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Wastewater surveillance of SARS-CoV-2 in Austria: development, implementation, and operation of the Tyrolean wastewater monitoring program

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ABSTRACT

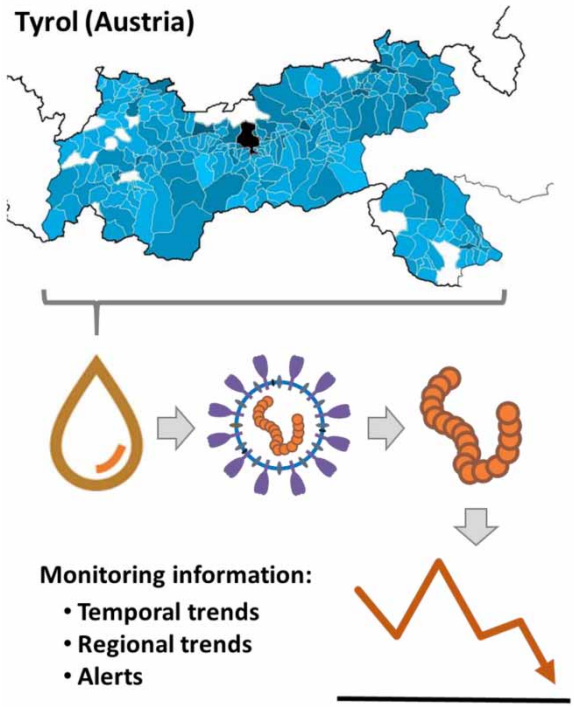
Wastewater-based epidemiology (WBE) is an effective approach for tracking information on spatial distribution and temporal trends of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) at the community level. Herein, the development, implementation, and operation of the wastewater monitoring program serving Tyrol – a federal province of Austria – are described. The development of this program was initiated by Tyrolean health authorities at the end of the first phase of the Coronavirus disease 2019 (COVID-19) pandemic (May 2020). In close co-operation with the water sector and academic institutions, efficient and effective workflows and processes for wastewater surveillance were established. The monitoring program went into operation in November 2020. By the end of July 2021, a total of 5,270 wastewater influent samples collected at 43 sites were analyzed. The monitoring program provided valuable insights into the development of the pandemic situation in Tyrol and fulfilled several tasks that are of importance in different phases of the pandemic. It represented an early-warning system, provided independent confirmation of temporal trends in COVID-19 prevalence, enabled the assessment of the effectiveness of measures, alerted about bursts of disease activity, and provided evidence for the absence of COVID-19. These findings underline the importance of establishing national wastewater monitoring programs as a complementary source of information for efficient and effective pandemic management.

Key words: COVID-19, SARS-CoV-2, sewage, Tyrol, wastewater-based epidemiology, wastewater-based surveillance

HIGHLIGHTS

- The development, implementation, and operation of the Tyrolean SARS-CoV-2 wastewater monitoring program are described.
- Health authorities are receiving regular updates on the pandemic situation in 43 Tyrolean regions.
- Turnaround times are <36 hours.
- Lead times of 3–7 days were observed.
- Ten positive cases per 120,000 persons were detectable.

GRAPHICAL ABSTRACT



INTRODUCTION

Wastewater-based epidemiology (WBE) is a competent and effective approach for tracking information on spatial distribution and temporal trends of biological and chemical agents at the community level (Wigginton *et al.* 2015; Choi *et al.* 2018; Mao *et al.* 2020; Medema *et al.* 2020a; Boogaerts *et al.* 2021a). WBE involves quantitative analysis of excreted biomarkers in population-pooled wastewater samples collected at or upstream of the influent to a wastewater treatment plant (WWTP). Measured loads are converted into information on exposure, use, or spread of agents in the monitored community providing an unbiased reflection of key aspects of public health.

WBE has successfully been used to detect polioviruses and inform eradication for several decades (Asghar *et al.* 2014). In the current pandemic of Coronavirus disease 2019 (COVID-19), the potential use of environmental surveillance in detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) circulation in communities has repeatedly been demonstrated (Ahmed *et al.* 2020a; Medema *et al.* 2020b; Wu *et al.* 2020). A summary of relevant publications is provided by a global collaborative at https://www.covid19wbec.org/publication-map (Bivins *et al.* 2020). The strengths of wastewater surveillance can be summarized as follows: (1) wastewater is a pooled sample providing information on virus circulation in the whole community served by the WWTP; (2) infected individuals, including symptomatic, presymptomatic, paucisymptomatic, and asymptomatic carriers, contribute to the total load of SARS-CoV-2 in wastewaters by shedding viruses or their RNA in stool, urine, or respiratory secretions (Jones *et al.* 2020; Weiss *et al.* 2020; Wölfel *et al.* 2020); (3) WBE provides information on spatial and temporal trends; (4) as infected individuals may shed the virus in stool before the infection is detected by a diagnostic test, wastewater signals precede the rise and fall of prevalences, hospitalizations, and intensive care unit admissions (Hillary *et al.* 2021; Wu *et al.* 2021; Rusinol *et al.* 2021); and (5) WBE is more inexpensive and less resource-intensive than diagnostic testing of individuals at a large scale (Thompson *et al.* 2020). Accordingly, wastewater surveillance may complement other well-established techniques for COVID-19 surveillance, such as clinical-based surveillance, hospital admission data, and mortality and morbidity rates.

Health authorities, such as the World Health Organization (WHO 2020), the United States Centers for Disease Control and Prevention (CDC 2021), and the European Commission (EC 2021), have recognized the potential usefulness of WBE

as an instrument for pandemic management. Accordingly, all around the world, monitoring programs have been initiated. One of the first monitoring programs in operation was established in Tyrol and is presented here.

Tyrol is a federal province of Austria located in the center of Europe and has a population of 760 thousand. Austria, and in due consequence Tyrol, operates a highly developed healthcare system, which includes an epidemiological surveillance program. Tyrol is one of the leading holiday destinations for winter sports. Approximately 5 million tourists have spent their winter holidays in Tyrol throughout the 2019/2020 season. During the first phase of the pandemic in Tyrol (February–May 2020), large number of tourists in combination with limited experience, information, and measures led to an increased risk of viral spreading in skiing resorts. Thus, Tyrol has potentially acted as a superspreading transmission hub (Popa *et al.* 2020) being responsible for cases exported to different European countries, including Germany (Correa-Martinez *et al.* 2020). A due consequence of these early events was an expansion of available surveillance and response capacities. One important activity initiated by the Tyrolean health authorities in May 2020 was the establishment of a wastewater monitoring program.

In this report, concepts, structure, and results of the Tyrolean wastewater monitoring program are presented. The health and water sector jointly worked together in the conception, development, and operation of this program. Further support was provided by academic institutions. The discovery phase started in May 2020. Feasibility of the approach was demonstrated by the end of August 2020. A first regional monitoring program involving nine WWTPs was launched in September 2020. The monitoring program involving 43 WWTPs was put into operation in mid-November 2020. By the end of July 2021, SARS-CoV-2 concentrations were determined in 5,270 Tyrolean wastewater samples. The existing data support the important role of SARS-CoV-2 wastewater monitoring in complementing clinical disease surveillance. WBE acts as an early-warning system indicating the resurgence of COVID-19 cases, provides independent confirmation of temporal trends in COVID-19 prevalence, and alerts about bursts of disease activity.

METHODS

The monitoring program is accompanied by research and development programs for providing new and advanced features with immediate application in the operational work. Consequently, the workflow was revised several times between April 2020 and March 2021. Important technological milestones included (1) the automation of RNA extraction from precipitates in May 2020, including a platform change in September 2020, (2) an increase in the processed wastewater volume from 50 to 250 mL by the end of July 2020, (3) the implementation of an internal amplification positive control (IPC) by the end of October 2020, (4) the implementation of a Pepper Mild Mottle Virus (PMMoV) target as a process control in mid-February 2021; (5) pipetting samples and reagents by a robotic system in mid-February 2021, and (6) quantification with linear RNA standards and reference material. In the following sections, only the final setup is described.

Sampling sites, wastewater sampling, and transport

Influent samples were collected from 43 Tyrolean WWTPs (Supplementary Material, Figure S1 and Table S1). Samples were collected as 24-hour composite samples using cooled autosamplers (4 °C) operated in a volume proportional mode. The daily flows of wastewater were measured online with sensors. The chemical oxygen demands (CODs) were directly determined on-site. About 500 mL aliquots were filled in polypropylene bottles, which were stored at 4 °C until transportation. The bottles were labeled with the name of the sampling location and the sampling date. The transport of the collected samples to the laboratory was accomplished by a courier service. Samples were transported in coolers (2–8 °C).

Wastewater concentration and automated nucleic acid extraction

Wastewater samples were processed within 24 h of arrival. They were stored at 4 °C until analysis. The protocols for wastewater sample pretreatment and PEG-NaCl-based precipitation of the virus fraction were based on the methods described by Ye *et al.* (2016) and Wu *et al.* (2020). For centrifugation, we used an Eppendorf 5910 R centrifuge equipped with a FA-6×250 fixed angle rotor (both Eppendorf, Hamburg, Germany) and 250 mL polycarbonate centrifuge bottles (TFS; Thermo Fisher Scientific, Waltham, MA, USA). The temperature was set at 4 °C, and the brake was deactivated unless stated otherwise. In a first step, 250 mL aliquots of the wastewater samples were cleared by centrifugation at 4,500×*g* for 30 min. In 225 mL of the resulting supernatant, 22.5 g of PEG 8000 and 5.1 g of NaCl (both Sigma-Aldrich, St. Louis, MO, USA) were dissolved by careful swirling. These solutions were allowed to sit at 4 °C for 15 min before they were spun at 12,000 × *g* for 50 min to pellet the precipitated virus fraction. After carefully discarding the supernatant, the pellets were re-centrifuged at 12,000 × *g*

for 5 min (brake set at level 4 on a scale from 0 to 9) and the residual liquid was removed with a pipette. Finally, the pellets were resuspended in 700 μL of buffer MTL (Qiagen, Hilden, Germany).

Four-hundred microliters of pellet suspension were supplemented with 60 μL of a solution comprising 60 ng of carrier RNA per microliter AVL buffer (both Qiagen). Subsequently, samples were subjected to an automated RNA extraction on a Biorobot EZ1 Advanced XL instrument (Qiagen) using the EZ1 Virus Mini kit (v2.0, Qiagen) according to the manufacturer's recommendations. Total RNA was eluted in 60 μL AVE buffer (Qiagen) and typically used immediately in quantitative reverse transcription real-time polymerase chain reaction (RT-qPCR) experiments. For long-term storage, the RNA extracts were kept at −80 °C.

Recoveries were determined by spiking chemically modified viral particles (NATtrol SARS-CoV-2 External Run Controls, 50,000 copies/mL, ZeptoMetrix, Buffalo, NY, USA) into wastewater samples.

Quantitative reverse transcription real-time polymerase chain reaction

Our *triplex* RT-qPCR approach amplified two previously published viral quantification targets – a 72-nt sequence stretch on the SARS-CoV-2 nucleocapsid gene (NC_045512.2:28,287–28,358) (Lu *et al.* 2020) and a 68-nt subsequence of the PMMoV replicase gene (AY859497.1:1878–1945) (Zhang *et al.* 2006; Haramoto *et al.* 2013) – as well as a 78-bp long, in-house developed synthetic IPC target. Single-tube reverse transcription and multiplexed quantitative real-time PCR amplification were performed with the Luna Universal Probe One-Step RT-qPCR kit (NEB; New England Biolabs, Ipswich, MA, USA). The 20-μL RT-qPCR cocktails contained 250 ng/μL of non-acetylated bovine serum albumin (Sigma-Aldrich), 1× of Luna Universal Probe One-Step Reaction Mix, 1× of Luna WarmStart RT Enzyme Mix (both NEB), and 6 μL of target RNA. Information regarding nucleotide sequences, fluorescent labels, and assay concentrations of PCR primers, TaqMan hybridization probes, and the synthetic PCR targets are provided in Supplementary Material, Table S2. To avoid potential contamination issues, we ordered the amplification primers and TaqMan hybridization probes from a different manufacturer (Microsynth, Balgach, Switzerland) than the synthetic PMMoV and IPC targets (Integrated DNA Technologies, Leuven, Belgium).

We used a Freedom EVO 100 robotic platform (Tecan, Männedorf, Switzerland) for an automated reaction setup in 96-well polypropylene PCR plates and two Applied Biosystems 7500 Fast Real-Time PCR System (all TFS) instruments for RT-qPCR amplifications.

The thermocycler protocol consisted of two initial holds at 55 °C for 10 min (reverse transcription) and 95 °C for 1 min (reverse transcriptase inactivation and initial template denaturation) and 40 PCR cycles of 95 °C for 15 s, 55 °C for 30 s, and 60 °C for 30 s. The speed of temperature transitions was set to 'standard'. For raw data acquisition and analysis, we used the 7500 Software (v2.0.6 or v2.3, both TFS). After automatic baseline correction and normalization to the ROX signal, the following fluorescence thresholds were used for Cq determination: 0.15 for SARS-CoV-2 N1 (FAM) and 0.05 for PMMoV (HEX/VIC) and the IPC system (Cy5). All samples, standards, and controls were run in duplicate. The difference of the Cq-values between the replicates was always < 0.5.

For RT-qPCR-based SARS-CoV-2 quantification, a linearized double-stranded plasmid DNA SARS-CoV-2 standard (Promega, Fitchburg, WI, USA) was used initially as reference standard to prepare calibration standards and quality control (QC) standards. This standard was subsequently replaced by an *in vitro* transcribed single-stranded partial RNA sequence of the SARS-CoV-2 N gene (IVT-T, Supplementary Material, Table S2). Furthermore, we used the National Institute of Standards and Technology (NIST)'s research grade test material 10169 – fragment 1 (NIST RGTM 10169, National Institute of Standards and Technology, Gaithersburg, MD, USA; https://bit.ly/3lSFGC6) as reference material. Fragment 1 is a synthetic sense strand RNA molecule corresponding to 3,985 nucleotides on the isolate USA-WA1/2020 SARS-CoV-2 genome (positions MN985325.1:25,949–29,698) (Harcourt *et al.* 2020).

As a quantification standard for the PMMoV genome, we used a synthetically prepared cDNA sequence (PMMoV-T, Supplementary Material, Table S2) that included nucleotide positions 1875–1948 of GenBank entry AY859497.1.

Calibration standards were prepared by diluting a stock solution that contained IVT-T RNA (16,667 copies/μL) and PMMoV-T (500,000 copies/μL; Supplementary Material, Table S2) in TLE buffer (pH 7.5) supplemented with 50 ng/μL yeast total RNA (Baoutina *et al.* 2019) within the range of 0.5–5,000 copies/μL for SARS-CoV-2 and 8×10^2–8×10^6 for PMMoV (in 1 mM Tris–HCl, 0.01 mM Na_2EDTA, pH 7.5). Initially, the calibration was included in every RT-qPCR run. After demonstrating its reproducibility, a master standard curve compiled from 12 independent experiments was used.

In RT-qPCR positive control reactions, 6 μL aliquots of stock dilutions (100- and 2,000-fold in 1 mM Tris–HCl, 0.01 mM Na_2EDTA, pH 7.5) were amplified in parallel to unknown samples.

The limit of detection (LOD) and limit of quantification (LOQ) of the SARS-CoV-2 quantification assay were determined by analyzing NIST RGTM 10,169 dilutions in 60 replicates. The LOD was defined as the lowest concentration where all replicates were positive. The LOQ was defined as the lowest concentration where the relative standard deviation was <30%.

Two negative controls (PCR-grade nuclease-free water) were included in each run. Extraction blanks were processed at least two times a week.

Data analysis and visualization

The SARS-CoV-2 concentration in wastewater was calculated based on the concentration factor, accounting for the effective volume analyzed in the RT-qPCR assay. These concentrations as well as key parameters of the influent wastewaters (CODs, daily flows of wastewater) were electronically submitted to the Tyrolean wastewater monitoring database. COD values were used to estimate the individual WWTP catchment populations. Per capita equivalents were calculated using 120 g/d/person.

Daily mass loads of SARS-CoV-2 were calculated by multiplying the measured concentrations in the 24-h composite samples with the corresponding daily flows of wastewater. Mass loads were converted into 'estimated numbers of positively tested persons' using an estimated average number of viruses that an infected person will contribute to the total load of viruses detectable in wastewater samples as divisor (Gerrity *et al.* 2021; Hasan *et al.* 2021; Saththasivam *et al.* 2021). The conversion factor (3.0×10^9 copies per infected person per day) was determined experimentally by fitting wastewater data to clinical test data.

The database also contained region-specific data on the actual numbers of confirmed COVID-19 cases obtained from clinical test results submitted to the epidemiological reporting system.

The two epidemiological parameters served as input for data visualization in the Tyrolean wastewater monitoring dashboard. The dashboard was created with ArcGIS (ESRI, Redlands, CA, USA).

RESULTS AND DISCUSSION

Vision and mission of the Tyrolean wastewater monitoring program

The monitoring program was designed to provide timely, comprehensive, exhaustive, and reliable information on the pandemic situation and trends in Tyrolean regions. To accomplish this task, samples from influents of Tyrolean WWTPs are regularly collected and analyzed. Less than 36 h after sampling, transformed SARS-CoV-2 loads in combination with other epidemiological parameters (i.e. region-specific prevalence) are made available to health authorities to provide guidance in the decision-making process.

Overview on the selected sampling sites

Tyrol has a population of 760,000 and consists of 279 municipalities. The most populous town is Innsbruck (132,000 inhabitants). There are nine further municipalities with >10,000 inhabitants, namely Kufstein, Telfs, Wörgl, Hall in Tirol, Schwaz, Lienz, and Imst. During the site selection process, utmost coverage of the Tyrolean population was the primary selection criterion.

Tyrol is a traditional tourism destination. In 2019, >12 million people spent their holidays in this country. Nine destinations reported >1 million overnight stays per year. These were Sölden, Innsbruck, Ischgl, Mayrhofen, Neustift im Stubaital, Serfaus, St. Anton am Arlberg, Seefeld in Tirol, and Eben am Achensee. The majority of them are internationally known ski resorts. On average, >100,000 visitors were present per day in Tyrol. During peak times, such as Christmas holidays, even more. Accordingly, tourism was the other factor that was considered during the program development.

There are 53 municipal WWTPs that serve populations ranging from 350 to 174,674 people (Supplementary Material, Figure S1). The WWTPs have a total capacity of 2.2 million population equivalents. The monitoring program covers all WWTPs with capacities >5,000 population equivalents (Supplementary Material, Table S1). With these 43 WWTPs, the coverage of 98% of the Tyrolean population and >90% of potential visitors was accomplished. Figure 1(a) summarizes the individual catchment population sizes. Innsbruck is the most populous region. Nauders can be found at the other end of the scale with 1,531 inhabitants. Figure 1(b) provides an overview on the overnight stays in January 2020 in the monitored regions. Strass reported >1.2 million and Niederndorf only 5,297. With the exception of Strass, where, for instance, Mayrhofen and Eben am Achensee drain their wastewater, the most populous regions are not found among the top touristic destinations and vice versa. Therefore, to cover both, residents and tourists, even rather small WWTPs were monitored.

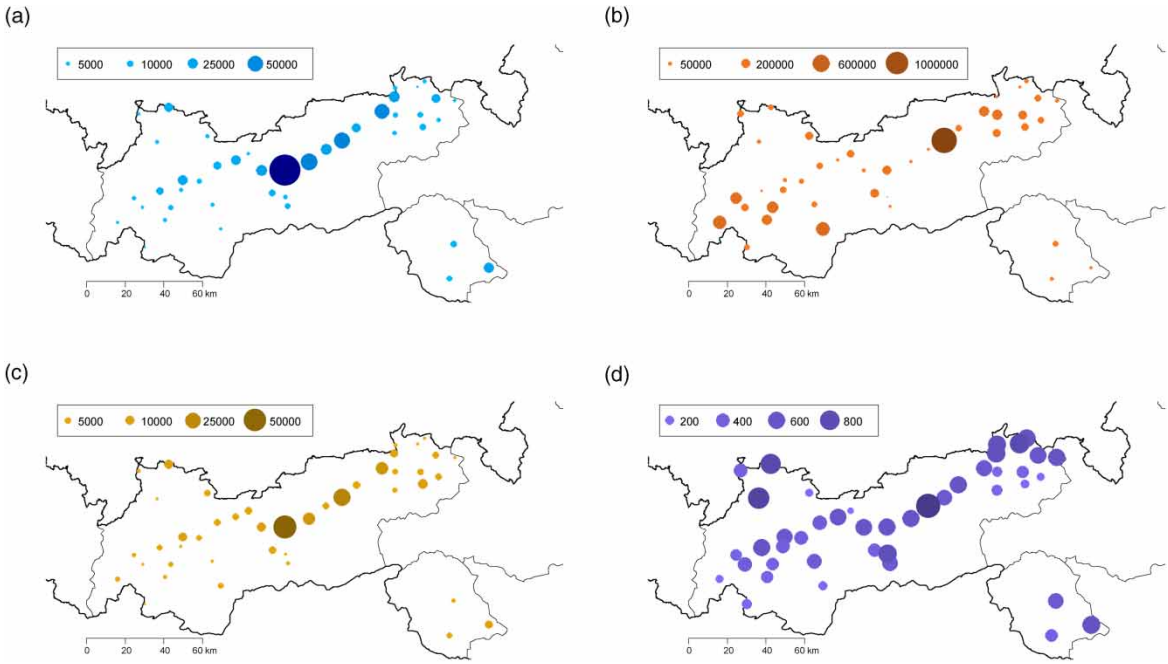


Figure 1 | Geospatial maps of Tyrol showing (a) the number of inhabitants living in the catchment areas of the 43 WWTPs, (b) overnight stays in the catchment areas in January 2020, (c) average daily flow rates (m^3/day) of the monitored WWTPs, and (d) average CODs measured in the collected 24-h composite samples (mg/L).

The project went through three phases with different numbers of WWTPs monitored (Supplementary Material, Figures S2 and S3). In the discovery phase (May–August 2020), Innsbruck was the primary target. By monitoring the region of Innsbruck, 23.1% of the total population and 3% of potential visitors were covered. The monitoring was extended to nine WWTPs in September 2020 (development phase). By monitoring Innsbruck, Fritzens, Schwaz, Strass, Radfeld, Kirchbichl, Kufstein, Niederndorf, and Ischgl, 55.4% of the total population and 33% of potential visitors were covered. The monitoring program with 43 WWTPs was put into operation in mid-November 2020 (delivery phase).

Developed workflow and processes

The workflows and processes established for the Tyrolean wastewater monitoring program are summarized in Figure 2.

Sample collection

In total, 43 Tyrolean WWTPs were included in the monitoring program (Figure 2(a); Supplementary Material, Table S1). Based on the results obtained during the first two phases of the project, we decided that the minimal sampling frequency for a single site should be two samples per week. This decision is in full agreement with published EC recommendations (EC 2021). Based on the available budget and laboratory capacity, however, it was possible to sample a restricted number of the 43 targeted WWTPs with a higher frequency. Here, the primary selection criterion was utmost coverage of the Tyrolean population.

Innsbruck was sampled daily. Twelve other WWTPs provided five samples per week (Sunday–Thursday). In the remaining WWTPs, two samples per week were collected (either Sunday/Tuesday or Monday/Wednesday). Samples were collected as 24-h composite samples using cooled autosamplers (4 °C) operated in a volume proportional mode.

Additionally, CODs and daily influent flows were provided by the WWTPs. Average daily flow rates and average CODs of the 43 WWTPs are summarized in Figure 1(c) and 1(d). The region with the highest average COD caused by a high content of industrial wastewaters was Schwaz. As a considerable number of samples from Schwaz showed inhibition, there might be a link between high COD values and PCR inhibition. Accordingly, the daily COD values were used to check plausibility of (negative) results. Lowest average COD values were observed for touristic destinations in lockdown (e.g. Ischgl, Sölden, and Nauders) and WWTPs with infiltration (e.g. Seefeld and Kitzbühel).

The hydrochemical parameters were electronically transferred to the Tyrolean wastewater database. They were used to calculate epidemiological parameters. Furthermore, they were used as plausibility checks for the analytical results obtained.

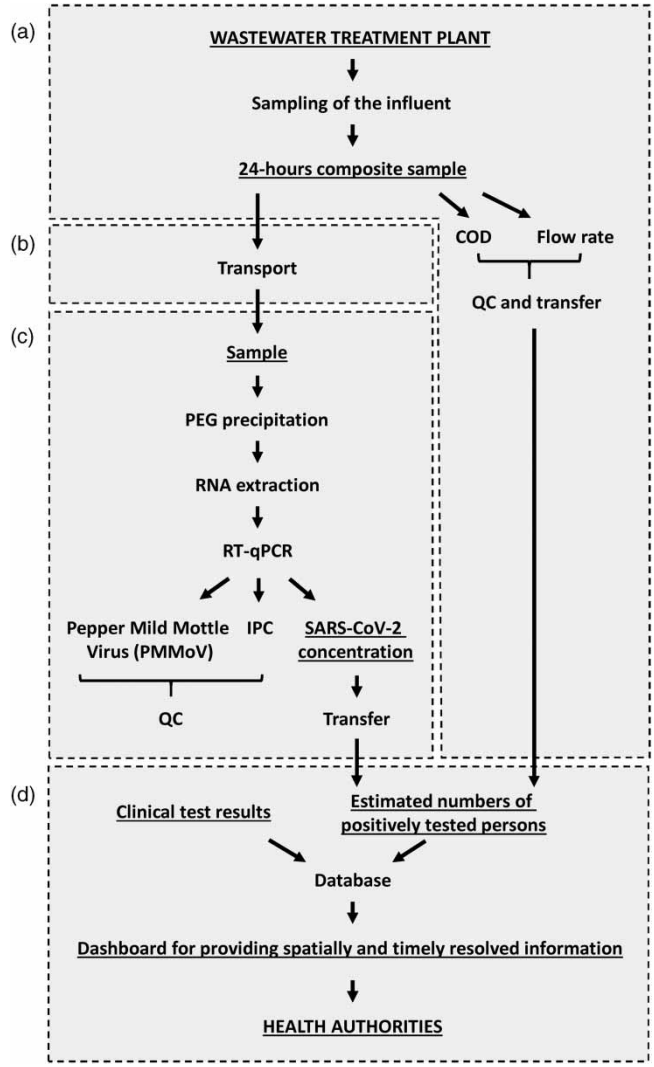


Figure 2 | Workflow established for monitoring SARS-CoV-2 loads in 43 Tyrolean WWTPs. The workflow involves (a) sample collection and measurement of hydrochemical parameters (flow rate and COD) at the WWTPs, (b) transport of the samples to the laboratory, (c) quantification of SARS-CoV-2 with a validated method, and (d) amalgamation of data sets in a single database with consecutive processing, visualization, and transfer of information to health authorities.

High CODs were often found as reasonable explanations for observed PCR inhibitions and, thus, for low and negative results. Increased daily influent flows also helped to determine additional outliers. Increased daily influent flows were usually measured on rainy days. Depending on the precipitation quantity and the sewer system characteristics (i.e. separate or combined sewer systems), sample composition might be altered by stormwater in a way that the measured SARS-CoV-2 concentration does not reflect the actual pandemic situation.

Beside the COD, several other hydrochemical parameters are regularly monitored in influent wastewaters. These include the biological oxygen demand, ammonia-nitrogen (NH_4-N), total-nitrogen (N_{tot}), and total-phosphorus (P_{tot}). However, particularly by small- and medium-sized WWTPs, only parts of these parameters are determined on a weekly or daily basis. The most frequently monitored parameter is the COD. Thus, to avoid extra work and to increase the willingness to co-operate, we decided to ask for the COD alone.

Sample transport and storage

During transport and storage, samples were kept at temperatures between 2 and 8 °C (Figure 2(b)). Storage and transport conditions can affect the detection and quantification of SARS-CoV-2 in wastewater samples because of stability. Various studies

provided sufficient evidence that SARS-CoV-2 is persistent in wastewater samples for several days if samples are stored at 4 °C (Ahmed *et al.* 2020b; Boogaerts *et al.* 2021b). As part of the validation process, we have evaluated sample stability. We observed that the N1-gene fragment showed high in-sample stability in wastewater samples for up to 7 days of storage.

We used thermally insulated boxes for shipping the cooled samples. Initially, transport was outsourced to parcel services. With the involvement of external services, at least 24 h of the total turnaround time were spent for transportation. The situation was unsatisfactory, because Innsbruck can be reached by car from any of the 43 monitored WWTPs within 3 h. Accordingly, a faster and more cost-effective transport concept was elaborated during the development phase. This included six collection routes with fixed pick-up and handover times. Sample transportation was organized by the administration of the province Tyrol using their drivers and carpool. With this internal courier service, samples were delivered to the laboratory on the same day of sampling usually before 2 pm. Typically, sample processing started within 1–3 h upon arrival.

We experienced that freezing is deleterious for SARS-CoV-2 recoveries from untreated wastewater samples. Therefore, this option was ruled out for storage and transport.

SARS-CoV-2 quantification

The rapid onset of COVID-19 and the urgency of the pandemic have forced us to provide quantitative data already in early phases of the monitoring program (Figure 2(c)). Factors like practicability, accessibility to instrumentation, supply of materials, and availability of knowhow and information had an impact on the selection of methods that were integrated into the initial and modified workflows. The monitoring program was accompanied by research and development programs for providing new and advanced features with immediate application in the operational work. Published recommendations represented further triggers for innovations (Bustin *et al.* 2009; Ahmed *et al.* 2020c). Important milestones included (1) the principle setup of the workflow in April 2020, (2) the first detection of SARS-CoV-2 in a wastewater sample collected on 16 April 2020, (3) automation of RNA extraction from precipitates in May 2020, including a platform change in September 2020, (4) increase of the processed wastewater volume from 50 to 250 mL by the end of July 2020, (5) implementation of an IPC by the end of October 2020, (6) implementation of the process control PMMoV in mid-February 2021; (7) pipetting samples and reagents by a robotic system in mid-February 2021, and (8) quantification with linear RNA standards and reference material.

The US CDC N1 RT-PCR assay was used for SARS-CoV-2 quantification. The assay was developed for clinical testing. Several studies have evaluated its performance for wastewater analysis (Ahmed *et al.* 2020a; Medema *et al.* 2020b; Gerrity *et al.* 2021; Pecson *et al.* 2021). So, there is sufficient evidence that the N1 RT-PCR assay performs equally or even better than other targets.

As reported by several other laboratories (Fomsgaard & Rosenstierne 2020), manufacturer shortages forced us to modify or exchange methods to accommodate supply issues. In May 2020, ultrafiltration devices were hardly available; hence, we decided to implement PEG-NaCl-based precipitation. In September 2020, reagents for running the initial RNA extraction could not be supplied. Therefore, the extraction platform was changed.

The laboratory responsible for SARS-CoV-2 quantification was accredited for genetic analysis according to ISO 17025. Accredited laboratories have to meet specific process and management system requirements. Process requirements are describing the activities to ensure that results are based on accepted science and aimed at technical validity. Management system requirements are those steps taken by the organization to give itself quality management system tools to support the work of its people in the production of technically valid results.

An important obligation for the analytical laboratory is validation of newly developed methods and workflows. We have performed a panel of experiments to verify that the developed SARS-CoV-2 quantification workflow is fit for the intended purpose. We evaluated the calibration model (assay efficiency of 100%, with an R^2 value of 0.996), determined the LOQ (27 copies per reaction) and LOD (nine copies per reaction), assessed recoveries ($20.6 \pm 12.2\%$), checked the intraday and interday reproducibilities (RSD $< 30\%$), and verified the stability of wastewater samples over several days at 4 °C. A publication summarizing these method validation efforts is in preparation.

Metrological traceability would require use of a certified reference material. Such material is currently not available. NIST RGTM 10169 is probably the best alternative. It differs from a NIST standard reference material in that it is not as highly characterized or traceable to the International System of Units. Nevertheless, it is homogeneous and undergoes continual stability testing. It contains synthetic fragments of the SARS-CoV-2 virus RNA, which is the target of molecular diagnostic tests. The RNA fragments were characterized for concentration using digital PCR methods.

A common way of checking the validity of test and calibration results is participating in appropriate proficiency testing. Inter-laboratory exercises are well established in the framework of WBE of illicit drugs (van Nuijs *et al.* 2018). They helped to improve the performance of participating laboratories and triggered harmonization and standardization of analytical methods. Despite their importance, inter-laboratory studies focusing on SARS-CoV-2 quantification in wastewater are not institutionalized so far (Chik *et al.* 2021; Pecson *et al.* 2021). Therefore, we have organized two such studies with Austrian and German colleagues. Obtained results clearly revealed a lack of standardization rendering a direct comparison of quantitative results from different laboratories nearly impossible (publication in preparation).

Importantly, the laboratory was designed with a one-way path from sample receipt to RNA extraction to PCR preparation to PCR analysis. Each step was accomplished in separate rooms. Owing to the strict separation, sample contamination indicated by positively tested blanks (i.e. no-template controls and extraction controls) was not observed.

Known PCR inhibitors found in wastewater, including bile salts, urea, phenol, ethanol, polysaccharides, sodium dodecyl sulfate (SDS), humic acids, tannic acid, melanin as well as different proteins, can lead to false-negative results if they are not effectively removed during sample preparation (Schrader *et al.* 2012). Their concentrations may vary within a single WWTP and between WWTPs. Furthermore, precipitation methods may enrich not only the target but also certain RT-PCR inhibitors. Accordingly, the presence of inhibitors in wastewater samples needs to be investigated. For this purpose, two controls were implemented in the process. With the IPC, the performance of the RT-qPCR assay is assessed. A sample was flagged if its IPC *Cq* was substantially delayed relative to that of the simultaneously amplified positive controls ($\Delta Cq > 2$). With PMMoV quantification, the overall process is monitored. A sample was flagged if the determined PMMoV load fell outside of the WWTP-specific average load $\pm$ standard deviation. If one of the controls indicates quality issues, either samples, precipitates, or extracts are reprocessed. Furthermore, dilution of precipitates and extracts was found to be a useful strategy to reduce inhibition. Overall, $<2.5\%$ of samples were reanalyzed.

At this moment, there is no ideal external control standard with the same properties of SARS-CoV-2 for quantification available (Boogaerts *et al.* 2021b; Chik *et al.* 2021). Therefore, no spike-in control was in use.

Owing to the optimization, automation, and parallelization of processes, we were able to analyze up to 42 samples per day. This capacity is sufficiently high to do service not only for the Tyrolean monitoring program, but also the Austrian monitoring program that is under-developed.

Turnaround times, defined as the number of hours between sample collection and the electronic transfer of quantitative results, ranged between 5 and 28 h. Including shipping times, results were reported to health authorities within 36 h.

Database, dashboard, and information transfer

All data from the WWTPs (i.e. daily influent flows and CODs) and the analyzing laboratory (SARS-CoV-2 concentrations) were collected from the Tyrolean wastewater monitoring database (Figure 2(d)). Transfer was accomplished electronically. The number of reported COVID-19 cases in the monitored regions represented additional entries to the database.

The wastewater data were used to provide estimated numbers of positively tested persons per region. A mass balance approach was used for this purpose (Gerrity *et al.* 2021; Hasan *et al.* 2021; Saththasivam *et al.* 2021). Individual viral loads were converted into estimated numbers of positively tested persons using an estimated number of viruses that an infected person will contribute on an average to the total load of viruses detectable in wastewater samples as a divisor. The divisor (3×10^9 copies per infected person per day) was determined by fitting data sets available in the discovery phase of this project (May–August 2020). It corresponds well to experimentally and theoretically determined values of virus shedding that were reported in other studies (6×10^8–2×10^{11} copies per infected person per day) (Gerrity *et al.* 2021; Hasan *et al.* 2021; Saththasivam *et al.* 2021).

Prevalence estimates offer several following advantages in comparison to the reporting of concentrations: (1) with these numeric values provided, even unexperienced users will learn very quickly the interpretation of wastewater data; (2) results from clinical and environmental surveillance can directly be compared with each other, which facilitates the assessment of the pandemic situation; (3) different regions can directly be compared with each other if population normalized loads are used; and (4) data from different regions can be aggregated to get representative situation pictures for territories and nations.

The applied mass balance approach has several following limitations: (1) fitting was accomplished without knowing the real numbers of cases; (2) this simple model neglected the fact that viral shedding is variable over time and between patients; and (3) the model does not correct for WWTP-specific parameters, such as in-sewer transportation and stability.

Clearly, with the developed mass balance approach, only a rough estimation of the actual number of infected persons can be provided. Nevertheless, based on the feedback provided by the health authorities, the above-mentioned advantages seem to outweigh the limitations.

The available epidemiological data served as an input for the Tyrolean wastewater monitoring dashboard. With this dashboard, the available temporal and geographic information was visualized to enable health authorities monitoring the pandemic situation in Tyrol as a whole and in each region individually. Multiple visualizations were integrated on a single screen. The dashboard included maps with region-specific prevalence numbers. Furthermore, time series were provided to see potential trends, including warnings and confirmation of absence.

The dashboard was built for internal use by health authorities only. Persons with access included decision-makers at the strategic and operational levels. The available information was used to assess the actual pandemic situation and its development, and in due consequence to provide justification for measures.

On a weekly basis, disclosure of the most relevant information to the general public was accomplished with short reports communicated via www.tirol.gv.at/covid-abwasser and press releases.

Representative monitoring results

Between June 2020 and July 2021, 5,270 samples were analyzed and the corresponding data were transferred to the Tyrolean wastewater monitoring database. The database and the corresponding dashboard represented effective and efficient tools for assessing the pandemic situation in Tyrol. In the following, representative monitoring results are discussed to demonstrate the vast potential and some limitations of the environmental surveillance approach.

Monitoring results from Innsbruck obtained between 2020-06-01 and 2020-09-27

In the discovery phase of the Tyrolean wastewater monitoring program, we have provided the proof-of-concept that WBE can be used to monitor viral circulation in the community (Figure 3(a)). Particularly, we have demonstrated its potential use as an early-warning system. As an early-warning system, wastewater surveillance should signal an initial outbreak or the recurrence of the virus, providing time for health authorities to prepare for the event and to minimize the impact.

Innsbruck was the first Tyrolean region with a regular SARS-CoV-2 monitoring. Monitoring started on 2020-06-01, right after the end of the first wave of COVID-19 in Tyrol. In June 2020, life was returning to relative normality, except for the mandatory wearing of a face mask and maintaining a safe social distance. Between 2020-06-15 and 2020-06-22, Innsbruck had no COVID-19 cases. In July 2020, the average number of cases was 14. Beginning with 2020-08-11, a rapid increase of positive cases was observed. At the end of August 2020, around 150 persons were positively tested.

In the first 2 weeks of June 2020, all tests of wastewater samples were negative. A first signal for resurgence of SARS-CoV-2 transmission in the community was obtained on 15 June 2020. This was 7 days ahead of reporting positive clinical tests. Until the beginning of August 2020, in all but one wastewater sample viral RNA was detected. The average of the estimated number of positively tested persons was 4.8. Taking in consideration that in July 2020, the average number of patients with confirmed SARS-CoV-2 infection was 14, the minimal number of clinical cases detectable by wastewater monitoring was 10 per 120,000 inhabitants. On 8 August 2020, a rapid increase of the viral load was observed. This 'burst' leading to 150 positively tested persons was observed 3 days earlier than the increase of positive clinical tests.

For the wastewater sample collected on 11 July 2020, a negative test result was obtained. We believe that high loads of stormwater entering Innsbruck's combined sewer system on that particular day were responsible for sample dilution and a negative test result. This hypothesis was supported by the fact that the PMMoV load determined by reanalyzing the extract of the sample collected on 11 July 2020 was 60–70% lower than the loads observed on rain-free days in July 2020.

Comparison of the graphs representing the wastewater and clinical testing results (Figure 3(a)) revealed that (1) wastewater monitoring complements established epidemiological monitoring tools, (2) wastewater leads clinical cases by 3–7 days, and (3) the minimum number of SARS-CoV-2-infected cases associated with a positive detection in Innsbruck's wastewater is 10 per 120,000 inhabitants.

The herein reported lead time and detection sensitivity are in full agreement with already published information (D'Aoust *et al.* 2021; Larsen *et al.* 2021; Olesen *et al.* 2021; Wu *et al.* 2021). However, the lead time of WBE is dependent on several factors, including diagnostic testing availability and reporting within the community, the sewage collection and conveyance systems, and the capacity and turnaround time of the WBE laboratory (Bibby *et al.* 2021). As resources allocated to diagnostic

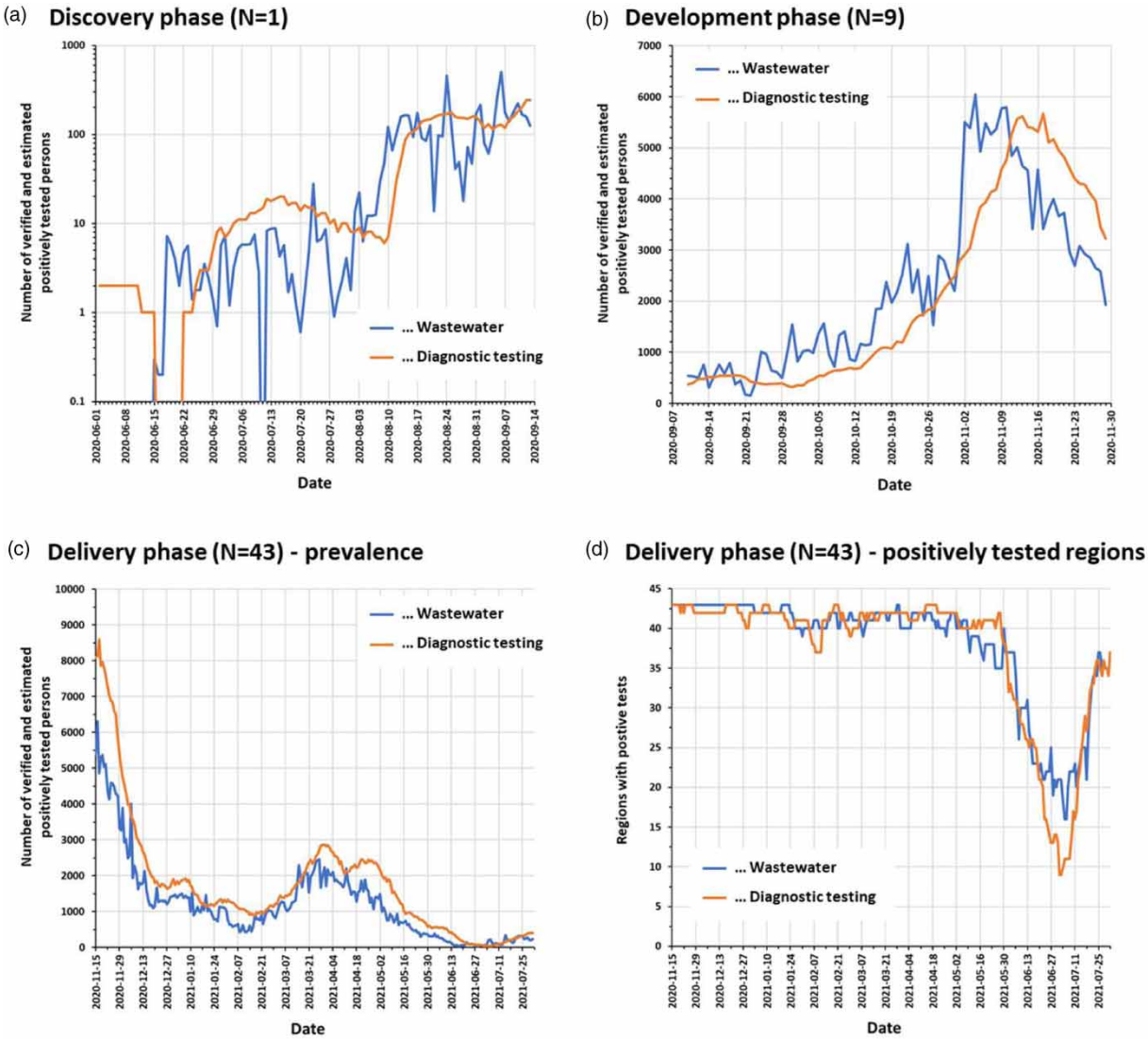


Figure 3 | A summary of important monitoring results obtained in (a) the discovery phase, (b) the development phase, and (c, d) the delivery phase of the project. For the delivery phase, temporal information on (c) the prevalence and (d) the number of positively tested regions is provided. In the discovery phase, only Innsbruck was monitored. In the development phase, Innsbruck, Fritzens, Schwaz, Strass, Radfeld, Kirchbichl, Kufstein, Niederndorf, and Ischgl were covered. In the delivery phase, 43 WWTPs were monitored. Accordingly, aggregated data are provided in (b) and (c).

testing and reporting are continuously expanding during this pandemic, it is possible that lead times might decrease accordingly.

Monitoring results from nine regions obtained between 10 September 2020 and 29 November 2020

After positive evaluation of the project, a monitoring program involving nine WWTPs was started in September 2020 (Figure 3(b)). The monitored WWTPs included Innsbruck, Fritzens, Schwaz, Strass, Radfeld, Kirchbichl, Kufstein, Niederndorf, and Ischgl. The monitoring program was intended to provide information on viral circulation in the community after returning from summer holidays.

On 10 September 2020, the number of positively tested persons was 374 in the nine monitored areas. The number of cases started to rise at the beginning of October 2020. The maximum number of cases was reached in mid-November 2020 (18 November 2020: 5,671 positively tested persons). After that, numbers started to decline.

To counteract the spread of the disease in the community, a number of non-pharmaceutical interventions were undertaken. The intention was to slow down the pandemic by restricting mobility and thus to preserve the capacity of the health systems

(Haug *et al.* 2020). Important measures included (1) limited opening hours (5 am to 10 pm) for restaurants, pubs, and bars (25 September 2020), (2) cancellation of public events, guest registration, and closure of clubhouses (16 October 2020), (3) nighttime curfew, closing of restaurants and hotels (3 November 2020), and (4) stay at home requirements, closing of schools and non-essential businesses (17 November 2020).

The clinical testing provided evidence for a steady increase of case numbers in autumn 2020. The wastewater monitoring results seem to indicate a different basis for the COVID-19 spread in the monitored communities at that time. We observed a stepwise increase of the total viral load. Rapid increases of viral loads in wastewater occurred on 24 September 2020, 16 October 2020, and 2 February 2020 (see also Supplementary Material, Figure S4). Interestingly, the three rises coincided with the implementation of reinforced measures. In each case, the stipulated restrictions were communicated to the general public several days before coming into force. Therefore, we speculate that a considerable part of the population used the days between announcement and reinforcement to cultivate social contacts, and this counteracted the effect of the measures taken.

Comparison of the graphs representing the wastewater and clinical testing results (Figure 3(b)) further indicated that already the lockdown measures from 3 November 2020 would have been effective for inducing a decline of positive cases. As the corresponding measures mainly targeted the gastronomy, we speculate that this sector represented an important driver for viral circulation in the monitored communities.

Monitoring results from 43 regions obtained between 15 November 2020 and 31 July 2021

The nationwide monitoring started at the peak of the second wave of COVID-19 infections in Tyrol (Figure 3(c)). The maximum number of COVID-19 cases in Tyrol were reported on 17 November 2020 ($n = 8{,}589$). Owing to the implemented lockdown measures, there was a steep decline in the number of cases until 20 December 2020 ($N = 1{,}793$). Owing to a partial withdrawal of measures before and during Christmas holidays as well as reduced acceptance, a slower decrease of cases was observed in the following weeks. After reaching a minimum number of cases on 17 February 2021 ($N = 895$), Tyrol entered into the third wave of infections. The peak number of infections in spring 2021 was observed on 1 April 2021 ($N = 2{,}845$). The lowest number of positive cases in 2021 were observed at the beginning of July ($N = 35$).

For the total viral load in Tyrolean wastewaters, similar trends were observed, which again substantiates that wastewater monitoring provides meaningful information on disease prevalence. In our opinion, wastewater monitoring and diagnostic testing complement each other. Wastewater analysis is particularly useful in verifying community-wide trends observed with clinical testing. Furthermore, the approach might be helpful in forecasting a potential rise and fall of case numbers. With wastewater monitoring trend reversals might be detected earlier than with clinical testing. For instance, the viral loads reached their minimum in February and June 2021 several days earlier than the clinical case numbers did (Figure 3(c)).

Another important feature of the wastewater monitoring is its ability to provide evidence for the absence of COVID-19 (Figure 3(d)). At the beginning of May 2021, the number of positively tested WWTPs started to decline. At the beginning of July 2021, SARS-CoV-2 was detected in <20 WWTPs. In all other regions, SARS-CoV-2 was not detectable in the collected wastewater samples. During July 2021, however, a turnaround was observed. Viral loads and the number of positively tested WWTPs started to rise again. Eventually, this is the beginning of the fourth pandemic wave.

CONCLUSIONS

In this report, we summarized concepts, structure, and results of the Tyrolean wastewater monitoring program. The development of the program was started after the first phase of the COVID-19 pandemic in Tyrol (February–May 2020) to expand the available surveillance and response capacities. The Tyrolean health and water sector jointly worked together in the conception, development, and operation of this program. The monitoring in Innsbruck started in June 2020. Eight more WWTPs were included in September 2020. The monitoring program involving 43 WWTPs was put in operation in mid-November 2020. By the end of July 2021, SARS-CoV-2 concentrations were determined in 5,270 Tyrolean wastewater samples.

The 24-h composite samples were collected at the influents of the monitored WWTPs 2–7 times a week. The samples were shipped at 4 °C by a courier service to the analytical laboratory in Innsbruck within 5 h upon availability. SARS-CoV-2 quantification was accomplished with a validated workflow, involving PEG-NaCl-based precipitation, automated lysis, and RNA extraction, as well as RT-qPCR of the N1 target. QCs included an IPC, quantification of PMMoV and positive control samples, as well as processing and quantification of blanks. The obtained results were submitted electronically to the Tyrolean wastewater monitoring database. Typically, results were available for review by health authorities in <36 h after finishing the

sampling process. The wastewater data together with the clinical test data served as an input for the Tyrolean wastewater monitoring dashboard. With this dashboard, the available temporal and geographic information was visualized to enable health authorities monitoring the pandemic situation in Tyrol as a whole and in each region individually.

The existing data support the important role of SARS-CoV-2 wastewater monitoring in complementing clinical disease surveillance. The monitoring program represents an early-warning system, provides independent confirmation of temporal trends in COVID-19 prevalence, enables the assessment of the effectiveness of measures, alerts about bursts of disease activity, and finally provides evidence for the absence of COVID-19.

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AUTHOR CONTRIBUTIONS

B.D. and H.N.: methodology, validation, investigation, writing – original draft, visualization, writing – review and editing. M.S.: methodology, validation, investigation, writing – review and editing, supervision, project administration. S.W. and M.K.: conceptualization, data curation, writing – review and editing, project administration. C.L.-F., W.P., and S.F.: methodology, resources, writing – review and editing. B.P.: methodology, formal analysis, data curation, writing – review and editing. A.H.: conceptualization, data curation, writing – review and editing, project administration. H.O.: conceptualization, methodology, validation, formal analysis, data curation, writing – original draft, writing – review and editing, visualization, supervision, project administration, funding acquisition.

DATA AVAILABILITY STATEMENT

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

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