Wild Type and Variants of Sars-Cov-2 in Parisian Sewage: Dynamics in Raw Water and Fate in Wastewater Treatment Plants

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Abstract

The presence of SARS-CoV-2 RNA has been extensively reported at the influent of wastewater treatment plants (WWTPs) worldwide and its monitoring has been proposed as a potential surveillance tool to early alert of epidemic outbreaks. However, the fate of the SARS-CoV-2 RNA in the treatment process of WWTP has not been widely studied yet; therefore in this study, we aimed to evaluate the efficiency of treatment processes in reducing SARS-CoV-2 RNA levels in wastewater. The treatment process of three WWTPs of the Parisian area in France were monitored on six different weeks over a period of two months (from April 14th to June 9th 2021). SARS-CoV-2 RNA copies were detected using digital polymerase chain reaction (dPCR). Investigation on the presence of variants of concern (Del69-70E484 and L452R) was also performed. Additionally, SARS-CoV-2 RNA loads in the WWTPs influents were expressed as the viral charge per population equivalent and showed a good correlation with French public health indicators (incidence rate). SARS-CoV-2 RNA loads were notably reduced along the water treatment lines of the three WWTPs studied (2.5-3.4 log). Finally, very low SARS-CoV-2 RNA loads were detected in effluents (non-detected in over half of the samples) which indicated that the potential health risk of the release of wastewater effluents to the environment is probably insignificant, in the case of WWTPs enabling an efficient biological removal of nitrogen.

I. Introduction

Surveillance of different markers in wastewater influents, has enabled in the past to characterize emerging chemicals, illicit drug use patterns and food consumption patterns (nitrogen and phosphorus)(Rocher et Azimi 2016; Bressy et al. 2016; Gasperi et al. 2008). Recently, the surveillance of SARS-CoV-2, contributed to the understanding on the disease spread within the communities. In August 2020 (WHO 2020), the World Health Organization confirmed the presence SARS-CoV-2 viral RNA in wastewater influent and in sewage sludge from several cities around the world (Milan, Paris, Murcia, Brisbane, Connecticut, Massachusetts, among others). Monitoring the behavior of SARS-CoV-2 RNA within sewer systems has rapidly appeared to be an interesting tool that provides precious information on the health of entire communities (Ahmed, Angel, et al. 2020; Medema et al. 2020; Wurtzer et al. 2020; Randazzo et al. 2020; S. Kumar et al. 2021). The efficiency of WWTP treatment processes and the potential health risks associated with the release of wastewater effluents containing SARS-CoV-2 into the environment remain to be verified. As the COVID-19 epidemic continues to spread all over the world, new variants of the SARS-CoV-2 virus are being detected. Those variants might be more transmissible or capable of evading immune response or their mutations might suppress diagnostic detection (Wurtzer et al. 2020; Ascoli 2021; Singh et al. 2021).

Digital PCR (dPCR) was rapidly pointed out as a good candidate regarding its sensitivity and quantification accuracy for SARS-Cov-2 monitoring (Staley et al. 2018) (Rački et al. 2014) (Hart et Halden 2020). Cao et al. have indeed shown in 2015 that dPCR exhibited higher precision and reproducibility than qPCR regarding quantification of human-associated fecal indicators in water (Cao, Raith, et Grith 2015). Besides quantification method, several authors have shown that virus concentration methods were also a critical aspect for an accurate and sensible quantification of SARS-CoV-2 in raw wastewater (Ahmed, Bertsch, et al. 2020) (Lu et al. 2020) (Jafferali et al. 2021). A EU patent from the 31th of December 2020 under the application number EP20306715.2 was thus developed by researchers of IAGE to have a reliable process for virus quantification in liquid matrices (comprising sampling, extraction and quantification steps). This method includes various optimization and quality control steps, which are crucial for generating reliable public health information as shown by (Ahmed, Bivins, et al. 2020) and (Berestycki et al. 2021). Furthermore, as variants of SARS-CoV-2 have emerged more recently (Del69-70,E484), L452R, among others), dedicated tools to target and monitor them were developed.
The present work intended to (i) provide an insight of the evolution of the main variants present in the raw waters coming from three different urban catchments and (ii) evaluate the presence of SARS-CoV-2 RNA using the new method of dPCR through the treatment process of three majors Parisian WWTPs.

II. Materials And Methods

1. Parisian WWTPs monitored in this study

The three studied plants are located upstream and downstream of the Parisian conurbation and their flows vary between 50,000 and 600,000 m\(^3\) per day (Figure 1). All of them are operated by the Greater Paris Sanitation authority (SIAAP) in charge of collecting, transporting and treatmening wastewater produced by close to 9 millions of inhabitants. Different technologies are used for the treatment of water and sludge. The SEG plant uses the biofiltration process for water treatment, the Seine Valenton plant (SEV) uses activated sludge treatment for water, and the Seine Morée plant (SEM) uses membrane filtration for water treatment. These WWTPs are designed to efficiently reach the European standards (« Directive 91/271/EEC » 1991; « Directive 2000/60/EC » 2000).

These three plants are fed by raw water coming from urban catchments presenting constrasted characteristics and differ mainly in their design capacity and treatment processes, as briefly described in Table 1.
Table 1
Data on catchment area and population of the three Parisian WWTP included in this study

|                      | SEV                  | SEG                  | SEM                  |
|----------------------|----------------------|----------------------|----------------------|
| Catchment area (km²) | 896                  | 225 (110⁹)           | 68                   |
| Density (inhab/km²)  | 2,760                | 22,531 (20 706 a)    | 4,016                |
| Nominal Population Equivalent (PE) | 2 618 000 | 1 000 000 | 52 300 |
| Treatment layout in nominal conditions | Pre-treatment - Primary settling - Extended aeration activated sludge - Tertiary physicochemical dephosphatation | Pre-treatment - Physico-chemical lamellar settling - 3 stages biofiltration | Pre-treatment - Primary settling - Membrane bioreactor (ultrafiltration) |

Average influent quality parameters in 2020

|                      | SEV                  | SEG                  | SEM                  |
|----------------------|----------------------|----------------------|----------------------|
| Wastewater flow [m³/d] | 514 844             | 254 942             | 16 175               |
| SS (mg/L) [%Removal] | 342 [97%]            | 226 [98%]            | 308 [99%]            |
| BOD₅ (mg O₂/L) [%Removal] | 276 [98%]         | 178 [97%]            | 298 [99%]            |
| TN (mg N/L) [%Removal] | 63 [70%]             | 46 [72%]             | 68 [84%]             |

SS: Suspended Solids, BOD₅: Biochemical oxygen demand – 5 days and TN: Total nitrogen

a Catchment area share with 2 other plants, SAV and SEC

b INSEE 2017 data: "https://www.insee.fr/en/statistiques/4516122"

The catchment area of SEG (located in the northwest area) is characterized by a very high urbanization level; by contrast, SEV and SEM (located in the southeast and east area) have a moderate urbanization level.

2. Sewage sampling and analytical procedures

Samples of raw (influent), settled and treated (effluent) waters were collected continuously (24-h). More precisely, a first campaign occurred from April 14th to April 28th a second campaign from May 26th to June 9th, corresponding to contrasting SARS-CoV-2 incidence levels (French National Health Agency). Sampling was performed using autosampler devices, during dry-weather conditions, from 7 am to 7 am the next day.

- Sample treatment

To detect SARS-CoV-2 RNA in wastewater samples, we relied on a diagnostic method recently developed to detect very low concentrations of SARS-CoV-2 in wastewater samples. This method combines an optimised extraction process (This method has been submitted by IAGE to the European Patent Office the 31st of December 2020 under
the application number EP20306715.2) with a DNA quantification based on dPCR. For each water sample of 1L, one RNA extraction was done.

- Sample analysis

The RT-dPCR reaction was performed following the manufacturer's instructions (QIAGEN, Germany) using the QIAcuity Eight Platform System, 5-plex (Cat. No. 911052), the QIAcuity One-Step Viral RT-PCR Kit (Cat. No. 1123145) and QIAcuity Nanoplate 26K 24-well (Cat. No. 250001). In total, 26000 RT-dPCR reactions by RNA extractions were performed.

The RT-dPCR reaction mixture for variant strain detection was prepared in a pre-plate as follows, depending on Nanoplate type. For Nanoplate 26K reactions, 10 µl of 4x One-Step Viral RT-PCR Master Mix, 0.4 µl of 100x Multiplex Reverse Transcription Mix, 5 µl of the primers/probes mix from the PENTA-CoV wastewater Kit (00283), 2 to 8 µl of RNA extract and RNase-free water were combined to reach a final reaction volume of 40 µl.

To screen for important SARS-CoV-2 variants, particular molecular signatures were developed using a 5-plex assay that takes full advantage of the five detection channels available on the QIAcuity One 5plex, QIAcuity Four and QIAcuity Eight instruments. The 5-plex assay uses one probe to detect SARS-CoV-2 wild type N1 region NC_045512v2, a second probe to detect the Del H69-V70 mutations associated with the so-called Alpha variant: 20I/501Y.V1 (B.1.1.7), a third probe to detect the L452R mutation associated mainly with global variants : VOC (B.1.617.2), Delta Variant, a fourth probe to detect the E484K mutation mainly associated with the Beta and Gamma variants:20H/501Y.V2 (B.1.351); 20J/501Y.V3 (P.1) and a fifth probe targeting PMMoV (NC_003630) serves as an internal control for wastewater RNA extraction. Limit of quantification was 550 UG/L.

Physico-chemical quality parameters were collected from the WWTPs through regulatory monitoring in raw and treated wastewater. These parameters were measured on a daily basis on 24-h composite samples collected with automated samplers. Analyses were performed by SIAAP central laboratory according to the following norms: NF EN 872 for suspendend solids-SS, NF EN ISO 5815-1 for Biochemical oxygen demand and BDO₅ and NF EN ISO 12260 for Total Nitrogen-TN.

### iii. Results And Discussion

#### 1. SARS-CoV-2 RNA concentration dynamic in raw waters

Table 2 summarizes the SARS-CoV-2 RNA loads obtained using RT-dPCR technique in the three studied sewage Parisian WWTPs.
Table 2
SARS-CoV-2 RNA loads in Parisan WWTPs.

| WWTP | Dates 2021       | Incidence rate\(^a\) per 10\(^5\) inhab. | Influent [UG/mL] | Variants [%] | Normalized\(^b\) [10\(^{10}\)UG/10\(^5\)PE] | Settled water [UG/mL] | Effluent [UG/mL] |
|------|------------------|------------------------------------------|------------------|--------------|----------------------------------------------|------------------------|-----------------|
| SEV  | April 14th       | 546                                      | 234              | 61% 5% 0% 34% | 494                                          | 199                    | 0               |
|      | April 21th       | 475                                      | 261              | 67% 28% 6% 0% | 624                                          | 290                    | 8               |
|      | April 28th       | 391                                      | 212              | 58% 4% 34% 4% | 548                                          | 215                    | 95              |
|      | May 26th         | 143                                      | 65               | 17% 17% 0% 67% | 248                                          | 8                      | 18              |
|      | June 2nd         | 104                                      | 47               | 53% 5% 0% 42% | 99                                           | 14                     | 50              |
|      | June 9th         | 66                                       | 11               | 9% 91% 0% 0% | 27                                           | 11                     | 0               |
| SEG  | April 14th       | 458                                      | 343              | 63% 17% 3% 17% | 1175                                         | 183                    | 9               |
|      | April 21th       | 387                                      | 227              | 60% 5% 0% 36% | 592                                          | 113                    | 17              |
|      | April 28th       | 317                                      | 250              | 41% 0% 38% 20% | 693                                          | 275                    | 37              |
|      | May 26th         | 121                                      | 26               | 0% 0% 24% 76% | 81                                           | 7                      | 0               |
|      | June 2nd         | 95                                       | 16               | 50% 50% 0% 0% | 51                                           | 3                      | 0               |
|      | June 9th         | 64                                       | 32               | 63% 0% 12% 25% | 101                                          | 6                      | 0               |

\(^a\) SEV (Department 94), SEG (Departments 75&92) and SEM (Department 93). Open acces data: https://www.data.gouv.fr/fr/datasets/synthese-des-indicateurs-de-suivi-de-lepidemie-covid-19/

\(^b\) Based on the organic biodegradable load having a BOD\(_5\) of 60 g of oxygen per day per inhabitant. See Table S1.
During the first sampling campaign period (from 14 April to 28 April 2021), high concentrations of SARS-CoV-2 RNA were detected in the inuents of Parisian WWTPs, with average values of 236 UG/mL for SEV, 273 UG/mL for SEG and 481 UG/mL for SEM. Lower concentrations were obtained during the second sampling campaign period (from 26 May to 9 June 2021) with average concentrations of 41 UG/mL for SEV, 25 UG/mL for SEG and 50 UG/mL for SEM. The decrease of the SARS-CoV-2 concentration from April to June 2021 is in good agreement with the reports of the French National Health Agency showing a decrease of the incidence rate of the epidemic.

The normalized SARS-CoV-2 RNA concentration (per 100 000 PE) and the incidence rate (per 100 000 hab.) for the departments corresponding to the catchment area of each WWTP are also presented in Table 2. The incidence rate data of departments 75 (Paris), 92 (Hauts-de-France), 93 (Seine- Saint Denis) and 94 (Val-de-Marne) were collected from the open data portal of the French National Health Agency. As broadly discussed in other studies, the correlation between the SARS-CoV-2 RNA concentration in raw wastewater and the incidence rate ($r^2 = 0.61$, Figure S1), confirms that SARS-CoV-2 RNA monitoring in wastewater is a good candidate indicator of the epidemic spread, as recently shown by (Wurtzer et al. 2020; Medema et al. 2020; Ahmed, Angel, et al. 2020; Balboa et al. 2020).

Besides the quantification of SARS-CoV-2, we determined the presence of variants of concern within the population: Alpha (Del69-70), Beta-Gamma (E484), Delta (L452R). The proportion of Del69-70 and E484 variants detected during the first sampling period is in good agreement with open data weekly released by French National Health Agency.
However, the Delta variant L452R was not a variant of concern during the sampling period, therefore, no data related to it were published at that time. It can be noted that the L452R variant was already present on influent samples from April 28th 2021 in the Parisian region; moreover, similar proportions of L452R variant (23 %) were reached among sequenced patients swabs only from June 20th 1-27th 2021, in the Parisian region. These results indicate that SARS-CoV-2 RNA isolated from WWTP influents is also a reliable tool to detect the introduction of variants of concern in the local population weeks before they appear at significant levels in either clinical or screening swab samples.

2. Efficiency of treatment process from WWTPs

SARS-CoV-2 RNA average removal efficiency of the global treatment process as well as of the settling and biological treatment steps between April, 14th and April, 28th are shown in Figure 2.a. The efficiency of WWTPs on reducing the values of main physico-chemical parameters is presented in Figure 2.b.

in Parisian WWTPs, for the first sampling campaign period between April, 14th and April, 28th

The three WWTP allows an efficient removal of particules, organic matter and nitrogen. Indeed, the treatment water lines enables the removal of 97-99% of suspended solids, 96-100% of BOD₅ and 73-85% of total nitrogen, as shown by Figure 2b. In these operating conditions, the average reduction of SARS-CoV-2 RNA was of of 2.4-3.3 log reduction (i.e. 85-97%) as it can be seen in Figure 2.a. Regarding the settling step, low to moderate reductions of SARS-CoV-2 RNA levels were observed (0.01-0.4 log) whereas for the biological treatment step higher removals were obtained (2.4-2.9 log). Low concentrations (<50 UG/ml) of SARS-CoV-2 RNA were detected in outlets of the studied WWTPs, in all cases except on April 28th in SEV (Table 2).

Recent studies on the reduction of SARS-CoV-2 RNA in WWTPs (M. Kumar et al. 2021; Serra-Compte et al. 2021; Hong et al. 2021) have reported lower removal efficiencies (0.5-1.98log) than the present study. However, no fair and deeper comparisons can be established since treatment processes and influent quality differ notably. Some authors (Kitamura et al. 2021; Balboa et al. 2020; M. Kumar et al. 2021; Kocacemi 2020; Li et al. 2021) have detected high concentrations of SARS-CoV-2 RNA in wastewater sludge, and hypothezised that viral material is mainly cumulated the solid fraction which implies that sludge treatment efficiently removes SARS-CoV-2 from wastewater.

By way of comparison with other viruses, previous study on the Parisian WWTPs shows that the overall efficiency of treatment process is equivalent for RNA-F bacteriophages (2.7-3.4 log reduction) (Mailler, Mèche, et Rocher 2021; Rocher et Azimi 2016). Results on the comparable removal of SARS-CoV-2 and RNA-F bacteriophages was recently reported (Serra-Compte et al. 2021; Montier et al. 2021). Further investigations should be performed to validate the use of RNA-F bacteriophages as indicators of SARS-CoV-2 removal along WWTPs.

Iv. Conclusions

The presence of SARS-CoV-2 RNA in raw wastewater (inluent), decanted and treated water (effluent) was quantified using RT-dPCR technique. SARS-CoV-2 RNA loads on influent showed good correlation with the incidence rate of COVID-19 in the Parisian area. The presence of variant L452R (Delta) was detected in samples from the present study from April 18th, a few weeks prior to its inclusion as a variant of concern by the French National Health Agency, which confirms the interest of using sewage analysis as a complementary approach to early detection of epidemics outbreaks.
Finally, the three standard sewage treatment processes (activated sludge, biofiltration or membrane bioreactor) in the Parisian area operated by SIAAP, with a complete treatment of carbon and nitrogen (removals of 96-100% of BOD5 and 73-85% of total nitrogen), are very efficient in eliminating SARS-CoV-2 RNA, with average removals of 2.4-3.5 log.

**Declarations**

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Figures
Figure 1

Location and nominal flow rate of WWTPs, in grey, plants sharing catchment area with the 3 studied in white

Figure 2

Removal of a.) Sars-CoV-2 (n=3) and b.) SS, BOD5 and Total Nitrogen (TN) (n=14) in Parisian WWTPs, for the first sampling campaign period between April, 14th and April, 28th

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