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Article

Wastewater Reveals the Spatiotemporal Spread of SARS-CoV-2 in the Canton of Ticino (Switzerland) during the Onset of the COVID-19 Pandemic

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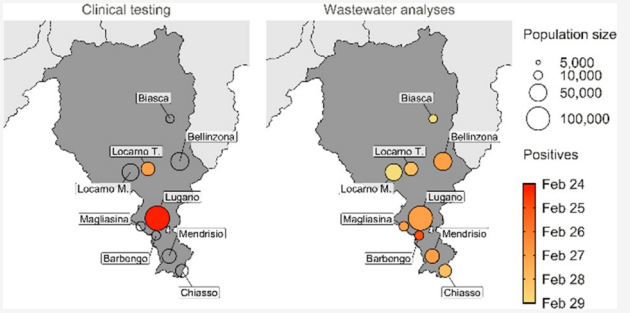
ABSTRACT: Wastewater-based epidemiology (WBE) has emerged as an effective tool for monitoring SARS-CoV-2 dynamics during the COVID-19 pandemic. Here, we add a spatial component to WBE and use it to investigate SARS-CoV-2 spread in the canton of Ticino during the onset of the pandemic in Switzerland (end of February 2020 to beginning of March 2020). Ticino is located at the border to Northern Italy, where a large COVID-19 outbreak occurred in February 2020. Not surprisingly, Ticino was the site of the first clinically confirmed COVID-19 case in Switzerland. We retrospectively analyzed daily influent samples from nine wastewater treatment plants in Ticino that jointly cover an area of 20 km × 60 km and 351,000 people (>99% of the population). Our result is a fine-grained view of the spatiotemporal evolution of the COVID-19 pandemic in this canton. The wastewater analysis revealed that by February 29, 2020, SARS-CoV-2 had already spread to all catchments. At the same time, only four individual cases had been clinically confirmed across the region served by the treatment plants investigated. Our results demonstrate that WBE could serve as a versatile tool to monitor the introduction and spread of an infectious agent on a regional scale. To fully exploit its utility, WBE should be implemented in real time and become an integral part of future disease surveillance efforts.



KEYWORDS: *COVID-19, sewage surveillance, first wave, spatiotemporal spread*

INTRODUCTION

The earliest European cases of COVID-19 were confirmed in France on January 24, 2020. Sequencing data suggested that the predominant lineage of SARS-CoV-2 that was spreading throughout the European continent had most likely originated in Italy.[1] Here, two imported cases from Wuhan were documented on January 30, 2020, whereas the first recognized autochthonous case was reported on February 21, 2020, in the town of Codogno (Lombardy), at 60 km southeast of Milan.[2] Soon, it became evident that sustained local transmission was taking place and eventually resulted in the then-largest COVID-19 outbreak outside China.

In Switzerland, the first confirmed case of COVID-19 was reported on February 25, 2020, in Ticino, a Swiss canton located at the border with Northern Italy, at just 100 km from the epicenter of the main Italian outbreak. This first positive case was detected in an adult who had attended an event in the Lombardy area less than 10 days before the test.[3] The Ticino region is characterized by a high influx of cross-border commuters, with around 70,000 workers traveling between both countries on a daily basis. Thus, the probability of virus diffusion from Italy to Switzerland was high. A study based on viral genome sequencing including geographical phylodynamic models supports this assumption.[1] The extent of this diffusion, however, remains unclear as clinical testing for SARS-CoV-2 was rare at the time.

As SARS-CoV-2 clinical cases were spreading across the world, wastewater-based epidemiology (WBE) soon emerged as a useful tool for monitoring the occurrence and dynamics of SARS-CoV-2 at a population level.[4−9] In contrast to clinical tests, which are biased toward symptomatic people, wastewater captures viruses shed by a large population in the sewershed, including asymptomatic, presymptomatic, and symptomatic cases. Since the onset of the pandemic, WBE has been implemented for real-time monitoring of the COVID-19 pandemic in many countries around the world.[10]

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In addition to real-time monitoring, retrospective analysis of biobanked wastewater samples offers an opportunity to assess historical viral spread across time and space. For example, two retrospective studies found that SARS-CoV-2 was already detectable in wastewater well before the first cases were officially confirmed. Specifically, La Rosa et al.[2] reported SARS-CoV-2 positive samples collected on December 18, 2019, in Italy, whereas Chavarria-Miró et al.[11] reported the presence of the virus in wastewater samples collected on January 15, 2020, in Spain. In both of these European countries, the first imported clinical cases were reported weeks later, on January 28 (Italy) and 30 (Spain), 2020.[12] Retrospective WBE thus has the potential to shed light on the origins and onset of viral epidemics and pandemics.

Here, we retrospectively analyzed wastewater samples collected during the onset of the first wave of the COVID-19 pandemic from different wastewater treatment plants (WWTPs) in the canton of Ticino, Switzerland. Our results suggest that SARS-CoV-2 was already widespread in this canton by February 29, 2020, whereas only a few localized clinical cases had been reported at this time, and a national lockdown was still two weeks away. Overall, this study demonstrates that WBE is a useful tool for tracking the entry and spatiotemporal spread of a pandemic in a given region. Our data adds to the existing body of evidence that supports the use of wastewater for better informed decision making by public health authorities. It furthermore demonstrates that systematic biobanking of wastewater samples may allow improved retrospective understanding of emergence and spread of novel infectious diseases in communities.

MATERIAL AND METHODS

Sewage Sample Collection and Storage. Here, 24-h composite influent samples were collected daily during the last week of February until March 8, 2020, from nine WWTPs in the Ticino region of Switzerland (Figure 1): Chiasso (population connected: 24,156); Mendrisio (population connected: 31,173); Scairolo (population connected: 11,178); Magliasina (population connected: 12,265); Lugano (population connected: 111,715); Locarno Ticino (population connected: 27,813); Bellinzona (population connected: 54,926); Biasca (population connected: 9767); and Locarno Maggia (population connected: 47,462). After collection, the wastewater samples were stored at −20 °C for up to 12 months to allow for the development of sufficiently sensitive sample concentration and virus detection assays. Sampling sites included in this study are shown in Figure 1.

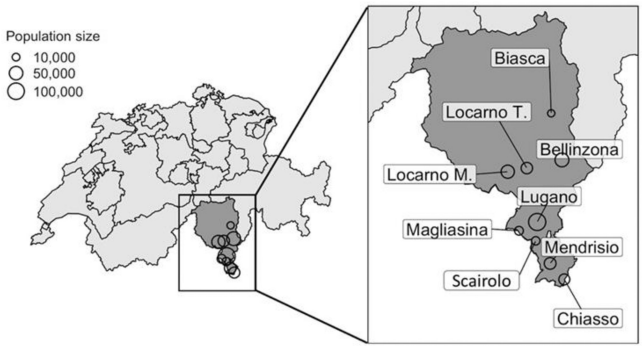

Figure 1. Location of the nine wastewater treatment plants investigated in the canton of Ticino (dark gray) of Switzerland (light gray). The sizes of the circles in the enlarged panel are proportional to the population in the catchment.

Sample Concentration and Nucleic Acid Extraction. Collected wastewater samples were processed in sequence, starting from the earliest sampling date available for each WWTP. Sample processing for each WWTP was continued until a positive signal of SARS-CoV-2 was obtained at least once by each detection method used (Reverse transcription-quantitative PCR (RT-qPCR) and nested reverse transcription PCR (nRT-PCR); see below).

Prior to processing, samples were thawed at room temperature overnight. For each sample, two biological replicates of 70 mL were concentrated. Specifically, samples were centrifuged for 30 min at 10,000*g* to pellet large particles and bacteria. Except for the slightly larger sample volume, viral concentration and nucleic acid extraction were then performed as described in detail in Fernandez-Cassi et al.[7] Briefly, wastewater supernatant containing viral particles was carefully collected while avoiding the inclusion of solid particles and was concentrated using centrifugal filter units with a cutoff size of 100 kDa (Centricon Plus-70; Millipore UFC701008). Nucleic acids were extracted in their entirety using a Qiagen RNA Viral Mini Kit (cat no. 22906, Qiagen, Valencia, CA, USA) and were eluted in 60 μL of AVE buffer. PCR inhibitors were removed using a Zymo OneStep PCR Inhibitor Removal column (Zymo Research, cat n° D6030). Our previous work has shown that this sample processing pipeline leads to minimal PCR inhibition and yields virus recoveries comparable to those found by others.[7] Extracted nucleic acids were stored for up to 2 months at −20 °C until further processing. Samples from Lugano were processed and analyzed by RT-qPCR as part of a previous study.[7] Extracted RNA from Lugano was kept at −20 °C and was reanalysed by nRT-PCR 8 months later as described below.

RT-qPCR Analysis of N1 and N2 Targets. To detect the presence of SARS-CoV-2 RNA, RNA extracts were analyzed by RT-qPCR with CDC N1 and N2 assays.[13] RT-qPCR amplifications were performed using the RNA UltraSense One-Step Quantitative RT-PCR System (Invitrogen Cat. No. 11732-927) on a Mic qPCR Cycler (Bio Molecular Systems) in 25 μL reactions containing 5 μL of RNA extract, 0.5 μM of the forward and reverse primers, and 0.125 μM of a probe. A summary of the primers and probes and thermocycling conditions are reported in Table S1. Bovine serum albumin (2 mg/mL) was added to each reaction to reduce inhibition. Two replicate analyses were performed for each biological sample, and each sample was analyzed by both undiluted and in 10-fold dilution with RNase-free water. A 2019-nCoV_N plasmid (Integrated DNA Technologies, Coralville, IA, USA) was used as the positive control. Cq values were determined using the micPCR software (v2; Bio Molecular Systems). Samples with a Cq ≤ 40 were considered presumptive positives for SARS-CoV-2.

We emphasize that RT-qPCR analysis was solely used for virus detection but not quantification. Previous work showed that quantification of SARS-CoV-2 RNA by the approach used herein required Cq values of less than 35 (N1) or less than 36.5 (N2),[7] levels that were rarely reached in this study. We therefore interpreted the data only as detectable or not detectable, and we did not convert Cq signals to RNA concentrations. RT-qPCR assays were nevertheless conducted following MIQE guidelines[14] where applicable (Table S2). The

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Table 1. RT-qPCR (N1, N2) and nRT-PCR (Spike Gene; S) Results, along with Number of New Cases (by Date of Specimen Collection) in Each Catchment[a]

	Chiasso				Mendrisio				Scairolo			
	population connected: 24′156				population connected: 31′173				population connected: 11′178			
	cases	N1	N2	S	cases	N1	N2	S	cases	N1	N2	S
24 Feb												
25 Feb										X	X	√
26 Feb										√	√	√
27 Feb						X	X	√		X	X	√
28 Feb		√	√	√		√	X	√		X	X	√
29 Feb		√	X	√		√	X	√		X	X	X
1 Mar		n.a.	n.a.	n.a.						√	X	√
2 Mar		n.a.	n.a.	n.a.		√	X	√		n.a.	n.a.	n.a.
3 Mar		n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.
4 Mar		n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.
5 Mar	4	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.
6 Mar	1	n.a.	n.a.	n.a.	2	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.
7 Mar	5	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.
8 Mar		n.a.	n.a.	n.a.		n.a.	n.a.	n.a.		n.a.	n.a.	n.a.
	Magliasina				Lugano				Locarno Ticino			
	population connected: 12′265				population connected: 111′715				population connected: 27′813			
	cases	N1	N2	S	cases	N1	N2	S	cases	N1	N2	S
24 Feb					1							
25 Feb												
26 Feb						X	X	X				
27 Feb		X	X	√		X	X	√	2			
28 Feb		X	X	√		√	X	√		√	√	√
29 Feb		X	X	√	1					X	X	√
1 Mar		√	X	√	1	X	X	√		X	X	X
2 Mar					2	√	X	√		X	X	X
3 Mar		√	√	X	6	n.a.	n.a.	n.a.		n.a.	n.a.	n.a.
4 Mar		n.a.	n.a.	n.a.	3	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.
5 Mar		n.a.	n.a.	n.a.	5	X	√	√		n.a.	n.a.	n.a.
6 Mar		n.a.	n.a.	n.a.	3	√	√	X		n.a.	n.a.	n.a.
7 Mar		n.a.	n.a.	n.a.	3	√	X	X	1	n.a.	n.a.	n.a.
8 Mar	1	n.a.	n.a.	n.a.	2	√	X	√		n.a.	n.a.	n.a.
	Bellinzona				Biasca				Locarno Maggia			
	population connected: 54′926				population connected: 9′767				population connected: 47′462			
	cases	N1	N2	S	cases	N1	N2	S	cases	N1	N2	S
24 Feb												
25 Feb												
26 Feb												
27 Feb		X	X	√								
28 Feb		X	X	X		X	X	X		X	X	X
29 Feb		X	X	X		√	√	X		X	X	√
1 Mar		X	X	X		X	X	√		X	X	√
2 Mar	1	X	X	X		X	X	√		X	X	√
3 Mar	1	X	X	√		n.a.	n.a.	n.a.		X	X	X
4 Mar						n.a.	n.a.	n.a.		√	X	X
5 Mar	1	√	√	√		n.a.	n.a.	n.a.				
6 Mar	1	√	X	X		n.a.	n.a.	n.a.		√	√	√
7 Mar	2	√	X	√		n.a.	n.a.	n.a.		√	√	X
8 Mar		n.a.	n.a.	n.a.	2	√	X	√	1	√	X	X

[a]If SARS-CoV-2 RNA was detected in at least one of two replicate samples, the sample is considered as positive, and the corresponding cell is marked with a check mark. Negative samples are marked with an X. Blank fields indicate dates for which samples were not available; n.a. indicates samples that were not analyzed. Cq values for each replicate and sequence accession numbers are given in Table S3.

LOD values for the N1 and N2 assays were calculated as described in Fernandez-Cassi et al.[7] and corresponded to 4.2 gc/mL wastewater and 2.6 gc/mL wastewater for the SARS-CoV-2 N1 and N2 genes, respectively.

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Nested RT-PCR (nRT-PCR) and Sanger Sequencing of the Spike Gene. As second approach for SARS-CoV-2 detection, we used an nRT-PCR assay targeting a small region of the spike gene, using the 329 amplicon base pair nRT-PCR spike gene assay described by La Rosa et al.[15] (Figure S1). Here, 11 μL of RNA extract was used to generate cDNA with the SuperScript IV First-Strand Synthesis System (Life Technologies), using random hexamers. Reverse transcription was performed at 50 °C for 30 min, followed by inactivation at 80 °C for 10 min. A summary of primers and thermocycling conditions used are reported in Table S1. PCRs were performed with Phusion Hot Start II DNA Polymerase with a High Fidelity (HF) buffer (Thermo Fisher Scientific, Waltham, MA, USA). For the first PCR (PCR ID 972; Table S1 and Figure S1), 8 μL of cDNA, 1 μL of primer 2319 (10 μM), and 1 μL of primer 2320 (10 μM) in a final volume of 50 μL were used. For the nested PCR (PCR ID 973; Table S1 and Figure S1), 4 μL of the product of the first PCR, 1 μL of primer 2321 (10 μM), and 1 μL of primer 2322 (10 μM) in a final volume of 50 μL were used.

PCR products were visualized by gel electrophoresis (agarose gel 1.7%). Amplification bands with the expected band size (328 bp) were excised and purified from the agarose gel using a NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagell) following manufacturer instructions. Each amplicon was Sanger sequenced on both strands (Fasteris, Geneva CH), using the nested primers ID 973 (Table S1).

Controls to Detect Contamination. Samples from each WWTP were processed independently on separate days to avoid cross-contamination. All Mastermix preparations, sample loading, retrotranscriptase reactions, and PCR runs were performed in separate areas to avoid potential sources of contamination. Each nucleic acid extraction batch included a negative extraction control (NEC) using molecular grade water (Invitrogen). The NEC was used as a negative control in subsequent RT-PCR reactions. Additionally, each PCR run included a no-template control (molecular grade water; Invitrogen) to rule out contamination during PCR preparation. All controls yielded negative results.

Bioinformatic Analysis and Nextstrain SARS-CoV-2 Clade Assignment. Raw FASTAq files with primers PCR ID 972 and 973 removed were queried for sequence similarity using BLASTN against the NCBI database to confirm that positive amplicons detected by nRT-PCR belonged to SARS-CoV-2. Subsequently, all FASTAq files were imported to Geneious (v 11.1.5). Files were trimmed on both 5′ and 3′ extremes by applying a phred cutoff score of Q20. Quality trimmed forward and reverse reads from the same sample were assembled when possible, and the consensus sequence was manually checked for nucleotide incongruences between both strands. Quality curated consensus sequences were submitted to GenBank under the accession numbers from OM501592 to OM501602. Finally, curated consensus sequences and forward or reverse chains with good quality were used to assign a SARS-CoV-2 clade by using the Nextclade web tool v1.7.1 (https://clades.nextstrain.org/).[16]

Clinical Case Data. The confirmed daily case numbers (organized by date of specimen collection) for each WWTP catchment were kindly provided by the Swiss Federal Office of Public Health.

RESULTS AND DISCUSSION

Detection of SARS-CoV-2 RNA by RT-qPCR. SARS-CoV-2 was detected by RT-qPCR in samples from all nine WWTPs (Table 1). For several of the RT-qPCR positive samples, Cq values were close to 40 (Table S3), suggesting that SARS-CoV-2 RNA concentrations in the samples were low. On the other hand, several samples also exhibited Cq values less than 36, particularly for N1 during the first week of March. These lower Cq values are surprising, given that the wastewater had been stored in the freezer for a year, with likely degradation of RNA prior to analysis.[7,17] The N1 RT-qPCR assay exhibited a higher sensitivity toward SARS-CoV-2 genome fragments compared to the N2 assay, and thus yielded a greater number of positive signals (37 and 10 positive samples for N1 and N2, respectively). Furthermore, for each sample positive in N1, the N2 assay typically exhibited a higher Cq value if positive (Table S3). These results are in agreement with our previous study where a high sensitivity for the N1 RT-qPCR assay was observed.[7] Other studies have also identified a better sensitivity for N1 assay compared to N2.[4,18]

The first positive signal was observed on February 26, 2020, in the Scariolo WWTP. The WWTP with the latest detection was Bellinzona, where a positive RT-qPCR signal was only observed on March 5, 2020. In two of the nine treatment plants (Lugano and Locarno Ticino), clinical reporting of SARS-CoV-2 occurred before the start of the wastewater sampling campaign, rendering a direct comparison of the timeliness of the two monitoring strategies impossible. In these two plants, clinical cases proceeded wastewater detection by 4 (Lugano) and 1 (Locarno Ticino) days. In six of the other seven WWTP catchments, a positive RT-qPCR signal in wastewater preceded the first clinical case confirmation with a lead time of 3 to 8 days. In the other plant (Bellinzona), clinical case confirmation occurred with an advance of 3 days. Overall, these findings indicate that SARS-CoV-2 had already spread through much of the canton by late February.

Detection of SARS-CoV-2 RNA by nRT-PCR. Because the concentrations of SARS-CoV-2 RNA in most samples were low, and the sensitivity of the RT-qPCR assay is limited,[7] samples were additionally analyzed by a nested RT-PCR approach targeting the spike gene. SARS-CoV-2 RNA was first detected by nRT-PCR in a sample of the Scairolo WWTP collected on February 25, 2020 (Table 1 and Figure S2). The spike gene RNA was furthermore detected in at least one sample of each WWTPs by March 1, 2020. In most WWTPs, the first detection of SARS-CoV-2 by nRT-PCR coincided with, or preceded, the detection by RT-qPCR, confirming that nRT-PCR is a more sensitive method compared to RT-qPCR to capture the onset of the pandemic. Only in one catchment (Biasca) did RTq-PCR yield the earliest positive result, by 1 day. Overall, the nRT-PCR analysis confirms that SARS-CoV-2 was already widely circulating in Ticino in late February.

Confirmation of SARS-CoV-2 Presence and Clade Assignment by Sequencing. Positive bands from the nRT-PCR electrophoresis gels presenting the expected band size (382bp) (Figure S2) were additionally Sanger sequenced to unambiguously confirm the presence of SARS-CoV-2. Of the sequenced bands, 11 contained at least a forward, reverse, or a consensus sequence of sufficient quality to confirm the presence of SARS-CoV-2 (Table S3). These bands represent samples from all but two WWTPs (Lugano and Locarno Ticino).

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To explore the identity of SARS-CoV-2 strain(s) circulating at that time, we performed an analysis of the curated Sanger sequences of the spike locus using Nextclade. The Nextclade tool assigned most of the consensus sequences to clade 19A, the same clade as the original Wuhan strain. This supports earlier findings based on sequences from clinical samples that the strain spreading in Europe at the beginning of 2020 was the same as the one isolated in Wuhan in December 2019.[1]

In conjunction with Sanger sequencing, nRT-PCR provided high confidence in positive signals, even if SARS-CoV-2 RNA concentrations were low.

Comparison of Spatiotemporal Spread of SARS-CoV-2 by Clinical and Wastewater Surveillance. Finally, we compared the spatiotemporal spread of SARS-CoV-2 based on WBE and clinical surveillance. Figure 2A shows the earliest date of SARS-CoV-2 detection by any of the three assays used (N1, N2, or S; see Table 1) in each WWTP. As is evident from this figure, SARS-CoV-2 spread rapidly throughout the canton, with detection of viral RNA in all WWTPs by February 29, 2020. Notably, SARS-CoV-2 was often detected in the earliest wastewater sample available, whereas nondetects were rare. We can therefore not exclude that the virus had spread even earlier than revealed in this work, which was limited by the onset of our sampling campaign.

Even when we apply more stringent criteria for SARS-CoV-2 confirmation (positive signals in two out of three assays on the same day; Table 1), it is evident that the pandemic had already widely progressed throughout the canton by the end of February (Figure S3). Wastewater from six WWTPs were found to be positive for SARS-CoV-2 by February 29, ranging from the north (Biasca) to the south (Chiasso), and including both large urban (Lugano) and small rural catchment areas (Biasca) WWTPs.

A different picture emerges when assessing disease spread based on clinical cases (Figure 2B). By the time the virus was already detected in all WWTPs (Figure 2A), clinical cases confirmations remained localized, with only two catchments (Lugano and Locarno Ticino) reporting cases by February 29. Combined, these two catchments confirmed a total of only four cases. On the basis of clinical data alone, a complete diffusion of the virus throughout the canton would only become evident a full week later, with Locarno Maggia and Magliasina reporting their first cases on March 8 (Table 1). When using only clinical case data, the spread of COVID-19 through Ticino is thus substantially underestimated.

CONCLUSIONS

WBE was found to be a useful tool to reveal the spread of SARS-CoV-2 in the canton of Ticino during the onset of the COVID-19 pandemic. Wastewater data point to a complete diffusion of COVID-19 throughout the canton as early as February 29, 2020, when clinical data still indicated small, localized outbreaks only. The virus had thus already reached all areas of the canton 2.5 weeks before a national lockdown was implemented on March 17. Had WBE data been available in real time, would public health authorities have mandated an earlier lockdown? Would this have achieved better protection of vulnerable populations and ultimately a lower death toll? We cannot answer this question for past events. Going forward, however, we suggest that WBE be included in the national tool set for disease surveillance to allow for prompt public health action. This requires that investments in wastewater-based surveillance be coincident to, rather than substantially later

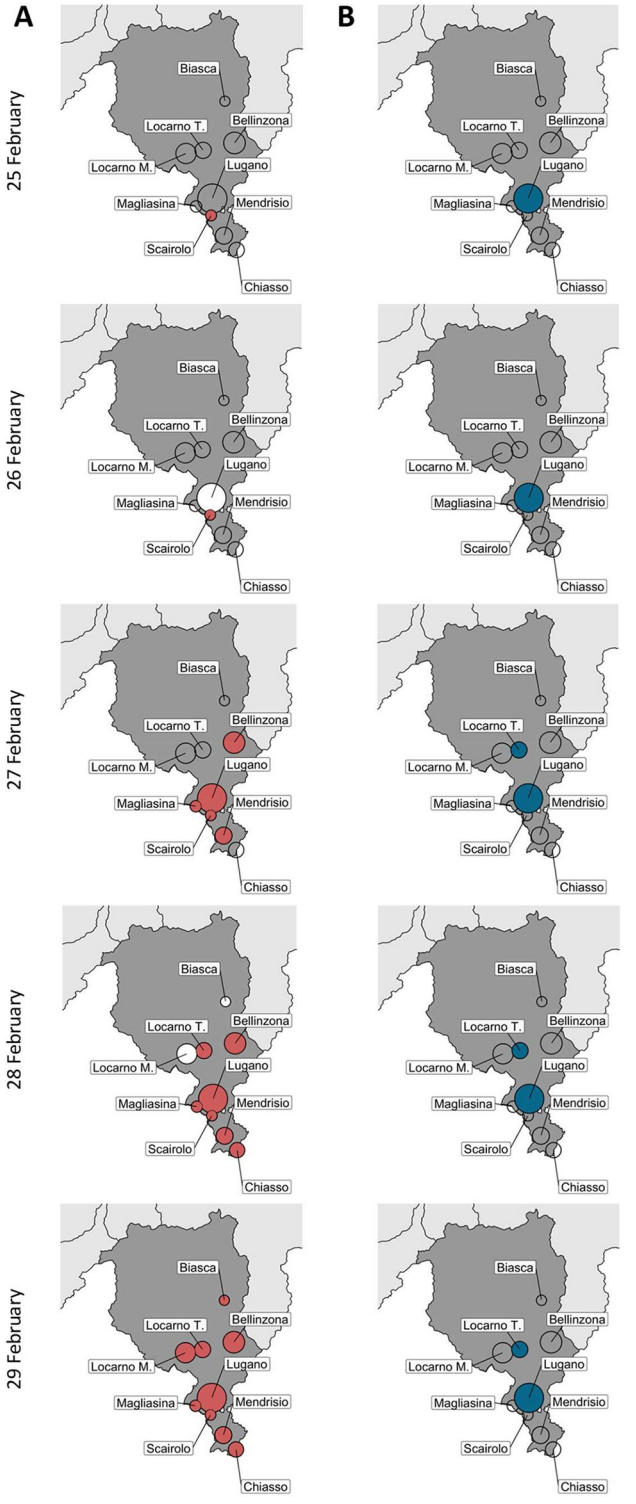

Figure 2. Spatiotemporal spread of SARS-CoV-2 in Ticino during late February 2020. The circle sizes are proportional to the populations in the WWTP catchment (Figure 1). (A) Spread determined by wastewater analysis. Circles are colored in red as soon as SARS-CoV-2 is first detected in wastewater by any of the three detection assays used (N1, N2, or S). White circles represent nondetects. Empty circles represent WWTPs for which samples were not available on the specified date. (B) Spread determined by clinical surveillance. Circles turn blue as soon as the first clinical case is confirmed in a given WWTP catchment. Note that the first case in Lugano stems from February 24 (Table 1), which is not included in this image.

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than, those in clincal-based surveillance. Given the rapid advancement in WBE methodology during the COVID-19 pandemic, researchers globally are now well prepared to start monitoring wastewater at short notice if given the necessary support.

WBE is particularly valuable during early stages, or even prior to, an outbreak or pandemic, when clinical testing is not yet implemented or widespread. Furthermore, WBE can capture pathogens that cause subclinical cases or spread by asymptomatic carriers and circulate unnoticed by clinicians. A coordinated global network of sentinel wastewater treatment plants could aid in the discovery of circulating pathogens with pandemic potential and help ensure future pandemic preparedness. Archived samples from such a network could further be useful in supporting retrospective analyses of the introduction and transmission of diseases into communities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00082.

> Images of agarose gels of nested PCR assays; visualization of the spatiotemporal spread of SARS-CoV-2 in wastewater when applying a stringent criterion for detection; additional details on RT-qPCR and nRT-PCR primers, probes, and protocols, including check list of experimental details; results of all RT-qPCR analyses and accession numbers for sequenced spike gene segments. (PDF)

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Notes

The authors declare no competing financial interest.

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