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Prediction of COVID-19 positive cases, a nation-wide SARS-CoV-2 wastewater-based epidemiology study

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ABSTRACT

Taking advantage of Estonia's small size and population, we have employed wastewater-based epidemiology approach to monitor the spread of SARS-CoV-2, releasing weekly nation-wide updates. In this study we report results obtained between August 2020 and December 2021. Weekly 24 h composite samples were collected from wastewater treatment plants of larger towns already covered 65% of the total population that was complemented up to 40 additional grab samples from smaller towns/villages and the specific sites of concern.

The N3 gene abundance was quantified by RT-qPCR. The N3 gene copy number (concentration) in wastewater fluctuated in accordance with the SARS-CoV-2 spread within the total population, with N3 abundance starting to increase 1.25 weeks (9 days) (95% CI: [1.10, 1.41]) before a rise in COVID-19 positive cases. Statistical model between the load of virus in wastewater and number of infected people validated with the Alpha variant wave (B.1.1.17) could be used to predict the order of magnitude in incidence numbers in Delta wave (B.1.617.2) in fall 2021. Targeted testing of student dormitories, retirement and nursing homes and prisons resulted in successful early discovery of outbreaks. We put forward a SARS-CoV-2 Wastewater Index (SARS2-WI) indicator of normalized virus load as COVID-19 infection metric to complement the other metrics currently used in disease control and prevention: dynamics of effective reproduction number (*Re*), 7-day mean of new cases, and a sum of new cases within last 14 days. In conclusion, an efficient surveillance system that combines analysis of composite and grab samples was established in Estonia. There is considerable discussion how the viral load in wastewater correlates with the number of infected people. Here we show that this correlation can be found. Moreover, we confirm that an increased signal in wastewater is observed before the increase in the number of infections. The surveillance system helped to inform public health policy and place direct interventions during the COVID-19 pandemic in Estonia via early warning of epidemic spread in various regions of the country.

1. Introduction

Surveillance of the infectious agents in wastewater (WW) is a surprisingly old concept. First failed attempts to detect poliovirus in WW were carried out in the beginning of 1930s and the approach was successfully implemented by the end of the decade (Paul et al., 1939).

Since then, molecular tools have transformed our capabilities and poliovirus is routinely monitored in WW far beyond just detection (Berchenko et al., 2017; Ivanova et al., 2019; Nakamura et al., 2015). Wastewater-based epidemiology (WBE) has been used for a wide variety of pathogenic organisms that pass through municipal WW treatment systems (reviewed by Sinclair et al., 2008). In addition, WW-based monitoring has been broadly deployed for surveillance of antibiotic resistance genes (Chow et al., 2020; Hendriksen et al., 2019; Hutinel et al., 2019; Majeed et al., 2021; Riquelme et al., 2022); xenobiotic and human biomarkers (Boogaerts et al., 2021); and anthropogenic

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psychoactive drugs (Zuccato and Castiglioni, 2009).

A global pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pushed WBE into a new gear, as researchers across the globe mobilised to monitor RNA levels of the virus in WW (reviewed by Bonanno Ferraro et al., 2021). Many types of infection within a community result in pathogen excretion in bodily fluids and/or solids and therefore, transported into the community sewage system (Sinclair et al., 2008). This includes even respiratory infections that are usually the result of replication of the pathogen in the nose, throat, or lungs. The SARS-CoV is excreted in the faeces and other bodily fluids (Leung et al., 2004; Wang et al., 2005) and the same was found to be valid in case of SARS-CoV-2 (reviewed by Cheung et al., 2020). COVID-19 WBE relies on the stability of the viral particles (and possibly free viral RNA). It is well-established that viral RNA (or particle) is stable in sewage systems despite they face the hostile environment (Karthikeyan et al., 2021a, 2021b; Larsen and Wigginto, 2020; Weidhaas et al., 2021).

After the efficacy of WW surveillance of SARS-CoV-2 was demonstrated by case studies, several nations started to monitor the virus spread using viral detection in wastewater as a proxy for SARS-CoV-2 prevalence in the general population (Shah et al., 2022). This effort has been extremely successful and in the case of the Netherlands, all of the wastewater treatment plants (WWTPs) were included, covering 99.6% of the Dutch population (van Boven et al., preprint). In Austria, viral variant-resolved WW based surveillance at national scale also reached high coverage (Amman et al., 2022).

In several regions the virus was detected in the WW before the appearance of COVID-19 symptomatic human subjects (Hernandez et al., 2021; Medema et al., 2020), with initial WW detection preceding the outbreak in the community by 7 to 10 days (Peccia et al., 2020). By now, many countries have established SARS-CoV-2 wastewater surveillance systems thereby also revealing factors on each step of COVID-19 WBE that influence its performance (Naughton et al., 2021). The main factors are: size of the WWTP, population density in catchment area and neighbourhood types in it (Fitzgerald et al., 2021; Haak et al., 2022); the fraction of WW - liquid phase, solid phase and bioaerosols investigated (Pourakbar et al., 2022); methods used for virus concentration and RNA extraction (Zheng et al., 2022; Pérez-Cataluña et al., 2021); biomarker used for normalization of SARS-CoV-2 signal (D'Aoust et al., 2021); the climatic conditions and WW systems (discussed by Carducci et al., 2020); quality assurance and control procedures for RT-PCR quantification (Ahmed et al., 2020).

Systematic meta-analysis of impact of these factors on WBE performance has been carried out by Li et al. (2022a), differences arising from methodological approaches have been reviewed by Kopperi et al. (2021), systematic review to assess the performance of WW surveillance as early warning system of COVID-19 community transmission was carried out by Shah et al. (2022), and uncertainties in estimating SARS-CoV-2 prevalence by WBE were explored by Li et al. (2022b). In general, many studies demonstrate the great potential of WBE as a public health tool, but also refer to the uncertainties and unexpected variability arising from factors such as the properties of the sewer network, sampling and quantification methods and approach for population normalisation.

Therefore, many variables are needed to be taken into account to adjust general approach for specific regions. In addition, the methodologies used for data collection and analysis differ. Therefore, exchanging experiences of different surveillance programs is essential. Here we describe the wastewater based SARS-CoV-2 surveillance program covering the entire country of Estonia that started in August 2020 (standard operational protocol delivered to Estonian Health Board and still in use), and outline how it was used in disease control and prevention in Estonia. The program developed several novel features for data normalisation, analysis and communication.

2. Materials and methods

2.1. Sampling from WWTPs- 24-hour composite samples

Automated samplers P6 Mini MAXX (Probenahmetechnik GmbH, Germany) and WS Porti 12 (WaterSam GmbH & Co, Germany) were used to collect 24 h composite influent wastewater (WW) samples, for which 96 subsamples (60–80 ml each) were collected every 15 min. Samples were kept at 4 °C until analyses, which were performed within a 24-hour period. ISO 5667–10 quality standards were followed during the sampling. Two subsamples were collected into 300 ml sterile PE bottles (VMK Trading) one to determine the viral load and another for *E. coli* abundance. Grab-samples were gathered from WWTP influent preferably from attenuation tanks or bar screen wells.

2.2. WW effluent characteristics and E. coli quantification

Most-Probable-Number (MPN) assay (Colilert-18, IDEXX Laboratories, Inc.) was used to quantify of *E. coli* (EVS-EN ISO 9308–2:2014). Plates were incubated at 36 ± 2 °C for 18–22 h. Blue fluorescence of hydrolysed 4-methylumbelliferyl-β-D-glucuronide (MUG) was measured under 365 nm ultraviolet light with Spectroline® CM UV-viewing cabinet.

Analysis methods and corresponding ISO standards were: chemical oxygen demand (COD), EVS ISO 15,705:2004; biological oxygen demand (BOD), EVS-EN ISO 5815–1:2019 and EVS-EN 1899–2:1999; total nitrogen concentration (Ntot); EVS-EN ISO 11,905–1:2003 and EVS-EN 12,260:2003; total phosphorus concentration (Ptot), EPA.134-C. Rev.0:2014, EVS-EN ISO 6878:2004; total suspended solids (TSS), EVS-EN 872:2005; pH, EVS-EN ISO 10,523:2012.

2.3. Concentration of virus fraction

After arrival to the lab, samples were preserved by fixation with 10% volume of stop solution (5% phenol: 95% ethanol) in order to conserve environmental RNA (Feike et al., 2012) and divided into three 50 ml aliquots in 50 ml centrifugation tubes. Larger suspended solids were removed by centrifugation at 4600 g for 30 min (4 °C). With syringes, the supernatant was carefully transferred and filtered manually through 0.22 μm Sterivex™ GP Sterile Filter Units (Merck KGaA, Germany) to eliminate non-viral particles from the solution. The filtrate (3 × 50 ml) was collected into new 50 ml centrifugation tubes and concentrated using Centricon® Plus-70 Centrifugal Filter Units with a molecular weight cut-off of 10 kDa (Merck Millipore, Burlington, USA) at 3500 x g for about 30 min each step (Medema et al., 2020). Each sample was concentrated down to about <600 μl solution, which was stored at −20 C° until RNA extraction for up to 24 h.

2.4. RNA extraction and qPCR

RNeasy® Mini Kit (Qiagen, Germantown, MD, USA) was used to extract RNA according to manufacturer's instructions with two exceptions. First, each volume of the individual samples was adjusted to 600 μl by either adding MQ water and, after adding 600 μl RTL lysis buffer, 800 μl of 96% ethanol was added. Second, the elution was carried out in two steps using 50 μl RNase Free Water (8000 g for 1 min). OneStep™ PCR Inhibitor Removal Kit (Zymo Research, USA) was used for additional removal of PCR-inhibiting compounds. RNA extracts were analysed by using one-step reverse transcription real-time PCR (RT-qPCR). The 10-μl reaction mixture contained 3 μl of the RNA extract, 1x One-step Probe CoV Mix, 1x One-step SOLIScript® CoV Mix (Solis BioDyne, Estonia), 200 nM of previously published primers for nucleocapsid genes primers ("Primers and Probes," 2019) and fluorescently labelled probe (Microsynth, Switzerland) adjusted to the final volume of 10 μl with molecular grade water (Solis BioDyne, Tartu, Estonia). To quantify SARS-CoV-2 RNA in the samples, a calibration standard curve was constructed for

each run using serial dilution series of calibrated EURM-019 single-stranded RNA (EC Joint Research Centre). All the reactions were performed in six replicates. Ultrapure molecular grade water was used as a negative control. RT-qPCR reactions were performed at 55 °C for 30 min, followed by 95 °C for 10 min and 45 cycles of 95 °C for 10 and 55 °C for 30 s on Roche LigthCycler 480 (Roche Life Sciences, Switzerland). Methods complied with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009), using the MIQE checklist (https://rdml.org/miqe.html).

2.5. COVID-19 positive cases

All COVID-19 positively tested (PCR based) personal data were collected centrally by Estonian Health Board and the data (August 1. 2020 to June 30, 2021) were used in this study. For the time period from July 1 to December 2021 open data provided by Health Board were used (https://opendata.digilugu.ee/docs/#/en/readme). To protect patient anonymity, data were provided connected to sewershed catchment using street names and regions ensuring that the identity of a case cannot be inferred from other publicly available information. Virus concentration in WW was compared to the local infection incidence rate in major test sites where the 24 h composite samples were collected. The main aim was to estimate the correlation and the time lag between number of COVID-19 positive cases and the copy number of viral genes in WW. Two parameters were derived from the data: i) scaling shift parameter, a conversion factor between virus volumetric concentration (gene copies ml^{-1}) and incidence rate (number of positively tested persons per week) and ii) the lag period, reflecting the time, in weeks, between the increase of the virus concentration in WW samples and the following increase in the infection incidence rate.

2.5. Statistical analysis and visualisation

Modelling of the association between the viral load and number of confirmed COVID-19 positive cases was carried out in R (R Core TEAM, 2020) using the extension packages "locfit" (Loader, 2020), "metafor" (Viechtbauer, 2010), and "plyr" (Wickham, 2011). The R script used is found in Supplemental Material (R script). Visualisation of the results was carried out using R version 3.6.3 (R Core TEAM, 2020) with the extension packages: ggplot2 and gganimate (Pedersen and Robinson, 2020).

To analyse the relationship between SARS-CoV-2 RNA concentration in the wastewater and inhabitants who were tested COVID-19 positive inhabiting the catchment area of the wastewater system, a two-step approach was used. In the first step, curve registration was applied separately for each site to accommodate differences in trajectories for copy numbers and the number of COVID-19 positive cases across sites. In the second step, estimates were combined across sites to obtain an overall country-wide estimate. In third step, estimated lag and shift parameters were used to predict COVID-19 positive cases from August 2021 to December 2021.

Step 1. For each site, curve registration was carried out by fitting non-parametric, local linear regression models to capture the highly non-linear trajectories in how copy numbers and number of COVID-19 positive cases developed over weeks (Loader, 1999). Specifically, separate local linear regression model fits were obtained for copy numbers and COVID-19 positive cases. The logarithm transformation was applied to both copy numbers and numbers of COVID-19 positive cases to achieve approximately constant variation across time as has been done previously (Cluzel et al., 2021), in effect rendering the scales for copy numbers and numbers of COVID-19 positive cases comparable. To accommodate for weekly variation in *E. coli* counts over time, copy numbers over time were scaled down or up depending on whether there was more or less *E. coli* compared to the mean level for the site. This adjustment factor (on a logarithmic scale) was also estimated using local linear regression.

Subsequently, it was assumed that the two trajectories for copy numbers and numbers of COVID-19 positive cases, respectively, only differed by a horizontal lag on the time scale and a vertical scaling shift on the two logarithmic scales. Based on this assumption, the two fitted local linear regression curves (through a grid of time points and corresponding predicted values) were used to estimate two parameters: a lag parameter and a scaling or shift parameter. To handle large variation between sites, lag and shift parameters were not estimated using the entire data available for 2020 and 2021. Instead, they were estimated for shorter, moving time intervals. Estimation was carried by means of least-squares estimation, which produced both estimates and corresponding approximate standard errors obtained through inversion of the Hessian.

These moving intervals could have widths between 5 and 13 weeks. The optimal width was determined using cross-validation using data from later weeks (which were not used for model fitting). For each width and each moving interval, which could be defined given the specified width, data were compared to the predictions based on the fitted local linear regression models using root mean squared errors that were obtained by averaging results from all moving windows with that width. Finally, the optimal width was the one resulting in the smallest average root mean squared error. Subsequently, the final site-specific estimated lag and shift parameters and their standard errors were obtained through averaging all interval-specific estimates for the chosen width. These estimates were also used for obtaining the final site-specific predictions.

Step 2. Separately for lag period and shift parameters, the site-specific estimated lag and shift parameter and their corresponding standard errors were combined in a meta-analytic approach. Estimates were averaged across sites, resulting in a single weighted average, where the weighting took into account the magnitude of their corresponding standard errors (Normand, 1999; Jiang et al., 2014). In this way the varying amount of data from the individual sites could be accommodated: estimates from sites with more data received more weight in the weighted average than sites with less data available. The weighted average could be conveniently estimated directly using a so-called random effect model where the imbalance across sites was automatically accommodated through site-specific random effects that carry the information about the imbalance.

Step3. The obtained estimated lag and shift parameters were used to predict COVID-19 positive cases using N3 gene copy numbers in WW from August 2021 to December 2021. Specifically, the following linear model, which defined on logarithmic scale, was used for prediction:

$$\log(\text{COVID} - 19\ \textit{positive cases}) = \textit{lag period} + \textit{shift parameter} \times \log(N3\ \textit{copy number})$$

Subsequently, the estimated logarithm-transformed incidence was back-transformed to obtain the estimated incidence.

In addition, linear modelling was used to associate basic variables measured from WW - total suspended solids (TSS), chemical and biological oxygen demand (COD and BOD), pH and total concentration of phosphorus and nitrogen (Ptot and Ntot),.

2.6. SARS-CoV-2 wastewater index (SARS2-WI)

SARS2-WI was calculated using site-specific N3 gene copy number concentration in wastewater normalised by MNP of *E. coli* and weighted with the populations size as following:

$$SARS2 = WI = \left(\sum_{i}^{i} \frac{N3_i\ copies/ml}{MPN\ E.coli/100\ ml} \cdot Population_i \right) \div Total\ population$$

where $N3_i$ is a site specific (i) virus concentration, MNP *E. coli*$_i$ is site specific MPN of *E. coli*, and Population$_i$ is size of the population in specific site, *Total population* is the sum of the inhabitants from the

specific sites.

3. Results

3.1. Quality assurance (QA)/quality control (QC) of PCR

Negative controls for nucleic acid extraction and PCR (at least 12 negative control samples) did not indicate amplification of the target material. Serially diluted (range from 1 to 10^7 copies per reaction) of standard samples (6 technical replicates) were used. Quality parameters of the qPCR were: standard curve intercept 42.8 (S.$E \pm 0.22$), and slope -3.56 (S.$E \pm 0.04$) with $R^2 = 0.99$ (S.$E \pm 0.004$). Efficiency of qPCR was 93.0% (S.$E \pm 2\%$), limit of detection (LOD) 44 (S.$E \pm 6$) copies per reaction, limit of quantification (LOQ) 128 (S.$E \pm 28$) copies per reaction.

3.2. Dynamics of the SARS-CoV-2 concentration in larger WWTPs

WWTPs in district centres and towns with population size over 10,000 individuals were tested weekly collecting 24 h composite WW samples from beginning of August to end of December 2021 ($n = 3555$). This makes a total of 20 continuous sampling points that cover 65% of the Estonian population (Fig. S1, Table S1). Initially in August 2020 when the viral spread was relatively low, the RT-qPCR analyses targeting four genes (N1, N2, N3 and S) were performed. While the sensitivity of detection was very similar for all the N genes, detection of the N3 gene was most reliable; detection of the S gene yielded highly variable results (Fig. S2). Therefore, only abundance of the N3 gene was estimated for the majority of collected samples.

In good agreement with the epidemiological situation based on SARS-CoV-2 testing of the population, no virus was detected at high concentration at the beginning of August 2020. In the middle of August 2020, there were outbreaks amongst nightclub clients of the second largest town of Estonia, Tartu (~100,000 inhabitants). According to the further epidemiological data, the outbreaks were isolated quickly. However, there was still a concern about the spread of the virus since a large event – WRC Rally Estonia – took place in Tartu and its surroundings. In this situation the WW surveillance gave important confirmation that the outbreak had ended.

In September and October 2020, the virus spread was low (Fig. 1). At the beginning of November, a considerable increase in the viral load was detected in wastewater all over the country, closely followed by a sharp increase in the number of infected people. In 2021, a similar correlation was observed between the WW data and number of infected people up to the end of June (rise and decline of Alpha variant wave (B.1.1.17) in Estonia). From August 2021, the next wave of Delta variant (B.1.617.2) developed, peaking in November 2021 (Fig. 1). This wave was also reflected in the concentration of the virus in WW. Dominating variants in the WW samples were determined using Ion AmpliSeq SARS-CoV-2 Insight Research Assay (detailed manuscript in preparation).

3.3. Targeted testing of the population using small scale grab samples

In places where a higher risk of the spread of the virus was considered, the grab samples were involved in the monitoring starting from

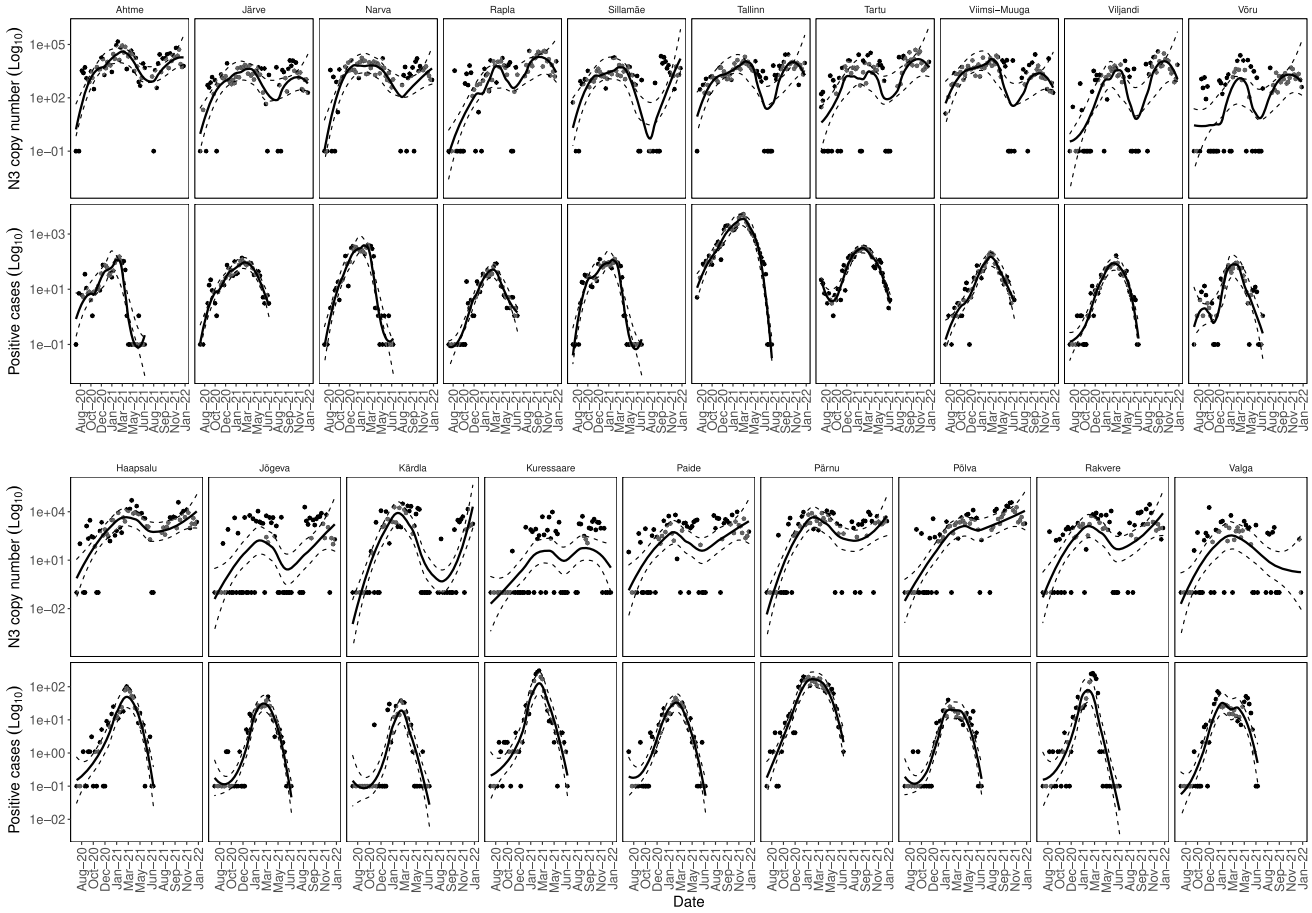

Fig. 1. Time series of N3 gene copy number (ml^{-1}) median values in raw wastewater and number of COVID-19 positive cases in 20 Estonian towns/cities covering ~65% of the total population from August 2020 to July 2021. Upper panel – WWTPs with a "good" relationship; and lower pane with "weak" relationship, solid line represent a smoothing line, dashed lines 95% CIs.

September 2020. Retirement and nursing homes for the elderly were the most serious concern as older people of the risk group are living in close contact with each other. Six nursing homes were tested from September to October 2020 (17 samples). In cases where the virus was found in the wastewater, its appearance was reported to the Health Board who organized immediate testing of clients and staff members for SARS-CoV-2 (data not shown). Prisons were in an especially complicated situation during the pandemic as there are limited options for isolation measures. Therefore, all three prisons in Estonia (~2500 prisoners) were involved in the weekly testing rounds, in one prison the total effluent of WW and effluent from one specific compartment was tested. Other critical points were/are the dormitories of vocational education schools and the dormitories of universities where the students live on weekdays and during the weekends many of them visit their homes all over the country. Therefore, WW of 28 dormitories was tested by grab samples (75 samples collected in total). In case of measuring SARS-CoV-2 copy number above detection limit (~70% samples), all inhabitants of the dormitory were tested for SARS-CoV-2. Occasionally WWs of small hospitals, day care centres, military barracks etc. were tested, data are summarised in Supplemental Table S2

The grab samples were also used to complement the 24 h sampling round of larger towns. Around 30–40 towns/villages were included in the weekly sampling plan. The information was used to estimate the overall epidemiological situation in the country and to target increased testing of people in affected sites/regions.

3.4. Associations between the viral concentration and WW characteristics

We analysed the linear relationship of TSS, BOD, COD, pH, Ptot and Ntot with N3 gene copy concentration in several sites. The overall relationships was weak, (adjR^2=0.18, p<0.0001). The strongest predictor to overall relationship was site (i.e. which WWTP) (6.7%, p<0.0001), Ntot (6.5%, p<0.0001) and Ptot (4.3%, p<0.0001) while TSS was weak predictor (0.5%, p = 0.048) similar to pH (0.5%, p = 0.059) and BOD was not significant. Relationship improved when nested design with specific site was used in model (adjR^2=0.50, p<0.0001): nested site variable predicted 12.3% (p<0.0001) of variability, followed by Ntot (7.2%, p<0.0001) and Ptot (5.1%, p<0.0001). Contribution of other variables was below 2%, while the interaction of TSS with site predicted 6.2% (p<0.0001) of the total regression, demonstrating importance of site for TSS concentration level. As conclusion, the effluent quality variables were weakly related SARS-CoV-2 levels within particular WWTP.

3.5. Modelling of the viral concentration to predict dynamics of infection

In the sampling period from August 2020 to June 2021 the viral abundance in wastewater started to increase in 1.25 weeks (9 days) (95% CI: [1.10, 1.41]) before the increase of COVID-19 positive cases. However, the variation between various cities was considerable (Table 2). Duration of the lag period varied from ~ 5 days to 2.5 weeks, while the model prediction correlates with the number of COVID-19 positive cases very well (Fig. 2). The lag time was reliable in most small/medium WWTPs but was twice longer in the largest town of Estonia (Tallinn, ~500,000 inhabitants, Table 1) compared to the average.

Finally, the obtained scaling shift parameter and the lag period specific to sites were used to predict incidence rate using N3 gene copy numbers in WW from August 2021 to December 2021. Predicted numbers were compared to county-based open data of COVID-19 positive cases from Estonian Health Board. The WW data are from the central towns of the counties but the numbers of COVID-19 positive cases were provided for the whole county. Therefore counties with a bigger share of the population in the county centre were selectively used (Fig. 3). The shape of infections was predicted very well for all these sites, indicating reliable lag period estimates, while the absolute number of COVID-19 positive cases was not predicted precisely, particularly for sites with < 50% share of population in the central town.

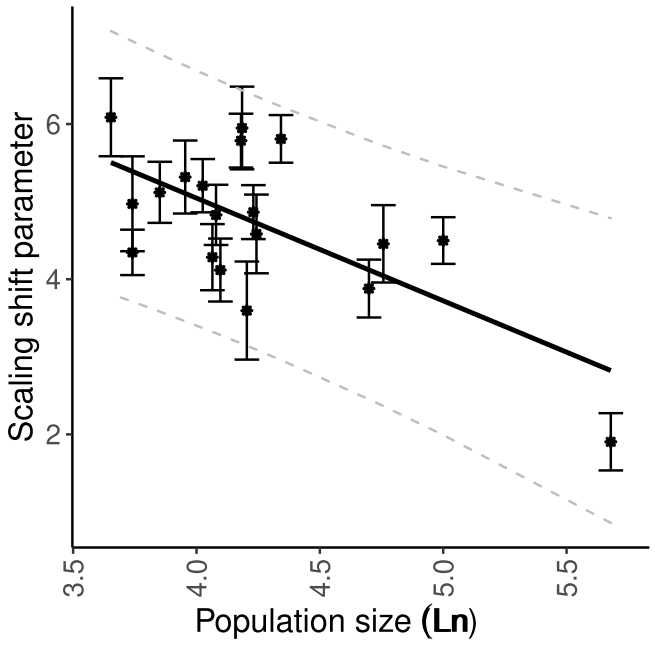

Fig. 2. Ln-ln relationship between scaling shift parameter and population size (R^2 =0.45, p<0.01), dashed line is 95% CI of prediction.

Table 1
Predictive power of SARS-CoV-2 genome copy number (N3 gene) in wastewater samples.

Site	Scaling shift parameter (Ln)	95% CI		Lag in weeks	95% CI	
Ahtme	5.8	5.4	6.1	1.3	0.9	1.8
Haapsalu	5.2	4.9	5.5	0.9	0.3	1.6
Järve	4.3	4.1	4.6	1.3	0.8	1.8
Jõgeva	5.2	4.7	5.7	1.0	0.4	1.6
Kärdla	6.1	5.6	6.6	1.0	0.5	1.5
Kuressaare	3.6	3.0	4.2	1.4	0.8	2.1
Narva	4.5	4.0	5.0	1.5	0.8	2.2
Paide	5.3	4.8	5.8	1.5	0.7	2.2
Pärnu	3.9	3.5	4.3	0.7	0.0	1.5
Põlva	5.1	4.7	5.5	1.1	0.6	1.7
Rakvere	4.6	4.1	5.1	1.5	0.9	2.1
Rapla	5.0	4.4	5.6	1.6	0.8	2.3
Sillamäe	4.1	3.7	4.5	1.2	0.3	2.2
Tallinn	1.9	1.5	2.3	2.5	1.7	3.4
Tartu	4.5	4.2	4.8	0.8	0.2	1.3
Valga	4.3	3.9	4.7	1.5	1.0	2.0
Viimsi-Muuga	5.8	5.5	6.1	1.1	0.5	1.6
Viljandi	4.9	4.5	5.2	1.5	0.9	2.1
Võru	4.8	4.4	5.2	1.7	1.1	2.3

Scaling shift parameter (log10) is the coefficient showing how many times is the N3 copy number higher than recorded cases. Lag in weeks indicates site specific lag time how much earlier the virus load increase before increase of COVID-19 positive cases.

3.6. SARS-CoV-2 wastewater index (SARS2-WI)

Due to differences in virus concentration in WW and the number of COVID-19 positive cases in various locations which depends on the population size, density and residence time of WW in the sewage system, we aimed to find a generalized parameter that can be used for the prediction of the incidence rate dynamics for the whole country. In

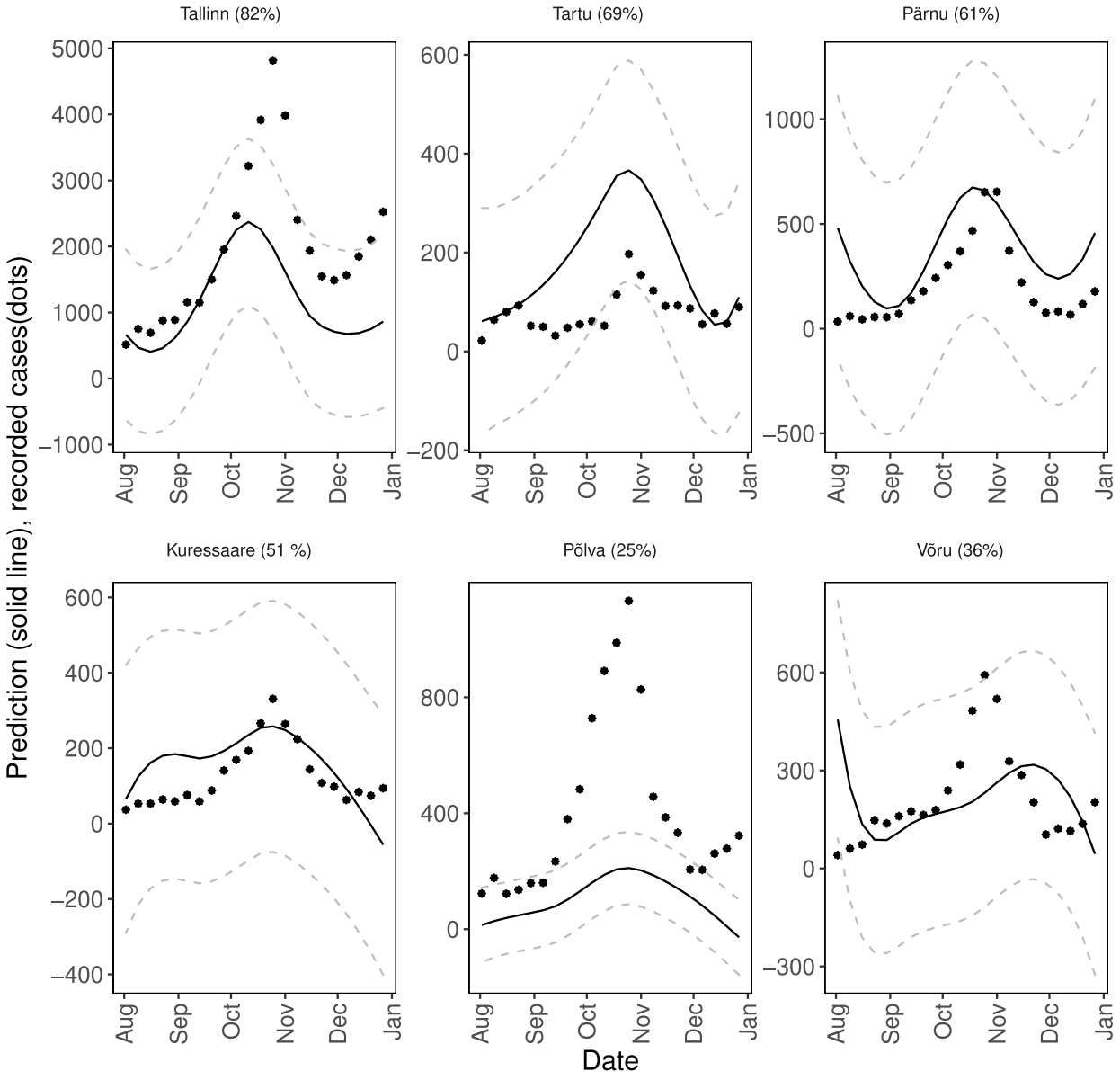

Fig. 3. Predictions of the incidence rates based on observed amount of N3 gene copy numbers in WW in selected sites with >25% of the population at the WWTP system and compared to COVID-19 positive cases in the whole county.

addition, the concentration (i.e. share) of faecal material can differ in different treatment plants and in time. There are multiple reasons for this variation, mainly the inflow of industrial WW and rainwater/melting snow. A standard method for estimating the concentration of faecal material is the MPN of *E. coli* count (EVS-EN ISO 9308–2). Measurement of this parameter is standardized and cheap. For estimating the average load of SARS-CoV-2 in 19 major sites (Fig. S1) we normalized the virus amounts with MPN counts of *E. coli* and weighted size of the population served by the treatment plant to obtain the SARS-CoV-2 Wastewater Index (SARS2-WI). The dynamics of SARS2-WI associates directly with the other epidemiological indices such as 7-day mean of new cases ($r = 0.73$, $p<0.001$) and sum of new cases within the last 14 days ($r = 0.80$, $p<0.001$) (Fig. 4). In contrast, the effective reproduction number (R_e) and SARS2-WI did not correlate directly but the increase in effective R and increase in SARS2-WI coincided before the accelerated increase of virus cases.

4. Discussion

We made two major observations in our study. First, how long was the lag period between the increase of virus abundance in WW and the increase in the number of infected people and, second, how the viral concentration was related to the incidence rate within communities of various sizes. Both these parameters can predict the real incidence rate per site in most cases.

Several previous studies have demonstrated the existence of up-to-one-week-long lag period between increase of the virus concentration in WW and the consequent increase in an incidence rate in the population (Karthikeyan et al., 2021b; Larsen and Wigginton, 2020). These estimates are in good agreement with our results. However, the strategy may vary how individuals were tested in other countries, nations or regions, depending what was the time difference between appearance of symptoms or contacts with positive individuals and performed test. Thus, if the frequency of WW sampling is sufficiently high (not less than

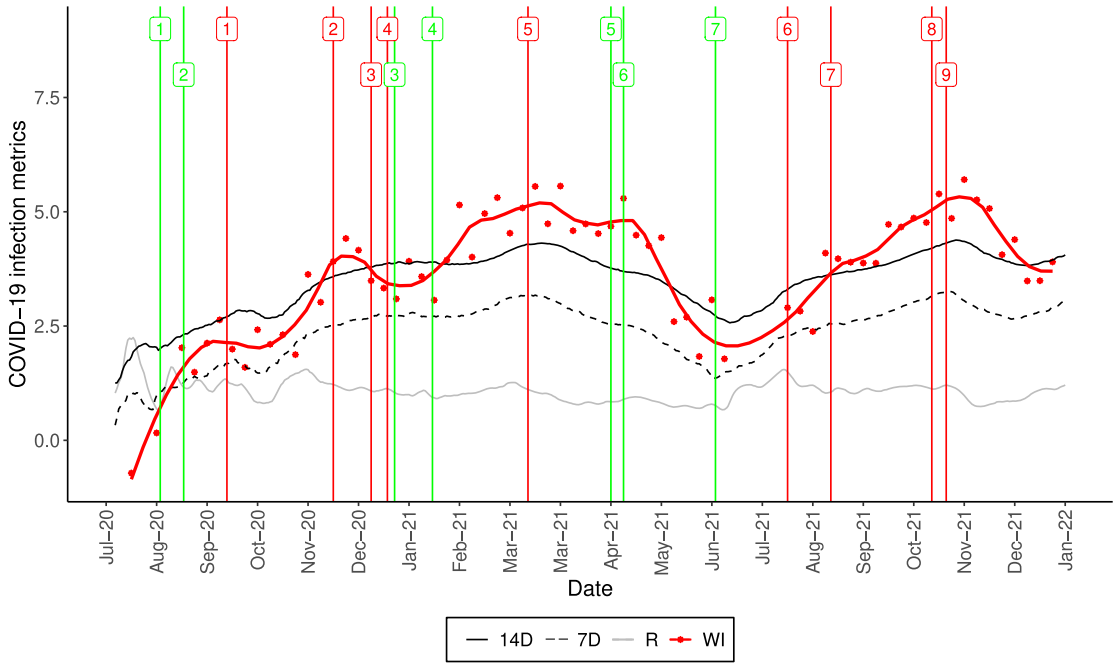

Fig. 4. Dynamics of normalized virus load - SARS-Cov-2 Wastewater Index (WI) in Estonia compared to effective reproduction (*Re*), total cases summed over last 14 days (14D) and moving mean of 7 days (7D). Applied restrictions (in red): 1) re-introduction of restrictions, mostly about maximum allowed people at events, including ban to alcohol sales during night hours; 2) face masks became obligatory, 2 + 2 rule in all indoor public places (appearing alone or in pairs while keeping at least 2 meters distance); 3) all schools to distance teaching; 4) temporary additional restrictions to regions with the highest spread of virus until January 17; 5) hardening of restrictions, shops closed, schools to distance teaching again, 2 + 2 rule everywhere; 6) masks again obligatory in public transport; 7) masks obligatory in all indoor rooms where no checking of vaccination passport; 8) hardening the rules, only vaccinated persons can enter public places (supermarkets were exception); 9) face masks became again obligatory to everybody.

once per week) and the time spent for sample analysis is sufficiently short (up to 2–3 days), the WBE constitutes an exceedingly useful early warning tool independent from individual testing strategies.

The lag between the increase of virus abundance and the number of infected people was most stable – and, therefore, likely the most reliable – in small-middle sized settlements (population size up to 100,000 individuals) with centralised sewer systems. The probable reason is that in larger sewer systems the wastewater is mixed on a larger scale before entering the WWTP. In a relatively large capital town of Tallinn (population size ~500,000 inhabitants) with a larger sewer system and sewershed catchment area (>1200-km-long pipe system in Tallinn as compared to few hundred km in most of the cities) the lag period, estimated 2.5 weeks (Table 1, Fig. 2), was outlying from the rest of sites. Reason of this observation remains unclear but indicates that reliable prediction is problematic in larger cities. However, WBE can be used for estimating the general spread in such sites. In comparison, a study covering ~50% of the population in Scotland demonstrated very strong correlation between levels of SARS-CoV-2 RNA in WW and recorded COVID-19 positive cases, while the exact relationships depended on the population size of the settlement (Fitzgerald et al., 2021). This observation is in a good concordance with our results (Fig. 2) but does not explain causality of such relationships. A detailed study using sampling at spatial resolution of the sewershed catchments in US Nevada indicated the importance of population density, urban centers, outlying suburban areas, and outlying urbanized districts. (Haak et al., 2022). In our study only Tallinn sewershed has similar contrasting areas of high density urban centre(s) versus urban sprawl areas. Considering the size of Estonian settlements (mostly below 100 000) single point (sewer system endpoint at WWTP) 24 h hour sampling seems to be reliable basis for WBE of this nation and outcompetes the usefulness of more detailed data which collection at many sampling points might be challenging and unreliable.

It has been reported that there is absent (or at least very weak) correlation between the viral concentration in wastewater and the incidence of infection in the catchment area (Karthikeyan et al., 2021b; Larsen and Wigginton, 2020). While in our dataset the correlation is also weak when the infection rates were low (Fig. 1, lower panel), it becomes significantly stronger at higher rates of infection (Fig. 1, upper panel). Moreover, the association between the viral concentration in WW and the number of COVID-19 positive cases strongly depends on the population size and, possibly, is also affected by the layout of the sewer system, while we cannot rule out the variability in the process of how the COVID-19 testing was implemented at specific sites (Fig. 2).

The WW based virus concentration can not only be used to predict the dynamics of epidemiology amongst specific sub-populations, but can provide a reliable estimate of the share of the population being infected (Fig. 3). Although we did not get access to the real numbers of infected people residing the catchment areas of studied WWTPs for the last 5 month of the study period, comparison with the open data per county were very reliable when the share of the population in the region was concentrated into the town feeding sewer system. However, the care should be taken with viruses evolving rapidly, this may change the shedding duration and proportion. In addition, testing virus load without discriminating the variants with various virulence cannot used to predict severity of epidemiological situation.

The WW surveillance data were produced very rapidly, i.e. analyses were ready on the next day upon collecting the samples, and were made immediately accessible to the Health Board and to the Government making the whole procedure suitable for operational WBE surveillance. This operational information was efficiently used to control the epidemiological situation via: i) identification of new cases in areas or sites such as nursing homes, prisons, dormitories etc., ii) analysis of the trends over longer periods to predict the increase or decrease of disease burden and iii) estimation the efficacy of interventions/restrictions in reducing disease spread as proposed by Sims and Kasprzyk-Hordern (2020).

Specifically, produced data were visualized on the dashboard in various ways: weekly dynamics in "raw" data as N3 gene copy numbers per ml of WW, dynamics of SARS2-WI and traffic light representation of the spread levels on the country map. Specifically, the SARS2-WI was a useful additional parameter for decision-makers complementing other common indicators of epidemiological situation i.e. 7-day average or last 14-day sum of COVID-19 positive cases and effective R (Fig. 4, Animation 1). Obviously, the 14-day sum of COVID-19 positive cases and SARS2-WI were correlated strongest, similarly to some other studies (Tiwari et al., 2022). Mechanism behind this observation can be related to knowledge that the viral shedding into faeces is estimate to happen more than 20 (up to 32) days (Miura et al., 2021). The long tail of shedding contributes significantly to the amount of virus particles in wastewater (Wölfel et al., 2020; Wu et al., 2022; Zheng et al., 2022). Hence, 14 day window gives better representation of how many people contribute to viral shedding SARS-CoV-2 in faeces and finally in wastewater. In addition, SARS2-WI is a balanced infection metrics that incorporates additional information about the ratio between human faecal waste (*E. coli* MPN count) and industrial grey water, additionally weighted with the population size. Other possible human faecal markers such as pepper mild mottle virus (PMMoV) or *Bacteroides* HF183 have been used in normalisation, but with little success (Feng et al., 2021). Some studies have managed to improve N1/N2 concentration relationship with COVID-19 positive cases using crAssphage abundance normalisation in large settings (>~400,000 inhabitants) with greater industrial and stormwater inputs. However, the most important was the number of COVID-19 cases (Nagarkar et al., 2022). Other studies report somewhat negative effect using similar normalisation - normalizing to a spiked recovery control (BCoV) or a faecal markers such as PMMoV or HF183 reduced correlations between viral load in WW and COVID-19 cases (Feng et al., 2021;Vadde et al., 2022). Currently, there is no standardized method to normalise the raw concentration of virus particles in WW and possible side effects by dilution of non-human-related WW. Further complicating the situation, SARS-CoV-2 concentrations in faeces vary by order of magnitude amongst infected individuals and over the course of infection (Kitajima et al., 2020; Wölfel et al., 2020).

5. Conclusion

Our study demonstrates that the WBE of SARS-CoV-2 covering the majority of the population is cost-effective and non-biased way to survey the transmission and dynamics of the epidemiological situation. Frequent WBE provides similar data comparable to random testing of thousands of individuals in a community as large as the whole country. In addition, the benefits of wastewater testing were extensively communicated to the general public via interviews in media broadcasting and focused popular science articles. These activities, targeted to wider public, received huge positive feedback helping to build a bridge between detailed technical information and general understanding.

CRediT authorship contribution statement

Veljo Kisand: Conceptualization, Methodology, Investigation, Visualization, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Peeter Laas:** Methodology, Investigation, Visualization, Writing – review & editing. **Kadi Palmik-Das:** Investigation, Writing – review & editing. **Kristel Panksep:** Methodology, Writing – review & editing. **Helen Tammert:** Methodology, Investigation, Writing – review & editing. **Leena Albreht:** Investigation, Writing – review & editing. **Hille Allemann:** Methodology, Investigation, Writing – review & editing. **Lauri Liepkalns:** Investigation, Writing – review & editing. **Katri Vooro:** Methodology, Investigation, Writing – review & editing. **Christian Ritz:** Methodology, Visualization, Writing – review & editing. **Vasili Hauryliuk:** Writing – review & editing. **Tanel Tenson:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Supplementary materials

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