






Detection and abundance of SARS-CoV-2 in wastewater in Liechtenstein, and the estimation of prevalence and impact of the B.1.1.7 variant

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ABSTRACT

The new coronavirus 2 (SARS-CoV-2) is known to be also shed through feces, which makes wastewater-based surveillance possible, independent of symptomatic cases and unbiased by any testing strategies and frequencies. We investigated the entire population of the Principality of Liechtenstein with samples from the wastewater treatment plant Bendern (serving all 39,000 inhabitants). Twenty-four-hour composite samples were taken once or twice a week over a period of 6 months from September 2020 to March 2021. Viral RNA was concentrated using the PEG centrifugation method followed by reverse transcription quantitative PCR. The aim of this research was to assess the suitability of SARS-CoV-2 fragments to relate the viral wastewater signal to the incidences and assess the impact of the emerging B.1.1.7. variant. The viral load in the wastewater peaked at almost 9×10^8 viral fragments per person equivalent (PE) and day on October 25, and showed a second peak on December 22 reaching a viral load of approximately 2×10^8 PE⁻¹ d⁻¹. Individual testing showed a lag of 4 days and a distinct underestimation of cases at the first peak when testing frequency was low. The wastewater signal showed an immediate response to the implementation of non-pharmaceutical interventions. The new virus variant B.1.1.7. was first detected in wastewater on December 23, while it was first observed with individual testing on January 13, 2021. Further, our data indicate that the emergence of new virus variant may change the wastewater signal, probably due to different shedding patterns, which should be considered in future models.

Key words: COVID-19, inflow, SARS-CoV, sewer, wastewater surveillance

HIGHLIGHTS

- Wastewater-based epidemiology covering an entire country (Liechtenstein).
- Lucrative lead of 4 days of SARS-CoV-2 gene copy numbers versus individual testing.
- Variant of concern B.1.1.7. detected in wastewater 2 weeks prior to individual testing.
- Variant of concern B.1.1.7. increased the ratio of wastewater signal per incidence.

1. INTRODUCTION

The new coronavirus respiratory disease (called COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that is known to be also shed through feces (Jones *et al.* 2020). Since disease outbreak is now globally widespread and had caused a pandemic, the need for fast, sensitive and reliable surveillance systems has become increasingly important. Thus, the detection of SARS-CoV-2 cannot solely rely upon individual testing, which cannot be seen as a long-term solution both from an economical and environmental perspective. As soon as disease prevalence reaches low levels, alternative options for disease surveillance must be adopted. One such option is wastewater-based epidemiology (WBE; Medema *et al.* 2020) that has been for many years a powerful and effective tool in controlling and eradicating diseases such as polio (Berchenko *et al.* 2017). For many years though, WBE has been neglected as a tool in disease surveillance;

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however, with the onset of the COVID-19 pandemic researchers started to reconsider this powerful tool for detecting SARS-CoV-2 in wastewater across the globe (e.g., [La Rosa et al. 2020](#); [Ahmed et al. 2021](#); [Gerrity et al. 2021](#); [Karthikeyan et al. 2021](#)).

The primary mode of transmission of SARS-CoV-2 is via respiratory droplets that people produce when they exhale, cough or sneeze ([Karia et al. 2020](#)). It has been shown that when sputum or saliva is swallowed (approximately $1.0\text{--}1.5$ L person⁻¹ d⁻¹), viral RNA enveloped in mucus may pass the stomach and intestines in a semi-protected state, thus avoiding degradation by gastric acid and pancreatic juices ([Hirose et al. 2017](#)). Apart from cells in the respiratory tract, SARS-CoV-2 may also infect other organs such as the gastrointestinal tract through the angiotensin-converting enzyme 2 receptor (ACE2), an important component of the angiotensin system ([Pola et al. 2021](#)). This results in diarrhea, vomiting and other gastrointestinal disorders ([Xiao et al. 2020](#)), which affect shedding viral particles through our wastewater system. It is estimated that up to 67% of infected persons shed SARS-CoV-2 viral particles with their stool ([Parasa et al. 2020](#); [Wu et al. 2020](#)). As SARS-CoV-2 particles in sputum and saliva may also directly contribute to the viral load in wastewater ([Wyllie et al. 2020](#)), given the abundance of the virus genetic material in nasopharyngeal fluids (approximately $10^5\text{--}10^{11}$ gc mL⁻¹) is much higher than in both urine (approximately $10^2\text{--}10^5$ gc mL⁻¹) and feces (approximately $10^2\text{--}10^7$ gc mL⁻¹) ([Jones et al. 2020](#)), it is important to say that the virus load in wastewater may not only be a result of feces and urine shedding but also of sputum and saliva spreading of infected people.

For the above reason, monitoring of SARS-CoV-2 RNA in wastewater may serve the purpose of following and predicting important infection trends ([Wu et al. 2020](#)) during the course of the COVID-19 disease. It is important that the degree of fecal release of viral particles is not related to the severity of the disease ([Zheng et al. 2020](#)) as SARS-CoV-2-RNA was detected in fecal probes despite negative PCR-testing result ([Chen et al. 2020](#)). Thus, WBE may be viewed as a monitoring and surveillance method independent of symptomatic cases and unbiased by any selected testing strategies and frequencies. Testing of wastewater may also be beneficial for seasonal outcomes as well as predicting travel and commuting effects ([Pearce et al. 2020](#)).

However, like any other monitoring system, WBE also has drawbacks such as sampling procedure, concentration steps and RNA quantification method. Commonly, 24-h volumetric sampling is preferred. While earlier studies often have relied on frozen wastewater samples, it has been found that freezing and thawing of samples may drastically lower the yield of intact RNA ([Markt et al. 2021](#)). In general, low viral loads in wastewater require elaborated and well-considered strategies to concentrate the RNA, such as the polyethylene glycol (PEG) precipitation method, which has been chosen in many laboratories despite the disadvantage of losing the viral fragments bound to solid matter (around 50%) ([Lu et al. 2020a, 2020b](#); [Pérez-Cataluña et al. 2021](#)). Extraction protocols and RT-qPCR bring additional uncertainties. However, it is clear and strongly recommended recently by the European Union, that WBE has huge potential to be used for COVID-19 surveillance ([European Commission 2021](#)). In particular, better estimates of the prevalence of COVID-19 in a population are required for optimizing and fine-tuning disease control strategies.

Smaller countries with only a few wastewater treatment plants (WWTPs) may particularly benefit from WBE during this pandemic. An example of such a country is the Principality of Liechtenstein which is here shown in our study. It is the fourth smallest state in Europe and geographically located in the alps between Switzerland and Austria. The country with approximately 39,000 inhabitants has only one WWTP (ARA – Abwasserreinigungsanlage Bendorf) serving the whole country. Thus, intensive wide-scale individual testing combined with wastewater surveillance on SARS-CoV-2, both detection and sequencing, offers Liechtenstein a unique opportunity to shed light on an entire country's viral prevalence on COVID-19. The aim of this study is to show the dynamics of COVID-19 fragments in the inflow of the WWTP of ARA Bendorf (technically configured for 105,000 population equivalents) and relate this to the COVID-19 disease incidence. It is hypothesized that WBE (i) shows rises in viral loads earlier than individual testing does, (ii) disease incidence is closely correlated with wastewater signal, (iii) new viral variants are detected earlier in wastewater than by individual testing and (iv) new variants change the viral wastewater signal.

2. MATERIALS AND METHODS

2.1. Sample collection and preparation

About 250 mL liquid influent samples were collected with a 24-h volume-equivalent sampler, cooled to 4 °C ([Markt et al. 2021](#)) and shipped to the laboratory in cooled packaging. In the laboratory, 70 g of the sample was transferred to an

80 mL centrifugation tube (Oak Ridge Type 3118, Nalgene™) and spiked with a transgenic virus-like particle based on the MS2-phage as internal control for sample preparation and RT-qPCR (real-time quantitative PCR) analysis. In order to remove particulate matter, the tube was then centrifuged for 30 min at 4,500 g (4508 R cooling centrifuge, Eppendorf, Hamburg, Germany) without brake (Medema *et al.* 2020). About 65 mL of the supernatant were then transferred to a centrifuge tube filled with 6.5 g of PEG (PEG 8000; ROTIPURAN®) and 1.46 g of sodium chloride. To dissolve the PEG, the tube was shaken with a tilt/roller mixer (RS-TR 05, Phönix Instrument, Garbsen, Germany) for approximately 10 min at room temperature. RNase-free water was used for balancing the centrifuge tubes. Samples were then further centrifuged at 12,000 g for 99 min without brake. The supernatant was carefully removed, leaving a small transparent pellet. About 800–1,000 µL of lysis buffer were added to the pellet, followed by vortexing for 15 s at 2,500 rpm and another centrifugation at 2,000 g for a few seconds (with stop) to collect all the lysis buffer droplets at the bottom of the falcon tube. The entire solution was then transferred to an Eppendorf micro reaction tube. RNA was then purified from the lysed pellet using the Monarch™ total RNA Miniprep Kit (New England Biolabs, Ipswich, USA).

2.2. RNA quantification

RNA copy numbers were determined using a duplex measurement assay for SARS-CoV-2 targeting the nucleocapsid (N1) gene primers (Lu *et al.* 2020a, 2020b) at the green channel and the process control based on MS2-phage at the yellow channel. A total of 20 µL RT-qPCRs contained 10 µL Luna Universal Probe One-Step Reaction Mix (2×), 1 µL Luna WarmStart® RT Enzyme Mix (20×) (both New England Biolabs), 0.8 µL primer (forward and reverse, final concentration 0.4 µM each target), 0.4 µL probe (final concentration 0.2 µM each target), 2 µL PCR grade water and 5 µL template. Analyses were conducted on a RotorGene cyler (Qiagen, Hilden, Germany). After an initial reverse transcription at 55 °C for 10 min followed by 95 °C for 1 min of denaturation, 45 cycles of 95 °C for 10 s and 60 °C for 40 s were performed running a two-step protocol. To calculate copy numbers, a plasmid standard containing the N-gene of SARS-CoV-2 (2019-nCoV_N_Positive Control, IDT, Leuven, Belgium) was used (Markt *et al.* 2021). Samples with dropouts or difference >2× the standard deviation per run of mean cycle threshold of the internal control were discharged.

2.3. Background data

The WWTP ARA Bendern (47.21202°N, 9.50106°E) serves and collects wastewater of all 11 communities of the Principality of Liechtenstein. The maximum length of stay of the wastewater within the catchment system is lower than 6 h, and the annual wastewater volume is $9.5 \times 10^6 \text{ m}^3$. The daily load of chemical oxygen demand and NH₄-N (ammonium nitrogen) sums up to 11,000 and 386 kg, respectively. Wastewater SARS-CoV-2-data were obtained on a weekly basis from September 20 to October 25, 2020; later, the inflow was sampled twice a week.

Individual testing was exclusively based on PCR tests, resulting in incidences on a daily basis. Almost all of the positive samples were sequenced to check for variants of concern. Data are available at <https://www.liechtenstein.li/land-und-leute/gesellschaft/>

2.4. Virus signal – data processing

Wastewater-based epidemiology encompasses a range of complex processes and variability, i.e., from viral shedding of infected persons to virus transport and degradation in the sewer system, sampling and quantification in the laboratory. Therefore, we outline a three-step procedure for data processing.

2.4.1. Outlier detection

Since the sewer system is a combined system, rain events dilute the wastewater and result in an increased runoff. For extreme discharge conditions, part of the wastewater will be immediately directed into the receiving water via combined sewer overflows. For these cases, a significant part of the viral load will not appear at the sampling point due to coincidental loss. Measurements taken under such conditions are thus likely to yield a false negative or lower signal and are treated as an outlier. We used the inflow data from the WWTP ARA Bendern over a period of 10 years (2010–2020) where flow is recorded daily (Q in $\text{m}^3 \text{ d}^{-1}$) and NH₄-N every 5 days (c_{NH_4} in $\text{g NH}_4\text{-N m}^{-3}$). In the following, we apply NH₄ as a proxy for the virus as we assume that both are diluted and thus behave approximately equal in the sewer system. In Figure 1, we use the relation of the NH₄ load ($Q \cdot c_{\text{NH}_4}$) to the mean NH₄ load during dry weather conditions (the mean dry weather flow in 2020 was $24,300 \text{ m}^3 \text{ d}^{-1}$). Under ideal conditions, this should be a unit fraction, but the influence of combined sewer overflows is visible for

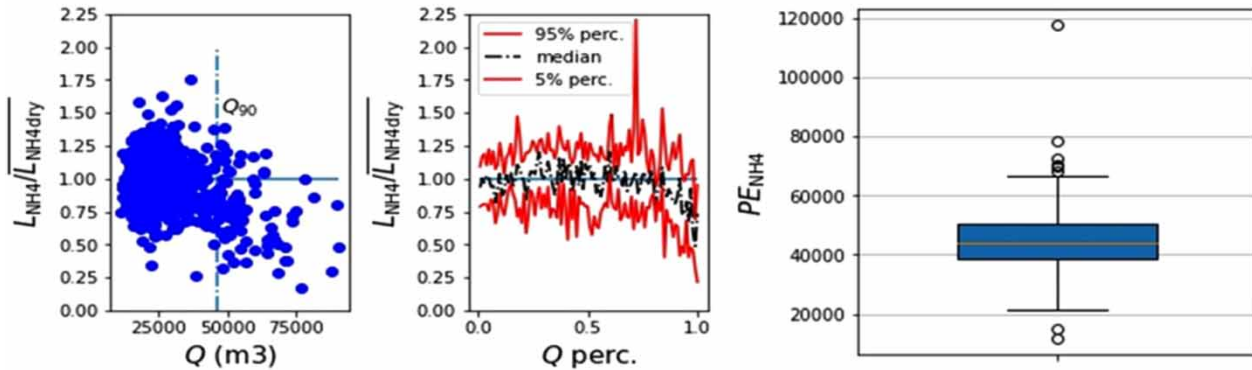


Figure 1 | Left – relative ammonium (NH_4) load versus inflow; center – relative NH_4 load plotted as the ordered list (percentiles) of inflow plus confidence intervals (5–95%); right – boxplot of PE computed with 8 g $\text{NH}_4/\text{PE}/\text{d}$ from the inflow data 2010–2020.

higher inflows. From this, we conclude that samples taken while the inflow is higher than the 90th percentile of the recorded inflow data (here $46,250 \text{ m}^3 \text{ d}^{-1}$) are potentially false and thus to be treated as outliers, which was the case for three samples.

2.4.2. Normalization

The use of population biomarkers in WBE is standard practice to account for the temporal variation in the population of the watershed (Been *et al.* 2014). While there are certainly more accurate biomarkers possible, in this case study we are restricted to the use of the parameter NH_4 in the inflow and apply a standard load of 8 g NH_4/PE (person equivalent)/day for an estimation such as:

$$\text{PE}_{\text{NH}_4} = \frac{c_{\text{NH}_4} * Q}{8}$$

Figure 1 depicts the variation of the PE discharging into the WWTP from the inflow data over the past 10 years. The median (44,100 PE) matches closely with the actual population, including regular incoming commuters who are common for the country. We further estimate realistic boundaries of the variation with the boxplot features as 20,900 PE minimum and 67,800 PE maximum. Samples outside of that range are either potentially false numbers or caused by external NH_4 sources that do not relate to the population and are removed as outliers.

2.4.3. Smoothing

Arabzadeh *et al.* (2021) describe the background and necessity of data smoothing in relation to SARS-CoV-2 signals. Following their procedure, we transformed the raw signal (c_{virus} in copies mL^{-1}) to the specific viral load (L_{virus}) using NH_4 -based normalization:

$$L_{\text{virus}} = \frac{c_{\text{virus}} * 8}{c_{\text{NH}_4}} \left[\frac{\text{Mega gene copies}}{\text{PE} * d} \right]$$

Mega gene copies (Mgc) refers to 10^6 gene copies. For data smoothing, we apply the SPLINE method (Arabzadeh *et al.* 2021).

2.5. Incidence modeling

Incidence is here defined as the epidemiological situation based on active cases documented by individual PCR tests. This measure is likewise common in pandemic surveillance as, e.g., 7-day incidence rate per 100,000. However, note that incidence deviates significantly from the actual infection status (usually denoted as prevalence) as there is always a significant number of undocumented cases which are not considered (Rippinger *et al.* 2021). Moreover, the incidence values are subject to test numbers and strategies and thus contain a significant uncertainty both in assessment and interpretation.

Despite the deficiencies, incidence modeling still serves as a valuable information and a prediction tool on epidemiological dynamics. In the following, we apply a simple regression model to estimate active cases (prevalence) from the viral load data

(as given after data processing and smoothing). For calibration, we apply the period of the second pandemic wave from November 16, 2020 to December 31, 2020 and used the model both retrospectively (the first pandemic wave) and predictively (until the end of March 2021). The underlying assumption is that the relation of undocumented cases to recorded cases in the calibration period applies also to the whole dataset.

2.6. SARS-CoV-2 whole genome sequencing

Extracted RNA was transcribed into cDNA and amplified according to the ARTIC Network (<https://artic.network/>) protocol (https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019/V3). Sample pooling and sequencing was performed on an Illumina NovaSeq 6000 where quality filtering of raw reads, demultiplexing, mapping and low frequency calling for single-nucleotide polymorphisms (SNPs) was conducted according to a published protocol by Popa *et al.* (2020). SNPs-calling for SARS-CoV-2 variant assignment to the B.1.1.7 lineage is based on the mutations listed in https://cov-lineages.org/global_report_B.1.1.7.html. Thereby, the median frequency of all B.1.1.7-specific SNPs covered and assigned was used to estimate the relative abundance of the B.1.1.7 variant in wastewater. Wet-lab library preparation and bioinformatic analysis was conducted at the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna. Using this method, we reached a median of 514 not sufficiently covered nucleotides per Sars-CoV-2 genome. Consensus sequences were deposited on GISAID (Liechtenstein, Environmental samples).

To investigate a variant-driven shedding behavior, we compared the ratio of virus load per 7-day incidence over two time periods using the Mann–Whitney *U*-test. The first time period (named wild-type) comprises data from November 6, 2020 to December 26, 2020, and the second time period (named B.1.1.7) comprises all data beginning with the occurrence of B.1.1.7 in wastewater starting on December 27, 2020.

2.7. Statistics

Single wastewater samples were taken weekly, respectively, twice a week. To be able to relate the wastewater data to daily incidences, the data were normalized on the basis of NH₄-N and data were smoothed as described above (Arabzadeh *et al.* 2021).

3. RESULTS

In Liechtenstein, over a period of 14 weeks from July 2020 to mid-September 2020, the number of tested individuals ranged around 200 PCR tests per week. During the following time, the PCR test frequency was sharply increased to an average of 800 PCR tests per week until the end of March 2021. The number of new positive-tested persons showed two peaks, one during the first week of November 2020 and a second during the last two weeks of December 2020 with 36 and 41 cases per day, respectively. Consistent with these numbers, the percentage of positive tests peaked during this same time, while later it decreased from the beginning of January onwards (Figure 2).

Based on the daily performed positive PCR tests, the 7-day incidence rate was calculated and showed two distinct ‘wave’ peaks (Figure 3). Similarly, the viral wastewater signals showed two distinct peaks. The first peak of the wastewater signal was about four times higher than the second one. While this first wastewater peak showed up earlier than the first peak of the 7-day incidence rate, which was not the case for the second peak. In contrast to the peaks, in both ‘waves’ the increase of the wastewater signal started earlier than the incidences did. During the two ‘waves’, the wastewater signal started to rise after September 28 and November 29, while the incidences started to rise around October 4 and December 17, respectively. Along with the incidences, the wastewater signal decreased in parallel as shown during the periods of November 2020 and January 2021. Effects of non-pharmaceutical interventions were reflected by both incidences and wastewater signals (Figure 3).

In Liechtenstein, most of the positive individual tests were analyzed for virus variant mutations through quality-tested, mutation-specific PCRs. This method allowed the detection of unique mutation constellations, such as the N501Y and E484 K mutations, within a few hours in contrast to genome sequencing, which requires several days (ÖGLMKC 2021). In the individual samples, the first variant of concern (VOC; variant B.1.1.7) was detected on January 13, 2021. The 7-day incidence rate of the VOC is shown (Figure 4). Conversely, sequencing of wastewater samples revealed a weak signal of the variant B.1.1.7, based on two of 16 marker mutations in late December 2020, followed by a higher than 20% signal in mid-January 2021 (12 of 16 marker mutations). A few weeks later the virus load signal was nearly up to 100%. The B.1.1.7 viral wastewater peaks on January 20 were followed by a peak based on individual testing 7-day incidence on January

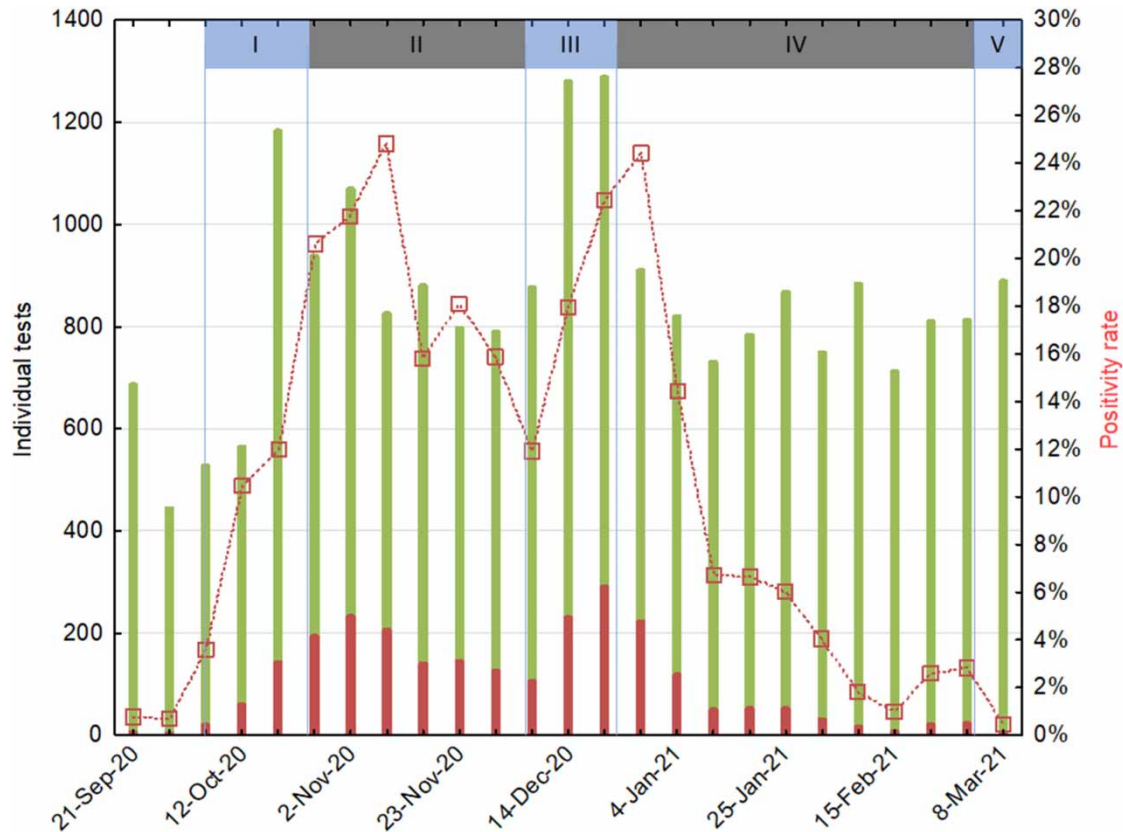


Figure 2 | Individual testing in Liechtenstein (weekly means). Number of positive (red bars), negative (green bars) and total number of tests (green+red bars) and positivity rate (red-dashed line). Non-pharmaceutical interventions in upper bar as: I. Oct 10 increased social distancing in public and private encounters <30 persons, II. Oct 24 gastronomy industry closed, III. Nov 30 gastronomy industry opened, IV. Dec 20 culture, sport, gastronomy industry closed, V. March 1 culture, sport reopened, encounters with 10 persons max.

24. While by February 18, the wastewater signal reached another peak, this was observed for the 7-day incidence only on March 8.

While the ratio of virus load per 7-day incidence was very high at the time when the test frequency was increased, this ratio decreased until the beginning of November 2020. Then, for nearly 2 months, the ratio of virus load per 7-day incidence remained constant at around 0.62 ± 0.14 Mgc PE⁻¹ 7-day incidence⁻¹ (Figure 5). During this episode, 992 ± 187 tests per week were conducted. From December 27, 2020, when the variant B.1.1.7 was detected for the first time in wastewater, the ratio increased to an average of 1.24 ± 0.76 Mgc PE⁻¹ 7-day incidence⁻¹ and showed two small peaks, one around mid-January and another one around the end of February 2021. Individual testing decreased during this period to 814 ± 64 tests per week. A comparison by the Mann–Whitney *U*-test indicated a statistically significantly higher ($n > 50$ days, $p < 0.005$) virus load per incidence for the B.1.1.7 period compared with the wild-type period (Davies *et al.* 2021).

Correlating active cases (proxied by the 14-day incidence) and the virus load in the wastewater, we derive

$$\text{Active cases}_{(t)} = L_{\text{virus}}(t-\text{lag}) * 1.773$$

as the regression model ($R^2 = 0.97$, standard error = 37.3). The lag of the virus signal has here been determined as 4 days. The upper and lower 95% confidence interval values of the regression parameter are computed as 1.676 and 1.869, respectively. While the statistical comparison of the virus load per 7-day incidence rate of the wild type and the variant B.1.1.7 periods indicate a statistically significant difference, the difference in the shedding dynamics is not reflected in the model.

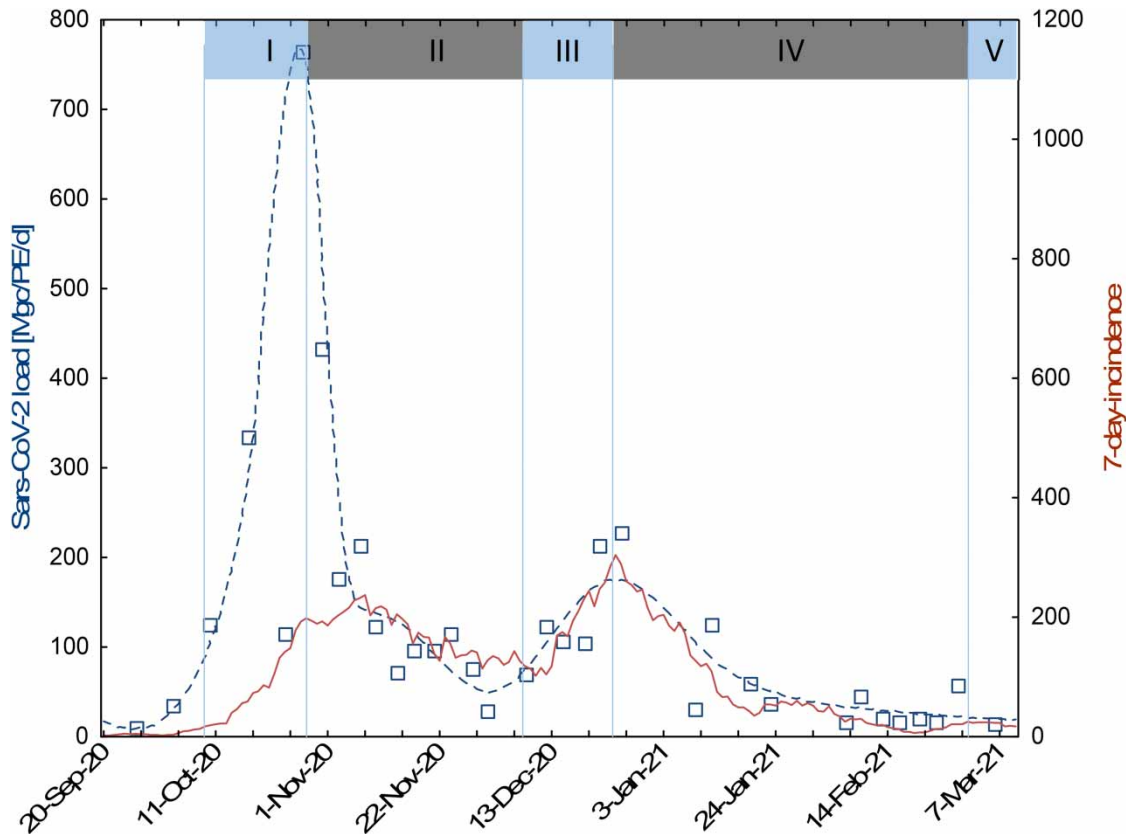


Figure 3 | Comparison of 7-day incidence from individual testing (red line) and the PE normalized virus load (blue squares) with its Spline-fit (dark blue-dashed line). Non-pharmaceutical interventions (shaded, header): see [Figure 2](#).

4. DISCUSSION

The principality of Liechtenstein is a small country sharing a strong connected economy with the neighboring countries such as Austria and Switzerland; thus, high commuter traffic and intermixing of nationalities can be well observed. Throughout the pandemic Liechtenstein's borders to Switzerland remained open, while the border to Austria was closed through times of lockdown on either side, except for labor commuters ([Regierung des Fürstentum Liechtenstein 2021](#)). The policy of flattening the incidence curve in Liechtenstein was very successful from the start of the pandemic. Concerning the first wave, it is known that the population responded well to the governmental COVID-19 safety and preventive measurements, although they were considered less strict than many other countries ([Thiel *et al.* 2020](#)). In this paper, we hence present data on the second wave of cases in which two major peaks were observed and give insights into the viral abundance and shedding in wastewater across the country.

The major non-pharmaceutical measures are indicated in [Figure 1](#), and both incidences and wastewater signals indicate their effectiveness. The response in the wastewater signal was faster than that observed in the 7-day incidence.

Confirming our first hypothesis (i), [Figure 3](#) shows that the SARS-CoV-2 viral loads in Liechtenstein wastewater increased prior to rising numbers in individual tests, which are in accordance with previous findings that suggested WBE could be used as an early warning tool for SARS-CoV-2 detection and its spreading (e.g. [Medema *et al.* 2020](#); [Ahmed *et al.* 2021](#); [Gerrity *et al.* 2021](#)). The clarity of the finding was to some extent blurred by a change in testing strategy during the period from the end of September to mid-October 2020 when testing effort was doubled. Except for considerable testing intensification in mid-November to December 2020, the testing strategy and frequency remained rather constant. Increased testing frequency is known to lead to an increased detection of incidences ([Wu *et al.* 2020](#)), while disease incidences derived from wastewater are unbiased. Disease prevalence, however, is hard to deduct from wastewater testing since reliable data on shedding are yet only available from animal models ([Mohandas *et al.* 2021](#)).

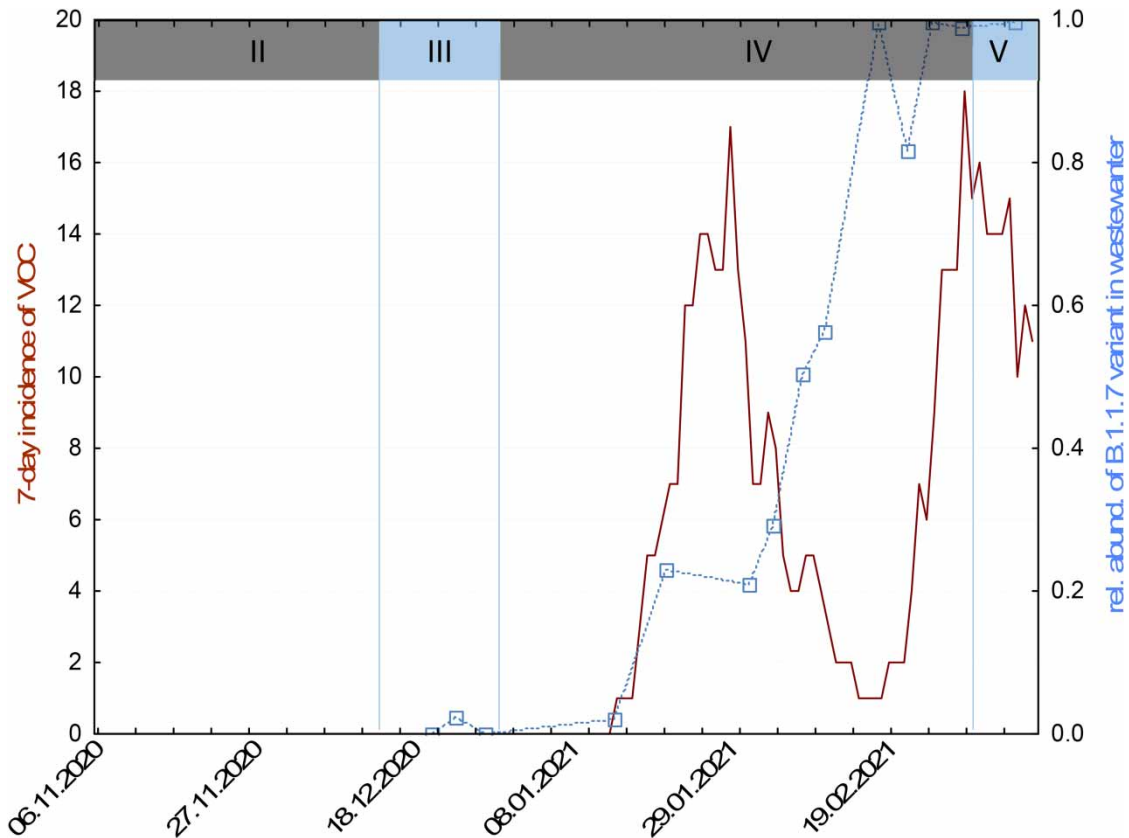


Figure 4 | Relative abundance of B.1.1.7 variant in wastewater based on median over found marker mutations (blue-dashed line) and 7-day incidence of VOC (red line). Non-pharmaceutical interventions (header, shaded): see Figure 2.

A linear regression model with an intercept of 0 was applied to correlate the incidences based on individual testing and wastewater signal. With a time-lag of 4 days, the highest correlations were achieved. Despite its simplicity, the incidence model exhibits a good prediction capability from the beginning of January 2021 onwards. While the spread of variants is likely to cause differences in the shedding dynamics, such effects are lost in the noise of the signal. Hence, the dynamics of both the wastewater signal and the epidemiologic situation are too low to identify such effects. The model clearly indicates a first pandemic wave approximately at the end of October 2020 that does not match the recorded active cases. However, since the predictions match the time period and also the severity (predicted 7-day incidence approximately 650 cases per 100,000) with the general pandemic situation in nearby countries (Austria/Switzerland), it is likely that the peak has been missed in the official recordings due to an insufficient number of tests. In summary, these data confirm both hypotheses (i) and (ii) by showing that the wastewater signal indicates changes in disease prevalence prior to individual testing and both signals are correlated as long as the testing strategy remains constant.

With the above presented results, the wastewater data suggest an underestimation of cases during the first peak of cases when the ratio of wastewater signal/incidence was extraordinarily high. Then, upon constant testing strategy, the ratio of wastewater/incidence remained constant. However, while testing frequency remained constant, the wastewater signal started to rise again in mid-January 2021. This was related to the beginning increase of the B.1.1.7 variant concentrations that were found both in individual tests and in the wastewater samples. It is estimated that the lineage B.1.1.7 has a 43–90% higher reproduction number than pre-existing variants, suggesting also higher shedding rates (Davies *et al.* 2021; Volz *et al.* 2021). Little is known about the behavior of B.1.1.7 in context with stool analysis, and there is a need for further research regarding this topic. Since the swallowing of sputum and saliva may considerably contribute to viral titers in stool, it seems evident that this also has an effect on viral wastewater load. Apart from the gut itself as a source of SARS-CoV-2 genes (Fitzgerald *et al.* 2021), viral genes contained in sputum and saliva may also ultimately contribute to the viral load in wastewater. The large amounts produced and swallowed per day (Rudney *et al.* 1995) and also directly excreted into

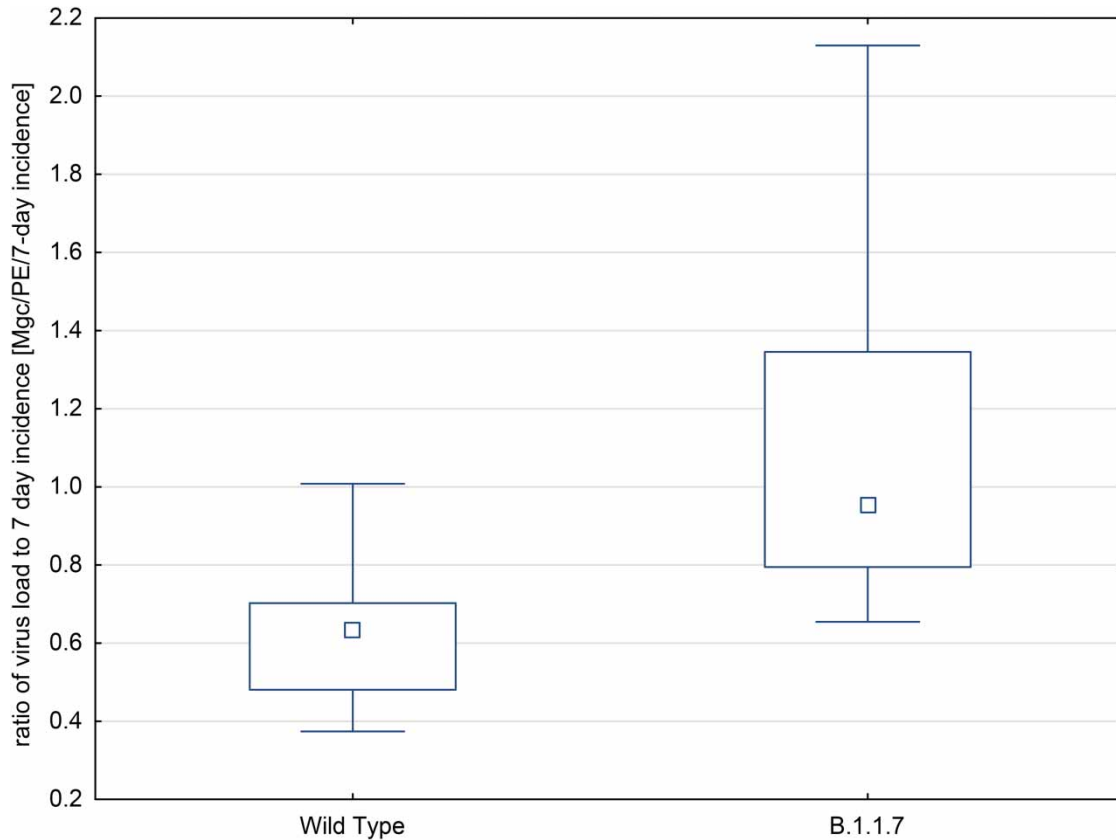


Figure 5 | Median of the ratio of the virus load per 7-day incidence during two periods of constant testing frequency: Nov. 6–Dec. 26 (wild-type period) and Dec. 27–Mar. 10, 2021 (B.1.1.7 period).

the sewer system, e.g. by toothbrushing, may considerably contribute to the gene copy numbers in wastewater. The observation of the early emergence of B.1.1.7 in wastewater as early as in December 2020 corroborates hypothesis (iii) in detecting new viral variants earlier than individual testing does. This is further supported by the emerging small peaks of the virus signal in wastewater per observed incidence around January 20 and February 19, 2021 (Figure 4).

The observations presented above could be challenged if there were changes in the wastewater temperature during the time of examination. The estimated half-life of SARS-CoV-2 ranges between 5 and 7 h at ambient (20 °C) conditions (Hart & Halden 2020). However, in the months of December 2020 to February 2021, the mean temperature of the inflow wastewater was as low as 10.9 ± 1.5 °C, and thus no temperature effect on virus fragment survival is expected to affect the results.

Finally, hypothesis (iv) is confirmed by the comparison of two periods characterized by constant testing frequency. After the first detection of the B.1.1.7 variant, the ration of WW signal per incidence increased and concurs with the view that variants like B.1.1.7 are characterized by enhanced fecal shedding dynamics (Davies *et al.* 2021; Volz *et al.* 2021). However, this could not be confirmed by our model that did not appear to be sensitive enough considering the low number of observations. This will have to be investigated in more detail with a broader dataset.

5. CONCLUSION

Presented data show a good correlation between the number of COVID-19 cases and SARS-CoV-2 gene concentration in the Liechtenstein wastewater system. Thus, the view that wastewater surveillance and monitoring offer an early indication for the spread of the SARS-CoV-2 is strongly supported. Particularly, it could be shown that wastewater data can better reflect changes in viral prevalence than individual testing. Similarly, wastewater viral loads may serve as an early indicator of mutations such as shown in the present case with the virus B.1.1.7 variant. For modeling prevalence and general interpretation of viral wastewater signals, it is suggested to accompany SARS-CoV-2 quantification in wastewater with genome sequencing. At low-case prevalence, wastewater monitoring and surveillance could serve as a crucial and efficient tool for

SARS-CoV-2 containment, requiring testing of asymptomatic individuals only when rises in the wastewater signal are evident. The lucrative lead of a couple of days would offer the opportunity to effectively set non-pharmaceutical interventions like social distancing or protective masks. To fully understand the changes of viral spreading and respond accordingly, it is important to identify where the virus variants are and thus, the inclusion of viral variants in predictive models will require a broader database and benefit the defeat of the virus across the globe.

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DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

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