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SHORT RESEARCH AND DISCUSSION ARTICLE

Wild type and variants of SARS-COV-2 in Parisian sewage: presence in raw water and through processes 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 was monitored on six different weeks over a period of 2 months (from April 14 to June 9, 2021). SARS-CoV-2 RNA copies were detected using digital polymerase chain reaction (dPCR). Investigation on the presence of variants of concern (*Del69-70*, *E484K*, and *L452R*) was also performed. Additionally, SARS-CoV-2 RNA loads in the WWTPs influents were expressed as the viral concentration in per population equivalent (PE) 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 reduction). 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 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.

Keywords Wastewater surveillance · SARS-CoV-2 · COVID-19 · Digital PCR · N1 gene · WWTPs

Introduction

Surveillance of different markers in WWTPs influents has enabled in the past to characterize emerging chemicals and illicit drug use patterns and food consumption patterns (nitrogen and phosphorus) (Bressy et al. 2016; Gasperi et al. 2008; Rocher and Azimi 2016). Recently, the surveillance of SARS-CoV-2 has contributed to the understanding on the disease spread within the communities (Ahmed et al. 2020a; Kumar et al. 2021a; Medema et al. 2020; Randazzo et al. 2020; Wurtzer et al. 2020). In August 2020, the World Health Organization confirmed the presence of SARS-CoV-2 viral RNA in wastewater influent and in sewage sludge from several cities around the world (Milan, Paris, Murcia, Brisbane, Connecticut, and Massachusetts, among others) (WHO 2020). Monitoring the behavior of SARS-CoV-2 RNA within sewer systems rapidly appeared to be an interesting tool that provides precious information on the health of entire communities, however, 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 (Giacobbo et al. 2021). As the COVID-19 epidemy continues to spread all over the world, new variants of the SARS-CoV-2 virus are being detected. These variants might be

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more transmissible or capable of evading immune response, or their mutations might suppress diagnostic detection (Ascoli 2021; Singh et al. 2021; Wurtzer et al. 2020).

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 and 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 et al. 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 et al. 2020b; Lu et al. 2020; Jafferali et al. 2021). A European patent from the 31st of December 2020 under the application number EP20306715.2 was thus developed by researchers of Ingénierie et Analyse en Génétique Environnementale (I.A.G.E) 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 et al. (2020c) and Berestycki et al. (2021). Furthermore, as variants of concern SARS-CoV-2 have emerged more recently (Del69-70, E484K, and L452R, among others), dedicated tools to target and monitor the variants were also 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 in three majors Parisian WWTPs.

Materials and methods

Parisian WWTPs monitored in this study

The three studied WWTPs are located upstream and downstream of the Parisian conurbation, and their flows vary between 50,000 and 600,000 m^3 per day (Fig. 1). All of them are operated by the Greater Paris Sanitation Authority (SIAAP) in charge of collecting, transporting, and treating wastewater produced by close to 9 millions of human population. Different technologies are used for the treatment of water and sludge. The Seine Gresillons (SEG) plant uses the biofiltration process for water treatment, the Seine Valenton plant (SEV) uses activated sludge treatment for water, and the Seine Morée

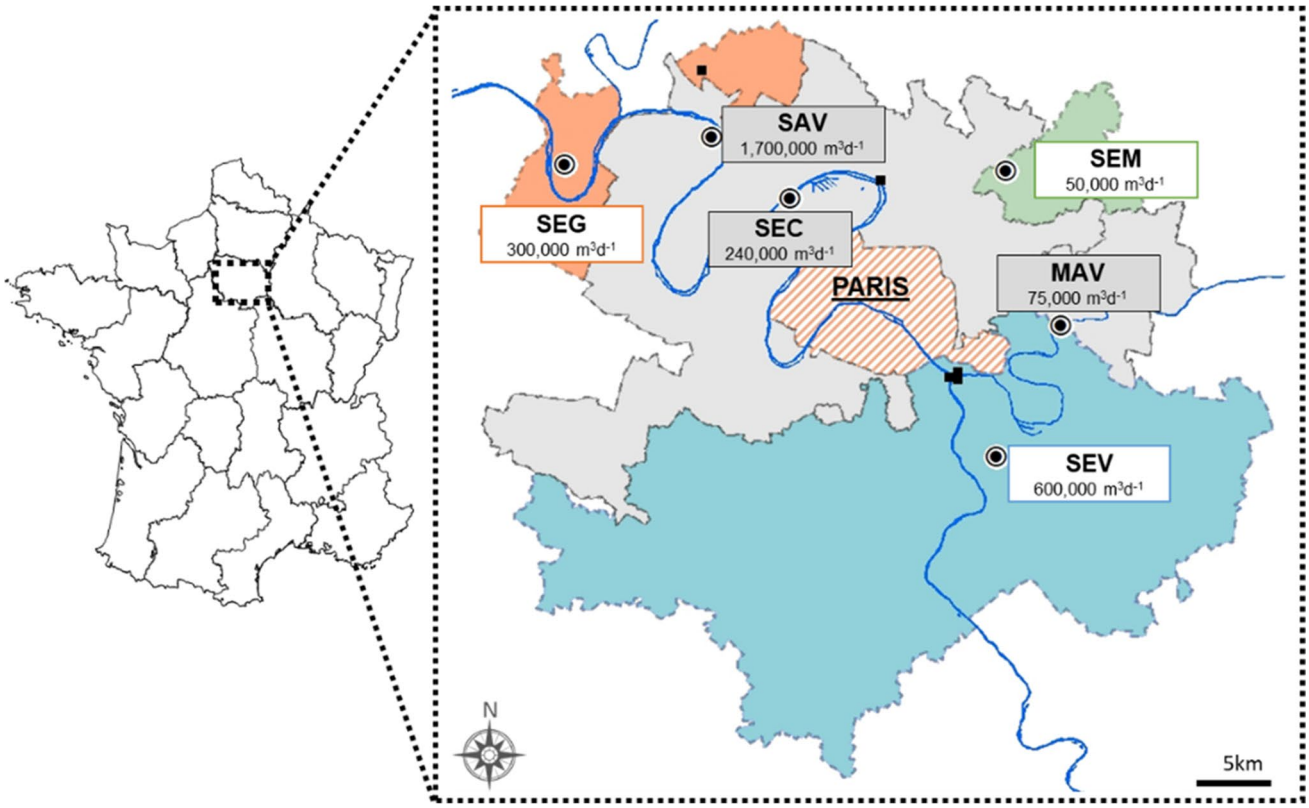

Fig. 1 Location and nominal flow rate of WWTPs

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 contrasted characteristics and differ mainly in their design capacity and treatment processes, as briefly described in Table 1.

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

Data treatment

Log removal values were calculated as follows:

$$\text{Global removal} = \log_{10}\frac{[SARS-CoV-2]_{Inlet}}{[SARS-CoV-2]_{Effluent}}$$

$$\text{Settling removal} = \log_{10}\frac{[SARS-CoV-2]_{Inlet}}{[SARS-CoV-2]_{Decantedwater}}$$

$$\text{Biological treatment removal} = \log_{10}\frac{[SARS-CoV-2]_{Decantedwater}}{[SARS-CoV-2]_{Effluent}}$$

Sewage sampling and analytical procedures

Samples of raw (influent), settled, and treated (effluent) wastewaters were collected using auto-sampler devices, with a flow-paced sampling program during dry-weather conditions, from 7 to 7 am the next day. A first campaign took place from April 14 to April 28, 2021, and a second campaign from May 26 to June 9, 2021, corresponding to contrasting SARS-CoV-2 incidence levels (French National Health Agency).

- Sample treatment

To detect SARS-CoV-2 RNA in wastewater samples, we performed a diagnostic method recently developed to detect very low concentrations of SARS-CoV-2 in wastewater samples. This method combines an ultrafiltration and an optimized extraction process (This method has been submitted by IAGE to the European Patent Office on the 31st of December 2020 under the application number EP20306715.2) with a DNA quantification based on dPCR. For each wastewater sample of 1L, one RNA extraction was done on 30 mL concentrate. The method parameters were optimized using different types of wastewater (from WWTP with nominal capacity from 10 000PE to 400 000PE) spiked with attenuated SARS-CoV-2.

- Sample analysis

The RT-dPCR (RetroTranscriptase-digital PCR) 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 26 K 24-well (Cat. No. 250001). A total of 26,000 RT-dPCR reactions by RNA extractions from 30 mL of concentrated wastewater were performed.

The RT-dPCR reaction mixture for SARS-CoV-2 variant strain detection was prepared in a pre-plate as follows: depending on nanoplate type. For Nanoplate 26 K

Table 1 Data on catchment area and population of the three Parisian WWTP included in this study

	SEV	SEG	SEM
Catchment area (km^2)	896	225 *(110[a])*	68
Density ($inhab/km^2$)[b]	2760	22,531 (*20 706* [a])	4016
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—Physicochemical lamellar settling—3 stages biofiltration	Pre-treatment—Primary settling—Membrane bioreactor (ultrafiltration)
Average influent quality parameters in 2020			
Wastewater flow [m^3/d]	514 844	254 942	16 175
SS (mg/L) *[%Removal]*	342 *[97%]*	226 *[98%]*	308 *[99%]*
BOD_5 (mg O_2/L) *[%Removal]*	276 *[98%]*	178 *[97%]*	298 *[99%]*
TN(mg N/L) *[%Removal]*	63 *[70%]*	46 *[72%]*	68 *[84%]*

SS suspended solids, ***BOD*$_5$** biochemical oxygen demand–5 days, *TN* total nitrogen

[a]Catchment area share with 2 other plants, Seine aval (SAV) and Seine Centre (SEC)

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

reactions, 10 µl of 4 × One-Step Viral RT-PCR Master Mix, 0.4 µl of 100 × Multiplex Reverse Transcription Mix, 5 µl of the primers/probes mix from the PENTA-CoV wastewater Kit (00,283), 4 µl of RNA extract, and RNase-free water were combined to reach a final reaction volume of 40 µl. The mixture was prepared in a pre-plate and then transferred into the 24-well 26 K Nanoplate. The latter was then loaded to the QIAcuity 8 instrument, which is a fully automated system. The workflow included (i) priming and rolling step in order to generate and isolate the chamber partitions; (ii) the amplification step under the following cycling protocol: 50 °C for 40 min for reverse transcription, 95 °C for 2 min for enzyme activation, 95 °C for 5 s for denaturation, and 58 °C for 60 s for annealing/extension for 40 cycles; and (iii) the imaging step was done by reading.

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), 20 J/501Y.V3 (P.1); and a fifth probe targeting *Pepper mild mottle virus* (PMMoV) (NC_003630) that serves as a human density control (see Table S1 in Supplementary information). Limit of quantification (LQ) was 550 GU/L.

Physicochemical 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 suspended solids, SS, NF EN ISO 5815–1 for biochemical oxygen demand and BOD_5, and NF EN ISO 12260 for total nitrogen, TN.

Results and discussion

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.

During the first sampling campaign period (from 14 to 28 April 2021), high concentrations of SARS-CoV-2 RNA were detected in the influents of Parisian WWTPs, with average values of 236 GU/mL for SEV, 273 GU/mL for SEG, and 481 GU/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 GU/mL for SEV, 25 GU/mL for SEG, and 50 GU/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 available as open access data: https://www.data.gouv.fr/fr/datasets/synthese-des-indicateurs-de-suivi-de-lepidemie-covid-19/.

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 ($r^2 = 0.61$, Fig. S1) confirms that SARS-CoV-2 RNA monitoring in wastewater is a good candidate indicator of the epidemic spread, as recently shown by Ahmed et al. (2020a), Balboa et al. (2020), Medema et al. (2020), and Wurtzer 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), and Delta (L452R). The proportion of Alpha and Beta-Gamma variants respectively associated with Del69-70 and E484 mutations detected during the first sampling period is in good agreement with the open data published weekly by the 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 28, 2021, in the Parisian region; moreover, similar proportions of L452R variant (23%) were reached among sequenced patients swabs only from June 20 to 27, 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 (Bar-Or et al. 2021; Buenestado-Serrano et al. 2021; Carcereny et al. 2022; Heijnen et al. 2021; Li et al. 2021; Peterson et al. 2022; Wurtzer et al. 2022; Yaniv et al. 2021a, b).

Table 2 SARS-CoV-2 RNA loads in Parisian WWTPs. Expressed in GU (genome unit)

			SARS-CoV-2 RNA load							
WWTP	Dates 2021	Incidence rate[a] per 10^5 *inhab*	Influent [GU/mL]	*Variants [%]* *A*	*B*	*C*	*D*	Normalized[b] [10^{10}GU/10^5 PE]	DE water[GU/mL]	Effluent [GU/mL]
SEV	April 14th	546	234	61%	*5%*	*0%*	*34%*	494	199	<LQ
	April 21st	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	<LQ
SEG	April 14th	458	343	63%	17%	3%	17%	1175	183	9
	April 21st	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	<LQ
	June 2nd	95	16	50%	50%	0%	0%	51	3	<LQ
	June 9th	64	32	63%	0%	12%	25%	101	6	<LQ
SEM	April 14th	624	858	70%	12%	5%	13%	2035	379	<LQ
	April 21st	538	187	63%	6%	0%	31%	524	NA	18
	April 28th	402	397	65%	5%	28%	2%	559	273	8
	May 26th	150	96	20%	20%	11%	50%	508	31	8
	June 2nd	114	28	25%	0%	0%	75%	48	27	<LQ
	June 9th	81	24	33%	0%	0%	67%	41	6	<LQ

[a]SEV (Department Val-de-Marne, Code: 94), SEG (Departments Paris & Hauts-de-Seine Codes:75&92), and SEM (Department Seine-Saint-Denis, Code: 93). Open access 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

A *= Alpha (Del69-70),* ***B*** *= Beta-Gamma (E484K),* ***C*** *= Delta (L452R),* ***D*** *= Total*

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 14 and April 28 are shown in Fig. 2a. The efficiency of WWTPs on reducing the values of main physicochemical parameters is presented in Fig. 2b.

The three WWTP allows an efficient removal of particles, organic matter, and nitrogen. Indeed, the treatment water processes enables the removal of 97–99% of suspended

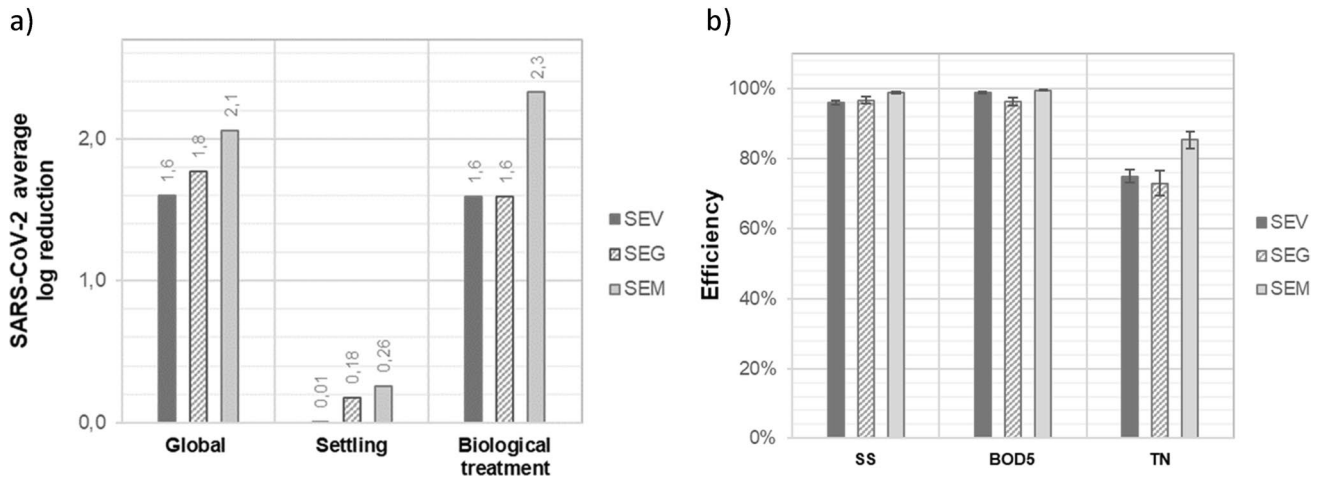

Fig. 2 Removal of **a** SARS-CoV-2 ($n=3$) and **b** SS, BOD_5, and total nitrogen (TN) ($n=14$) in Parisian WWTPs, for the first sampling campaign period between April 14 and April 28, 2021

solids, 96–100% of BOD_5, and 73–85% of total nitrogen, as shown by Fig. 2b. In these operating conditions, the average reduction of SARS-CoV-2 RNA was of 1.60–2.06 log reduction as it can be seen in Fig. 2a. Regarding the settling step, low to moderate reductions of SARS-CoV-2 RNA levels were observed (0.01–0.26 log reduction), whereas for the biological treatment step, higher removals were obtained (1.6–2.3 log reduction). Low concentrations (< 50 GU/ml) of SARS-CoV-2 RNA were detected in outlets of the studied WWTPs, in all cases except on April 28 in SEV (Table 2).

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

Conclusions

The presence of SARS-CoV-2 RNA in raw wastewater (influent) and settled 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 18, 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, are very efficient in eliminating SARS-CoV-2 RNA, with average reductions of 1.60–2.06 log.

By way of perspective, a comparison with other viruses on previous study on the Parisian WWTPs shows that the overall efficiency of treatment process is equivalent for F-specific RNA Bacteriophages (2.7–3.4 log reduction) (Mailler et al. 2021; Rocher and Azimi 2016). Results on the comparable removal of SARS-CoV-2 RNA and F-specific RNA Bacteriophages were recently reported (Montier et al. 2021; Serra-Compte 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.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-022-22665-x.

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Author contribution All authors contributed to the study conception and design. Experimental and data collection were performed by Franz Durandet and Elodie Pichon, while data analysis was done by Melissa Lopez Viveros, Sam Azimi, and Vincent Rocher. The first draft of the manuscript was written by Melissa Lopez Viveros, and all authors commented on previous versions of the manuscript. Sam Azimi and Vincent Rocher were in charge of supervision and validation. Celine Roose-Amsaleg and Ariane Bize greatly contributed to final review and editing. All authors read and approved the final manuscript.

Data availability All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests Not applicable.

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