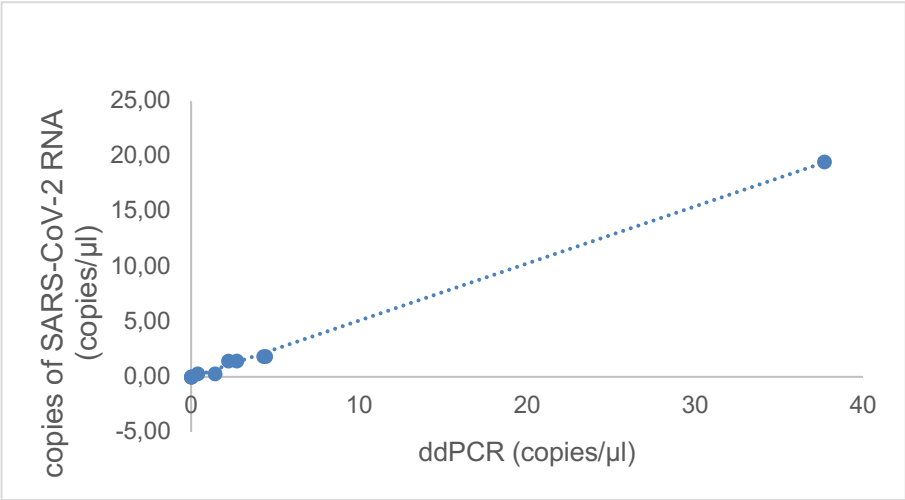


Figure 11. *Correlation plot between the copy number of viral RNA and the ddPCR results.*

6.6 Correlation of COVID-19 cases and SARS-CoV-2 in wastewater

The ddPCR method was selected for the correlation of SARS-CoV-2 in wastewater and COVID-19 cases in the population, because the method gives the absolute quantification of the copy number of the SARS-CoV-2 virus. The qPCR without quantitative control shows only the relative quantification within a sample series. Viral loads of the wastewater samples were detected by ddPCR using E and N1 primers. The SARS-CoV-2 was detected in 8 locations in both time points but, the virus was not detected in Seinäjoki and Pietarsaari (Table 14)

Table 14. *The wastewater treatment plants and the presence of the SARS-CoV-2 in their wastewater.*

WWTP	22.2.2021	22.3.2021
Seinäjoki	negative	negative
Oulu	positive	positive
Helsinki	positive	positive
Rovaniemi	positive	positive
Turku	positive	positive
Kuopio	positive	positive
Espoo	positive	positive
Lappeenranta	positive	positive
Pietarsaari	negative	negative
Tampere	positive	positive

The viral copies of the wastewater samples were analyzed by ddPCR with N1 primer and E primer (*Table 15*). The virus load in the wastewater was highest in Helsinki, Turku and Espoo in both February and March. In Seinäjoki and Pietarsaari virus was not detected. Other cities showed moderate virus load. The original data of ddPCR is shown in the *Figures 14* and 15.

Table 15. *SARS-CoV-2 copies from WWTP samples analyzed by ddPCR.*

WWTP	22.2.2021		22.3.2021	
	N1	**E**	**N1**	**E**
Seinäjoki	0	0	0	0
Oulu	0	0.17	1	0
Helsinki	1	0.9	0.36	0.6
Rovaniemi	0.22	0.09	0.14	0.13
Turku	1	0.9	1.4	0.8
Kuopio	0.08	0	0.13	0.11
Espoo	1.5	1.2	1.2	1.9
Lappeenranta	0.29	0.15	0	0.8
Pietarsaari	0	0	0	0
Tampere	0.1	0.07	0.5	0

The number of COVID-19 cases varies between cities significantly. In Helsinki the number of diagnosed cases is highest (1537-1720 individuals) followed by Espoo and Turku. Rovaniemi and Seinäjoki have few diagnosed cases and in Pietarsaari has no cases. Other cities have from 13 to 128 of COVID-19 cases. The SARS-CoV-2 copy number in wastewater per COVID-19 case in the area of the treatment plant was correlated using *Formula 1.*

$$\frac{ddPCR\ result\ (\frac{copies}{\mu l})}{N(number\ of\ COVID{-}19\ cases\ in\ the\ area\ of\ WWTP)} \qquad (1)$$

In some WWTP area has more higher copy number of virus per case than in others (Figure 12 and 13). This difference is particularly significant in Rovaniemi. The reason for this is unknown, but reason may be, for example domestic tourism, in which case some of infection would be recorded as cases of the place in residence of the infected individuals. Another possible reason may be that infected individuals in those areas have

undergone fewer test than elsewhere. The reason may also be related to the better persistence of the viral RNA in wastewater in those areas. In addition, the number of COVID-19 cases in some study cities are low. Therefore, the possible variation in the secretions of virus in different stage of disease process and different individuals may have an effect to the results. The sampling of the wastewater and dilution of wastewater may have an effect to the results

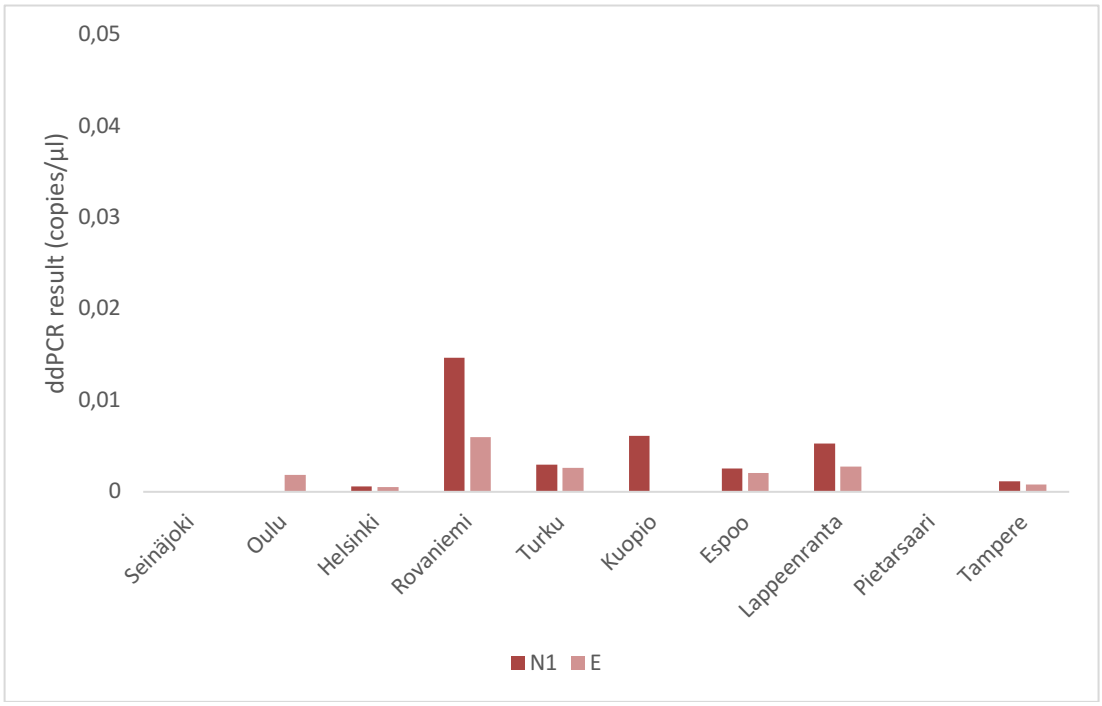


Figure 12. *The copies of the virus divided by the number of COVID-19 cases in the WWTP areas on 21.-22.2.2021*

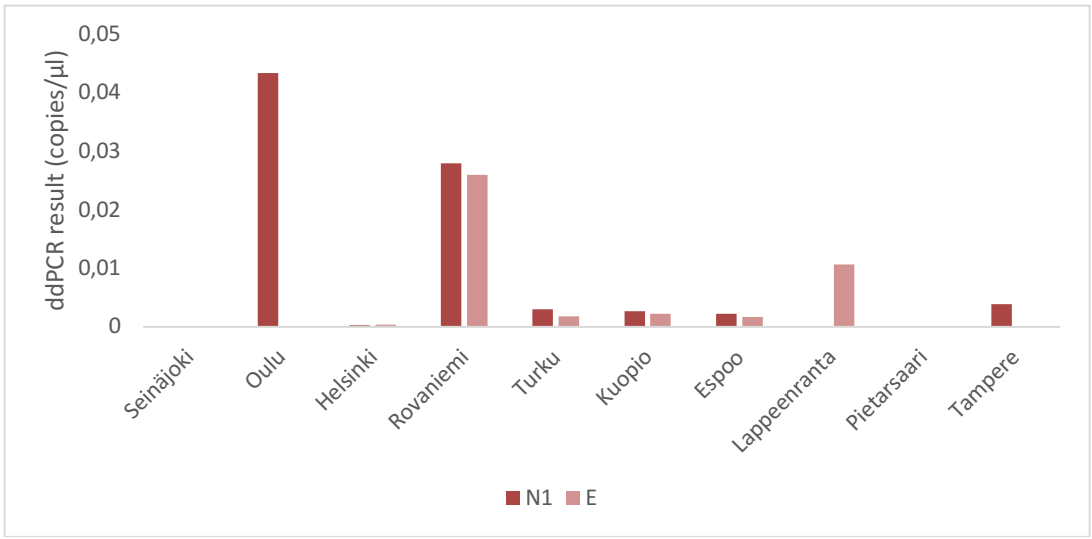
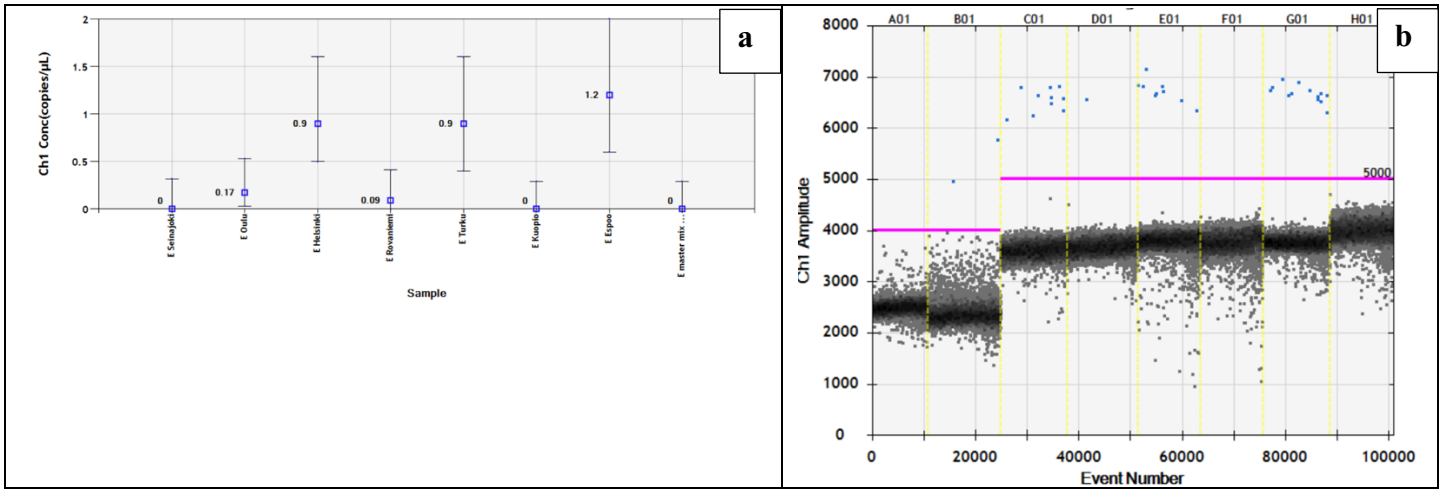


Figure 13. *The copies of the virus divided by the number of COVID-19 cases in the WWTP areas on 21.-22.3.2021*

WWTP specific COVID-19 cases were divided by WWTP specific population size in *Table 16.* The table shows that the areas of some WWTPs had more COVID-19 cases in proportion to the population than others. In week 8 the 10^{-4} value was highest in Helsinki (20.0), Espoo (15.1) and Turku (11.3). The value was zero in Pietarsaari and 1.27 in Seinäjoki. In week 12 the 10^{-4} value was highest in Helsinki (17.9), Turku (15.23), Espoo (13.85) and Lappeenranta (11.9). The value was zero in Pietarsaari and Seinäjoki and 0.19 in Rovaniemi.

Table 16. *The WWTP specific COVID-19 cases divided by WWTP specific population size and ddPCR results.*

WWTP	week 8 (10^{-4})	ddPCR result copies/µl	week 12 (10^{-4})	ddPCR result copies/µl
Seinäjoki	1.27	0	0	0
Oulu	4.55	0.17	1.15	1
Helsinki	20.0	1	17.9	0.6
Rovaniemi	2.73	0.22	0.91	0.14
Turku	11.3	1	15.23	1.4
Kuopio	1.43	0.08	5.27	0.13
Espoo	15.1	1.5	13.85	1.2
Lappeenranta	8.73	0.29	11.9	0.8
Pietarsaari	0	0	0	0
Tampere	4.45	0.1	6.4	0



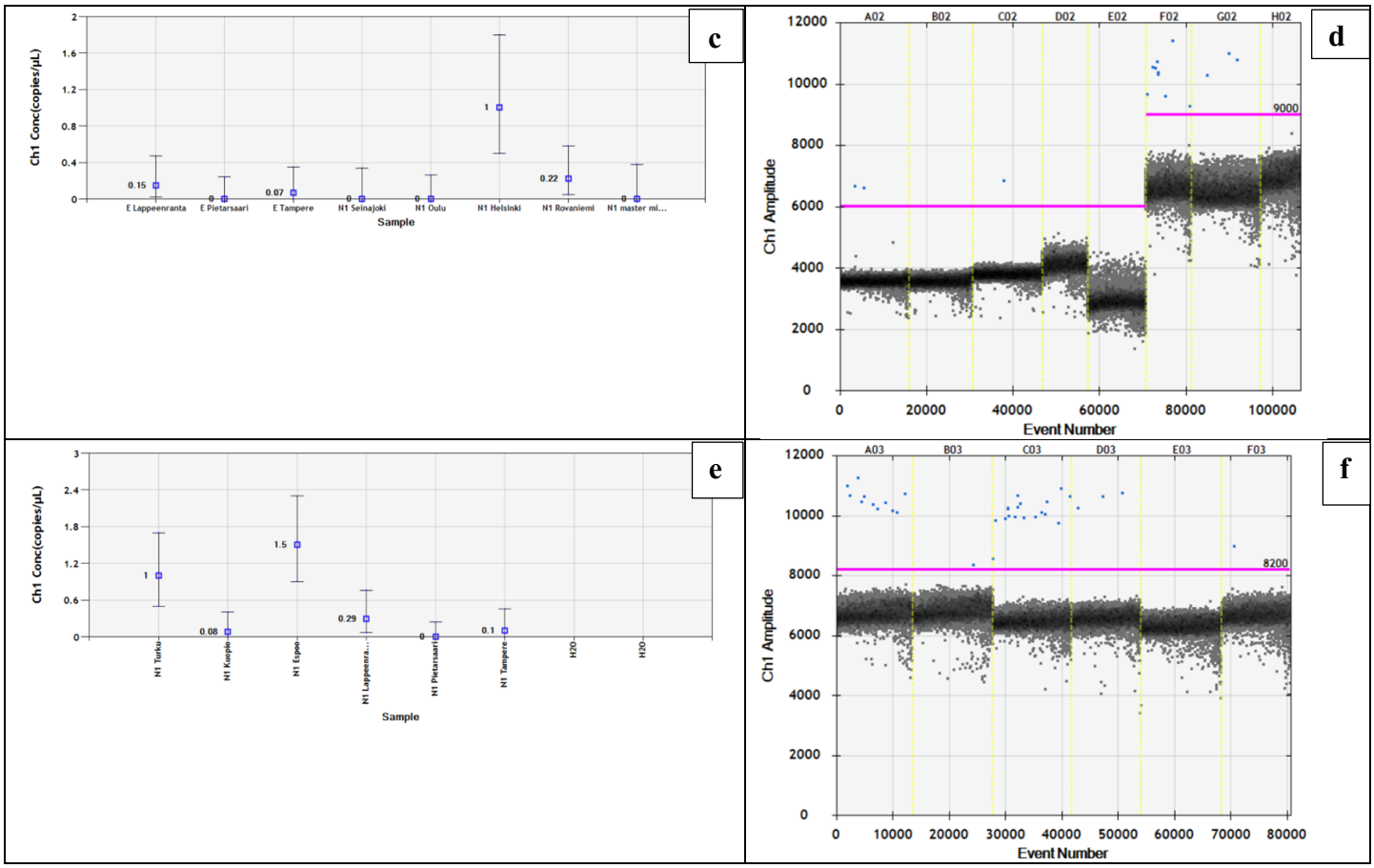


Figure 14. *On 21.-22.2.2021 collected wastewater samples analyzed by ddPCR.*

Concentrations and amplitudes: (a&b) E primer and Seinäjoki, Oulu, Helsinki, Rovaniemi, Turku, Kuopio, Espoo and NTC. (c&d) E primer and Lappeenranta, Pietarsaari, Tampere and N1 primer and Seinäjoki, Oulu, Helsinki, Rovaniemi and NTC. (e&f) N1 primer and Turku, Kuopio, Espoo, Lappeenranta, Pietarsaari, Tampere and 2 x blank.

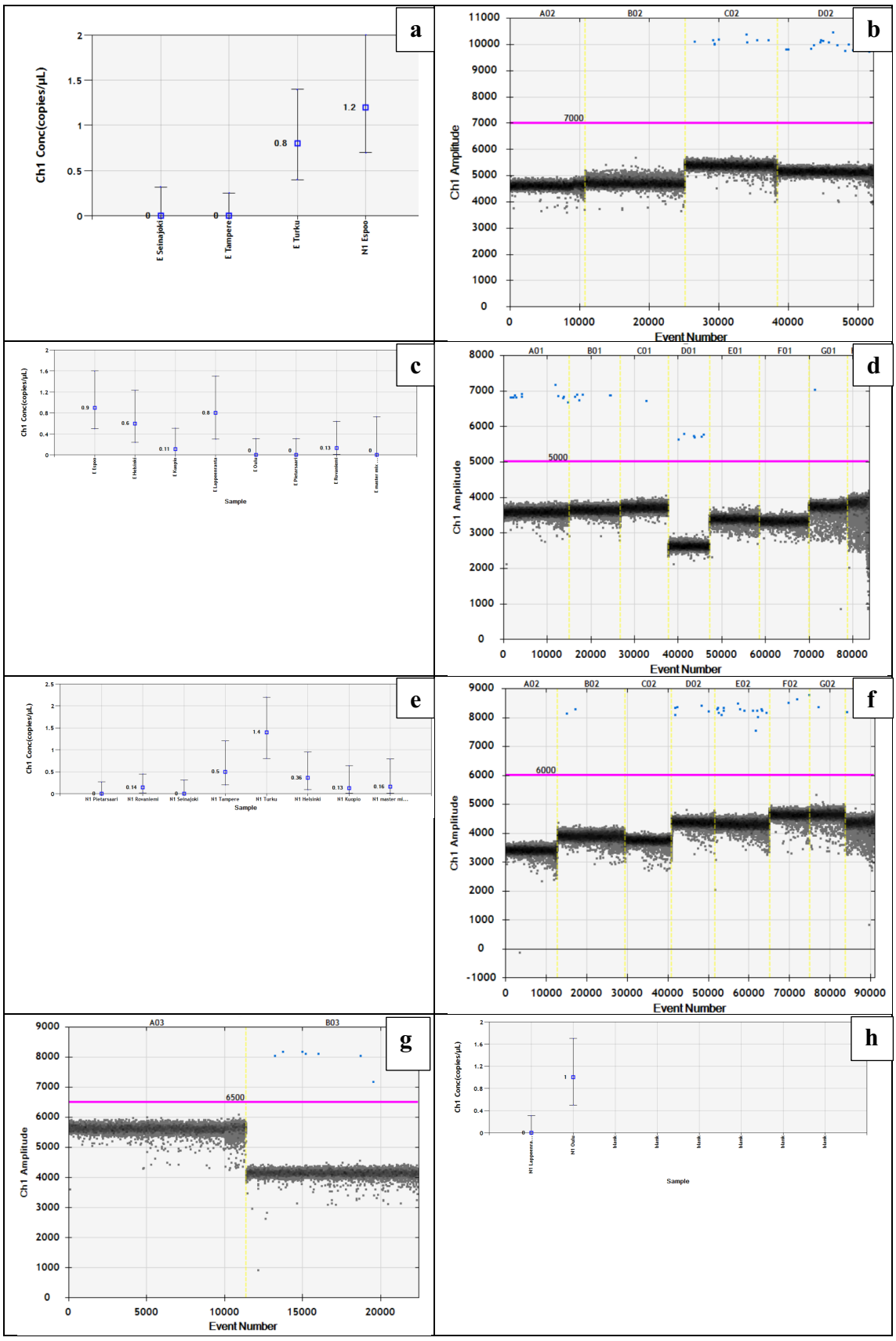

a
Ch1 Conc(copies/µL)
E Seinajoki
E Tampere
E Turku
N1 Espoo
b
Ch1 Amplitude
Event Number
7000
c
Sample
d
5000
e
f
6000
g
6500
h

Figure 15. *On 21.-22.3.2021 collected wastewater samples analyzed by ddPCR.*

Concentrations and amplitudes: (a&b) E primer Seinäjoki, Tampere, Turku and N1 primers Espoo. (c&d) E primer Espoo, Helsinki, Kuopio, Lappeenranta, Oulu, Pietarsaari, Rovaniemi and NTC. (e&f) N1 primer Pietarsaari, Rovaniemi, Seinäjoki, Tampere, Turku, Helsinki, Kuopio and NTC. (g&h) N1 primer Lappeenranta, Oulu and 6 x blank.

6.7 Data from wastewater treatment plant, population data and prevalence of COVID-19 cases

The correlation between copy number of SARS-CoV-2 virus in wastewater and COVID-19 cases was further improved by taking account also the population size of the wastewater treatment plant areas. However, the wastewater treatment plant areas and hospital district areas do not cover same areas and that had to be taken into account.

In 2019, 4 046 754 Finnish people were added to the sewage network (Suomen ympäristökeskus, Vesihuollon tietojärjestelmä, Liittyjämäärät) and the number of population were 5 525 292 (Suomen virallinen tilasto (SVT), Väestö kielen mukaan 1980–2019). 73,24% of the population in Finland were users of the sewage network. In Finland, almost 60% of population is subject to wastewater monitoring (THL Väestötason huumeiden käyttö). *Table 17* shows the wastewater treatment plants and the cities whose viral load has been detected in this thesis work. The samples used in this work, represent 2 245 000 person and 40,6% of Finnish population (THL Koronaviruksen jätevesiseuranta).

68% of wastewater from Vantaa goes to Viikinmäki wastewater treatment plant in Helsinki and 32% of wastewater from Vantaa goes to Suomenoja wastewater treatment plant in Espoo. For this reason, in this thesis work it was decided to divide the cases of infection in Vantaa as follows: 2/3 of the cases were included in the cases of Viikinmäki wastewater treatment plant areas and 1/3 of the cases were included in the cases of Suomenoja wastewater treatment plant areas. Mäntsälä is a second special case: southern part of Mäntsälä, residental areas Hyökännummi and Ohkola, belongs to the area of Viikinmäki wastewater treatment plant and wastewater of other areas of Mäntsälä goes to other wastewater treatments plants that are not included in this thesis work. Thus, the number of cases of Mäntsälä in Infectious diseases register of Finland has been divided by two.

Table 17. *Wastewater treatment plants, their population and cities*

WWTP	Population	Cities
Helsinki; Viikinmäki	860 000	Helsinki, Järvenpää, Kerava, Mäntsälä (southern), Pornainen, Sipoo, Tuusula, Vantaa (eastern and central)
Espoo, Suomenoja	390 000	Espoo, Vantaa (western), Kirkkonummi, Kauniainen, Siuntio
Lappeenranta, Toikansuo	63 000	Lappeenranta, Lemi, Taipalsaari
Kuopio, Lehtoniemi	91 000	Kuopio
Oulu, Taskila	200 000	Ii, Muhos, Oulu, Utajärvi
Rovaniemi, Alakorkalo	55 000	Rovaniemi
Turku, Kakolanmäki	300 000	Aura, Kaarina, Lieto, Marttila, Masku, Mynämäki, Naantali, Nousiainen, Oripää, Paimio, Pöytyä, Raisio, Rusko, Turku
Pietarsaari, Alheda	31 000	Pietarsaari, Pedersörenkunta, Uusikarlepyy, Luoto
Tampere, Viinikanlahti	200 000	Kangasala, Lempäälä, Pirkkala, Tampere
Seinäjoki, keskuspuhdistamo	55 000	Seinäjoki

The prevalence of COVID-19 cases was calculated per 100 000 individuals for each city. The prevalence was then correlated with SARS-CoV-2 copy number and scatter plot was done (*Figure 16)*. The analysis shows that the virus load in the wastewater correlates with the prevalence of the COVID-19 cases in different cities and analyzed time points. Only clear outlier is one of the two Oulu samples. The explanation may be that the analysis of the virus load was not accurate: N1 primer set showed 1 copy/µl of SARS-CoV-2 gene in the sample and E primer set was negative. This is clearly the largest variation between the two methods. Prevalences of the cities in this work varies between 9 and 200. Westhaus et al. (2021) monitored wastewater of 9 cities in Germany in April 2020 and they reported that prevalence of COVID-19 cases per 100 000 individuals varies between 30 and 174.

Table 18. *Number of COVID-19 cases and ddPCR results at the selected wastewater treatment plants. A higher positive value of the ddPCR results has been selected for this table.*

WWTP	**week 8** number of COVID-19 cases	ddPCR result (copies/µl)	**week 12** number of COVID-19 cases	ddPCR result (copies/µl)
Seinäjoki	7	0	0	0
Oulu	91	0.17	23	1
Helsinki	1720	1	1537	0.6
Rovaniemi	15	0.22	5	0.14
Turku	340	1	457	1.4
Kuopio	13	0.08	48	0.13
Espoo	589	1.5	540	1.2
Lappeenranta	55	0.29	75	0.8
Pietarsaari	0	0	0	0
Tampere	89	0.1	128	0.5

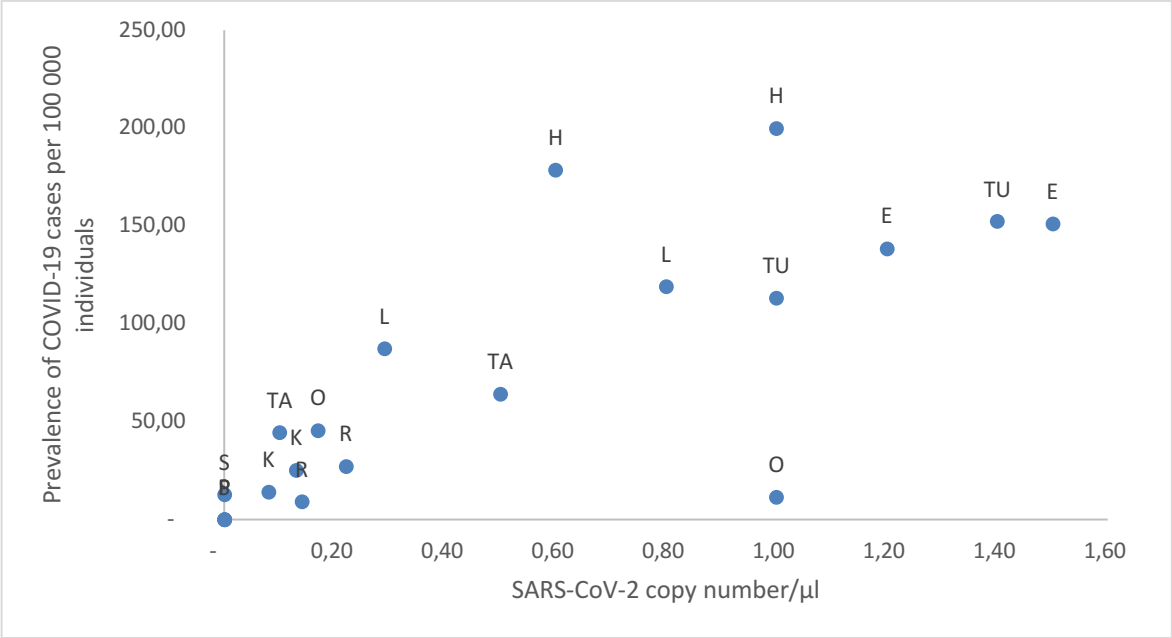


Figure 16. *Scatter plot between the prevalence of the COVID-19 cases per 100 000 individuals and the SARS-CoV-2 copy number based of ddPCR analysis.*

S=Seinäjoki, O=Oulu, H=Helsinki, R=Rovaniemi, TU=Turku, K=Kuopio, E=Espoo, L=Lappeenranta, P=Pietarsaari, TA=Tampere

The wastewater monitoring has been suggested for a tool to monitoring the SARS-CoV-2 pandemics in the world (Langone et al. 2021). This study showed that this is highly possible, but the model has to be done more precisely. It can be improved by collecting samples from a longer period of time with different epidemic situations. It is also important to collect background information that may have an effect to survival of SARS-CoV-2 in wastewater such as wastewater flow, pH and temperature. Methodological development is also needed, for example to assess the faecal content of wastewater using for example *E. coli* or crAsspages. Chemical composition of the wastewater varies between treatment plants and therefore the monitoring the effects of PCR inhibitors is important.

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7. CONCLUSIONS

Primer optimization for ddPCR

N1 was the most sensitive PCR primer sets of the primers used with the clinical samples and the wastewater samples. Difference between the primers is smaller when the sample was more diluted. The second most sensitive primer was E primer. These primer sets were selected for the SARS-CoV-2 monitoring of wastewater.

PCR inhibition in ddPCR

No inhibition was observed with the wastewater samples by ddPCR. 1:5000 pure water diluted SARS-CoV-2 RNA was compared to 1:5000 wastewater diluted samples and the presence of wastewater did not decrease the detected concentration of the samples. However, inhibition may have an effect. Wastewater treatment plants are different, there may be a difference in the intensity of inhibition between them. Also, composition of the wastewater of same WWTP can varies if timepoint varies.

RNA extraction method optimization in ddPCR

With ddPCR, the RNA extracted with Qiagen Viral RNA Mini Kit gave little higher copy numbers of SARS-CoV-2 virus than Chemagic Viral DNA/RNA Kit but difference between these kits was not large. On the contrary, Chemagic Viral DNA/RNA Kit worked with qPCR than Qiagen Viral RNA Mini Kit.

Specificity between RT-qPCR and RT-ddPCR

Both methods detected SARS-CoV-2 RNA from QCMD samples. They did not detect 229E and OC43 coronaviruses and they detected different copy numbers SARS-CoV-2 RNA, indicating that both methods were specific, and no false positive was found. In summary, the two tested methods analyzed all QCMD samples correctly. The same QCMD series was analyzed by hundreds of laboratories in the world and 497 datasets were reported. About 84% of the laboratories analyzed all samples correctly showing that the optimized methods are high quality.

Sensitivity between RT-qPCR and RT-ddPCR

The lowest detected amount by ddPCR was 1:80000 diluted RNA at 0.56 copies/µl with N1 primer and 1:40000 diluted RNA at 0.59 copies/µl with E primer. The results are in line with LoDs reported in the literature (0.09-1.05 copies/µl with N primer) (Suo et al.

2020). The lowest detected amount by qPCR was 1:5000 diluted RNA at Ct-value 38.91. The ddPCR method detected the virus well in wastewater samples and only one of the WWTP samples were negative even though cases had been reported in the area of that wastewater treatment plant. For the other samples, the result was positive if cases were reported to the area except the one WWTP (Seinäjoki) where seven cases occurred, but no viral RNA was detected in the wastewater sample.

SARS-CoV-2 in wastewater

The virus was found in 8 WWTPs: Oulu, Helsinki, Rovaniemi, Turku, Kuopio, Espoo, Lappeenranta and Tampere. Detected viral RNA copies per volume were not consistent with the reported COVID-19 cases of an area and some WWTPs obtained higher viral concentrations per reported COVID-19 case. The reason for this variation is unknown and it can be due to many factors, such as the fact that infected asymptomatic individuals may not apply for the test. Due to domestic tourism, cases may have been reported in a different city than where the person has excreted the virus in the wastewater. However, when the ddPCR results were correlated to the prevalence of COVID-19 cases per 100 000 individuals for each city, the correlation was seen. This thesis showed that the monitoring SARS-CoV-2 in wastewater is a promising tool for monitoring SARS-CoV-2 epidemics.

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