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## First evidence of SARS-CoV-2 genome detection in zebra mussel (*Dreissena polymorpha*)

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1 Full title: **First evidence of SARS-CoV-2 genome detection in zebra mussel (*Dreissena***  
2 ***polymorpha*).**

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4 Antoine LE GUERNIC <sup>1\*</sup>, Mélissa PALOS LADEIRO<sup>1</sup>, Nicolas BOUDAUD<sup>2</sup>, Julie DO  
5 NASCIMENTO<sup>1</sup>, Christophe GANTZER<sup>3#</sup>, Jean-Christophe INGLARD<sup>4</sup>, Jean-Marie  
6 MOUCHEL<sup>5#</sup>, Cécile POCHET<sup>4</sup>, Laurent MOULIN<sup>6#</sup>, Vincent ROCHER<sup>7</sup>, Prunelle  
7 WALDMAN<sup>5</sup>, Sébastien WURTZER<sup>6#</sup>, Alain GEFFARD<sup>1</sup>.

8

9 1: Université de Reims Champagne-Ardenne, UMR-I02 SEBIO, Moulin de la Housse  
10 BP1039, 51687 Reims, France.

11 2: Food Safety Department, Actalia, Saint-Lô, F-50000, France

12 3: Université de Lorraine, LCPME, Laboratoire de Chimie Physique et Microbiologie pour  
13 l'Environnement, UMR 7564, Institut Jean Barriol, Faculté des Sciences et Technologies,  
14 Vandœuvre-lès-Nancy F-54506, France

15 4: Grand Reims Communauté Urbaine, Direction de l'eau et de l'assainissement, CS 80036  
16 – 51722 Reims Cedex

17 5: Sorbonne Université, CNRS, EPHE, UMR 7619 Metis, E-LTER Zone Atelier Seine, Paris,  
18 F-75005, France

19 6: Eau de Paris. Direction de la Recherche, du Développement et de la Qualité de l'Eau,  
20 94200 Ivry-sur-Seine, France.

21 7: Syndicat Interdépartemental pour l'Assainissement de l'Agglomération Parisienne  
22 (SIAAP), Direction de l'Innovation, 82 avenue Kléber, Colombes, 92700, France

23

24 #: Member of the Obepine's steering committee

25 \* Corresponding author: [antoine.le-guernic@univ-reims.fr](mailto:antoine.le-guernic@univ-reims.fr)

26 **Abstract**

27 The uses of bivalve molluscs in environmental biomonitoring have recently gained  
28 momentum due to their ability to indicate and concentrate human pathogenic  
29 microorganisms. In the context of the health crisis caused by the COVID-19 epidemic, the  
30 objective of this study was to determine if the SARS-CoV-2 ribonucleic acid genome can be  
31 detected in zebra mussels (*Dreissena polymorpha*) exposed to raw and treated urban  
32 wastewaters from two separate plants to support its interest as bioindicator of the SARS-  
33 CoV-2 genome contamination in water. The zebra mussels were exposed to treated  
34 wastewater through caging at the outlet of two plants located in France, as well as to raw  
35 wastewater in controlled conditions. Within their digestive tissues, our results showed that  
36 SARS-CoV-2 genome was detected in zebra mussels, whether in raw and treated  
37 wastewaters. Moreover, the detection of the SARS-CoV-2 genome in such bivalve  
38 molluscans appeared even with low concentrations in raw wastewaters. This is the first  
39 detection of the SARS-CoV-2 genome in the tissues of a sentinel species exposed to raw  
40 and treated urban wastewaters. Despite the need for development for quantitative  
41 approaches, these results support the importance of such invertebrate organisms, especially  
42 zebra mussel, for the active surveillance of pathogenic microorganisms and their indicators in  
43 environmental waters.

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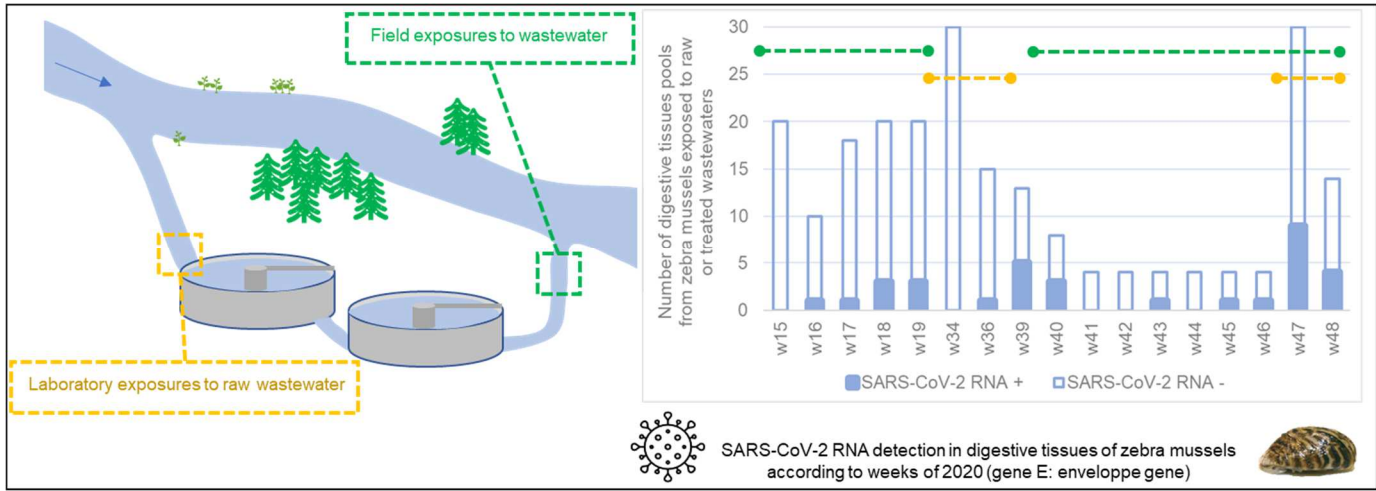
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46 **Keywords:** Bivalve; COVID-19; RT-qPCR; wastewater.

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48 **Graphical abstract**

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## 64 1. Introduction

65 Since several months, the world has been facing a historic viral pandemic. This pandemic  
66 was declared by the World Health Organization (WHO) on March 11, 2020 and has a known  
67 origin in the Wuhan region in China (Langone et al., 2021; WHO, 2020a). Since then, this  
68 disease has spread around the world, causing many victims, and disrupting daily life. This  
69 pandemic (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2  
70 (SARS-CoV-2). The *Coronaviridae* includes seven virus species that infect humans, among  
71 them SARS-CoV and Middle East respiratory syndrome-related coronavirus (MERS-CoV),  
72 which appeared in the 2000s, and therefore SARS-CoV-2, discovered in December 2019 (Lu  
73 et al., 2020; Yang et al., 2020). In France, human infections were focused on two waves of  
74 contamination, from early March 2020 to mid-May 2020 and from mid-October 2020 to the  
75 end of 2020.

76 This virus is mainly transmitted by direct contact with an infected person or indirectly via  
77 infected droplets (Langone et al., 2021; Salinas-Ramos et al., 2021). These droplets are  
78 found in the air or on surfaces whose nature greatly varies the lifespan of the virus (Ren et  
79 al., 2020). However, since the SARS-CoV-2 can infect and replicate both gastrointestinal  
80 glandular epithelial cells and respiratory system, the faecal-oral contamination cannot be  
81 excluded (Amirian, 2020; Heller et al., 2020; Peng et al., 2020). The occurrence of SARS-  
82 CoV-2 genome in the faeces is about 43% of COVID-19 patients and can be detected longer  
83 in digestive tract than in the respiratory one (Amirian, 2020; Kitajima et al., 2020; Zhang et  
84 al., 2021). This virus can therefore reach wastewater via sewages from cities and hospitals.  
85 The presence of SARS-CoV-2 genome has been detected in many raw wastewaters  
86 worldwide, especially during intense epidemiological phases (Balboa et al., 2020; Guerrero-  
87 Latorre et al., 2020; Kitajima et al., 2020; Nemudryi et al., 2020; Randazzo et al., 2020;  
88 Rimoldi et al., 2020; Wang et al., 2020; Wurtzer et al., 2020a). Some of these studies have  
89 reported the presence of SARS-CoV-2 in the raw wastewater but no longer observed it after  
90 biological treatments performed by some wastewater treatment plants (WWTPs) (Balboa et

91 al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020; Singer and Wray, 2020). Nevertheless,  
92 others have detected SARS-CoV-2 genomes in treated wastewaters at wastewater treatment  
93 plants in France and Germany (Westhaus et al., 2021; Wurtzer et al., 2020b). This virus can  
94 also be detected in rivers in many developing countries, with rudimentary or in the absence  
95 of water treatment systems (Guerrero-Latorre et al., 2020), but also in developed countries  
96 (Polo et al., 2021; Rimoldi et al., 2020). However, there is still little knowledge concerning the  
97 survival of infectious SARS-CoV-2 in this aquatic environment. During laboratory experiment,  
98 Desdouits et al. (2021) demonstrated the accumulation of inactivated SARS-CoV-2 genome  
99 in different shellfish tissues of oysters (*Crassostrea gigas*). Wurtzer et al. (2021) have shown  
100 the presence of numerous forms of SARS-CoV-2 genomes in wastewaters including a small  
101 part of infectious and encapsidated particles using RT-qPCR and infectivity assays. Traces  
102 of SARS-CoV-2 genome were assessed in digestive tissues of *Ruditapes philippinarum* and  
103 *R. decussatus* taken from several coastal sites in Spain (Polo et al., 2021).

104 For several years, the use of sentinel species (i.e. bivalve molluscans) of the microbiological  
105 contamination of the environmental waters has intensified. The detection for many pathogens  
106 in filter-feeding and sessile organisms have many advantages and can complement the  
107 direct analyses of water matrices. Bivalve molluscans can indicate bacterial, protozoan or  
108 even viral presence in water (Bighiu et al., 2019; Capizzi-Banas et al., 2021; Kerambrun et  
109 al., 2016; La Rosa et al., 2021). The high filtration capacity of bivalves allows them to filter  
110 large volumes of water (Palos Ladeiro et al., 2018; Polo et al., 2021). These invertebrate  
111 organisms can therefore be exposed to a panel of contaminants potentially more  
112 representative of their environment than that found in a water sample. Indeed, Bighiu et al.  
113 (2019) pointed to bacterial indicators 132 times higher in zebra mussels than in wastewater.  
114 Correlatively, the hepatitis A virus was detected in 16% of bivalve samples (*Mytilus*  
115 *galloprovincialis*, *Solen vagina*, *Venus gallina*, and *Donax trunculus*) against 9% in all water  
116 samples (La Rosa et al., 2021). This has also been demonstrated in zebra mussels for the  
117 Low Pathogenic Avian Influenza virus, but to a lesser extent (Stumpf et al., 2010). This  
118 filtration capacity is supplemented by an interesting bioaccumulation kinetics since the filter-

119 feeding bivalves rapidly accumulate biological pollutants while being able to keep them  
120 several days (or even weeks) after the pressure in the environment has disappeared (Bighiu  
121 et al., 2019; Capizzi-Banas et al., 2021; Stumpf et al., 2010). This allows to have an  
122 integrative approach of water contamination over time. Also, the possibility to perform active  
123 biomonitoring through the caging allows a temporal and spatial assessment of the  
124 contamination, comparing different geographical sites or hydrosystems (Capizzi-Banas et al.,  
125 2021). Among indicator species, the zebra mussel, *Dreissena polymorpha*, has many  
126 advantages for biomonitoring programs under biological pressure (Kraak et al., 1991; Palos  
127 Ladeiro et al., 2014). This species is quite resistant to environmental pressures, is easy to  
128 handle and can be used in laboratory studies or in the field through the caging technique  
129 (Bervoets et al., 2005; Capizzi-Banas et al., 2021; Géba et al., 2020; Kerambrun et al., 2016;  
130 Le Guernic et al., 2020; Palos Ladeiro et al., 2018). The digestive tissues of bivalve molluscs  
131 is generally used to detect the presence of enteric viruses, especially enteroviruses, since it  
132 is the main site of contamination within the bivalve maybe due to specific receptors (Fuentes  
133 et al., 2014; Le Guyader et al., 2006; Lees and TAG, 2010; Suffredini et al., 2020). Desdouits  
134 et al. (2021) have reported accumulation of inactivated SARS-CoV-2 genome in digestive,  
135 mantle, and gill tissues of oysters (*Crassostrea gigas*). This highlighted the potential interest  
136 of using digestive tissues of *D. polymorpha* for detecting SARS-CoV-2 genome in  
137 environmental waters.

138 In this context, the objectives of this study were: i) to know if SARS-CoV-2 genomes can be  
139 detected in zebra mussels at the inlet and / or at the outlet of two French wastewater  
140 treatment plants (WWTPs), namely Reims and center Seine (Ile-de-France public sanitation  
141 service, SIAAP), and ii), to determine if this organism can be used as a bioindicator of water  
142 contamination by this virus in field and laboratory exposures. These objectives are tested  
143 during the two waves of contamination observed in France.

144

## 145 **2. Materials and methods**

146        *2.1. Zebra mussels*

147        Zebra mussels ( $2.98 \pm 0.38$  g;  $2.51 \pm 0.19$  cm) were collected during October and November  
148        of 2019 from Der lake (51290 Giffaumont-Champaubert, France, N 48°33'35"; E 4°45'11")  
149        and brought back to the laboratory, where they were maintained in 1,000 L aerated tanks  
150        with 750 L of municipal drinking water ( $13.46 \pm 1.77^\circ\text{C}$ ; pH  $8.15 \pm 0.17$ ;  $597 \pm 27$  mS/cm;  
151         $0.21 \pm 0.05$  mg/L nitrites;  $58.05 \pm 13.54$  mg/L nitrates;  $0.14 \pm 0.42$  mg/L ammoniac). Mussels  
152        were kept several months before the experiments, under these acclimation conditions.  
153        Throughout this acclimation step, mussels were fed *ad libitum*, twice per week, with  
154        *Nannochloropsis* (Nanno 3600, Planktovie, Marseille, France).

155

156        *2.2. Reims and SIAAP WWTPs*

157        The Reims WWTP is located at 16 chemin des Temples, 51370 Saint-Thierry (49°16'49.566"  
158        N, 3°59'32.625" E) and is managed by Grand Reims. The center Seine WWTP is located 5  
159        Boulevard Louis Seguin, 92700 Colombes (48°55'57.936" N, 2°14'38.58" E) and is managed  
160        by the SIAAP. Their characteristics are summarized in Annex 1. Briefly, the two treatment  
161        plants have common characteristics, namely physical and chemical treatment of wastewater  
162        and sludge, as well as biological treatment of wastewater. The biological treatment of the two  
163        WWTPs is mechanical (anaerobic and aeration tanks, biofilters, etc.) and does not include a  
164        step with chlorine. At the end of the water treatment process, this water is discharged into the  
165        Vesle for the Reims WWTP, and into the Seine for that of the SIAAP.

166

167        *2.3. Experimental designs*

168                *2.3.1. In situ exposures to treated wastewaters*

169        Two exposures to effluent were performed on dates corresponding to the two first  
170        epidemiological waves observed in France. The first one was performed from 07<sup>th</sup> April 2020  
171        to 07<sup>th</sup> May 2020, while the second one was performed from 25<sup>th</sup> September 2020 to 27<sup>th</sup>



172 November 2020. These cages were deposited into the 1,000 L acclimation tanks.  
 173 Polyethylene cages, having a volume of 931 cm<sup>3</sup>, and exhibiting 5 x 5 mm mesh, contained  
 174 150 mussels, and were then deposited by two at the study sites. For the site of Reims, cages  
 175 were placed on the sediment with a water column height of at least 40 cm above them and  
 176 were connected to the bank with a cable, while for the center Seine site, cages were placed  
 177 inside the WWTP in a tank receiving treated wastewaters.

178 For the earlier experiment (April and May 2020), mussels were caged at the exit of the Reims  
 179 WWTP (49°16'39.5" N, 3°59'06.5" E) and inside that of center Seine (48°55'57.936" N,  
 180 2°14'38.58" E). Caging and sampling kinetics are described in Table 1. The digestive glands  
 181 of three zebra mussels were grouped together to have enough biological material for the  
 182 analyses. At each sampling time, ten pools of three digestive glands are recovered for Reims  
 183 WWTP, and five pools of three digestive glands for SIAAP WWTP. The samples were then  
 184 directly frozen by liquid nitrogen vapours and then stored at -80°C before the analyses.  
 185 Concerning the experiment conducted during autumn 2020, mussels were only exposed to  
 186 the exit of Reims WWTP. As previously described, dissections were performed at laboratory  
 187 and pools of digestive glands were then stocked at -80°C until RT-qPCR.

188 For each different caging periods, less than 10% mortality was reported.

189

190 *Table 1: Caging and sampling kinetics for exposures to treated wastewaters.*

Experiment	Locations	Start	Sampling times	End
Spring 2020	Reims WWTP	07th April	D0 / D1 / D3 / D7 / D14 / D21 / D28	05th May
	Center Seine WWTP	16th April	D4 / D7 / D11 / D14 / D18 / D21	07th May
Autumn 2020	Reims WWTP	25th September	D3 / D7 / D14 / D21 / D28 / D35 / D41 / D49 / D56 / D63	27th November

191

### 192 2.3.2. Laboratory exposures to raw wastewater

193 Four laboratory experiments were performed on dates corresponding to the second  
 194 epidemiological wave observed in France. The first one was performed from 18<sup>th</sup> August to

195 22<sup>nd</sup> August 2020, the second from 02<sup>sd</sup> September to 05<sup>th</sup> September 2020 (September 1),  
196 the third exposure was realised from 24<sup>th</sup> September to 27<sup>th</sup> September 2020 (September 2),  
197 while the fourth one was performed on one week from 16<sup>th</sup> November 2020 to 23<sup>rd</sup> November  
198 2020.

199 The experimental procedure is identical for all four experiments, as described below. Before  
200 the experiments, mussels were placed in 10 L aerated glass tanks in the dark with control of  
201 the temperature at 13°C. Four tanks containing each 30 *D. polymorpha*, were implemented:  
202 i) with 100% (4 L) of Cristaline Aurele drinking water (spring Jandun, France); ii) 10% of raw  
203 wastewater coming from the WWTP of Reims and collected the day before (drinking water  
204 q.s. 4 L); ii) 33% of raw wastewater (drinking water q.s. 4 L); and iv) 100% of raw  
205 wastewater. These waters were changed every day, and the input of raw wastewater came,  
206 each experiment day, from a sample the day before. Concerning the first exposure (August  
207 2020), samples were collected on D1, D2, D3 and D4. For both September exposures,  
208 samples were collected only on D3, and mussels were not fed during the experimentation  
209 step, while concerning the last exposure (November 2020), that lasted longer (sampling time  
210 on D1 and D7), mussels were fed every day with Nanno 3600 algae (Planktovie, Marseille,  
211 France) before the water change. For this last exposure, two tanks containing 30 zebra  
212 mussels were placed for the 100% raw wastewater condition. As previously described,  
213 dissections were performed at laboratory and pools of digestive glands were then stocked at  
214 -80°C before RT-qPCR analysis. During the exposures carried out at the end of September  
215 and in November, mussels in 100% and 33% raw sewage conditions could be dissected  
216 respectively before D3 and D7 according to their general condition (in particular the time  
217 required to close the valves). For these experiments, 15 pools of 3 mussels were dissected  
218 before D3 (September) or D7 (November) because of the toxicity of untreated wastewater,  
219 undiluted or two-thirds diluted.

220

221 *2.4. SARS-CoV-2 genome detection in wastewater*

222 Analyses of SARS-CoV-2 genome in raw wastewater were realised by the Obepine group  
223 (Réseau Obepine, 2021). Obepine is a French consortium born at the start of the SARS-  
224 CoV-2 health crisis, using wastewater as an epidemiological surveillance tool. For many  
225 months, they follow the evolution of the contamination of wastewater by this virus at the level  
226 of around thirty WWTPs located in France. Data collected by Obepine are summarized in  
227 indices which are available on their web site (Reims WWTP: [https://www.reseau-](https://www.reseau-obepine.fr/reims/)  
228 [obepine.fr/reims/](https://www.reseau-obepine.fr/reims/); and center Seine WWTP: <https://www.reseau-obepine.fr/seine-centre/>).

229 Briefly, virus particles were concentrated by ultracentrifugation of 11 mL of wastewater  
230 sample and RNA genome were extracted according to Wurtzer et al. (2020a). SARS-CoV-2  
231 genes RdRp (RNA-dependent RNA polymerase), E (envelope protein) and N (nucleocapsid  
232 protein) were assessed and quantified by RT-qPCR according to Pasteur Institute protocol  
233 (WHO, 2020b), Corman et al. (2020) and CDC protocol (U.S. Department of Health and  
234 Human Services, 2020) respectively (Table 2). Then these data were synthesized into an  
235 indicator obtained by data assimilation with a digital model of the Kalman filter type (Forward-  
236 Backward). This graph was constructed only with envelop protein gene. Data for the Reims  
237 and center Seine (SIAAP) WWTPs were collected from April 2020 to January 2021, and  
238 compared to periods of confinement and curfew observed in France (Réseau Obepine,  
239 2021). This information is available on the Obepine network site (Réseau Obepine, 2021).

240

## 241 *2.5. SARS-CoV-2 GENOME detection in digestive tissues of D. polymorpha*

### 242 *2.5.1. RNA quantification*

243 After thawing of samples, 200 µL of proteinase K for 200 mg of digestive tissues was added  
244 (3 U/ml, Euromedex, Souffelweyersheim, France). Samples were then homogenized several  
245 seconds with an ultra-turrax (Ika-Werk, Janke & Kunkel, Staufen im Breisgau, Germany).  
246 Then, cells were lysed by adding trizol reagent and the whole was vortexed (Molecular  
247 Research Center Inc., OH, USA). Chloroform (VWR) was added and vortexed 30 seconds  
248 with samples and then incubated 15 minutes at room temperature. The aqueous phase

249 containing the nucleic material was recovered after centrifugation (12,000 g, 15 min, 4°C).  
250 The following steps of RNA extraction were realised using the PureLink™ RNA mini kit  
251 (Invitrogen, ThermoFisher Scientific, MA, USA) following the manufacturer  
252 recommendations, until recovering RNA in RNase free water. RNA samples were frozen (-  
253 20°C) until reverse transcription polymerase chain reaction.

254

#### 255 2.5.2. Reverse transcription polymerase chain reaction and RNA detection

256 SARS-CoV-2 RNA detection was based on works of Corman et al. (2020), and performed  
257 with SuperScript™ III one-step RT-PCR with platinum™ Taq (Invitrogen). Genes tested in  
258 this article were: RdRP: RNA-dependent RNA polymerase gene; E, an envelope protein  
259 gene and N, nucleocapsid protein gene. Primers and probes used come from the study of  
260 Corman et al. (2020), were provided by Eurogentec (Liege, Belgium) and are described  
261 below (Table 2). Unlike water samples, the viral load within the digestive gland mash cannot  
262 be preconcentrated. Characteristics of RT-qPCR were: 10 min at 55°C (RT) / 3 min at 95°C /  
263 50 cycles of 15 sec at 95°C / 30 sec at 58°C (CFX96 Touch Real-Time PCR System,  
264 BioRad, CA, USA). No template controls (NTC) were realised by adding molecular-grade  
265 water, positive controls were performed by adding SARS-CoV-2 positive control (COV019  
266 batch number 20033001, Exact Diagnostics, TX, USA) before RT-qPCR, and extraction  
267 controls were performed by adding 10 µL of this positive standard to digestive gland pools  
268 from mussels not exposed (between dissection and freezing). This positive extraction control  
269 allowed the obtention of an extraction yield between initial and final concentration of 70% for  
270 the E and N genes, and of 28% for the RdRp gene. The positive detections of the SARS-  
271 CoV-2 genome in the digestive tissues of zebra mussels were validated by a second  
272 passage of these samples in reverse transcription polymerase chain reaction.

273

274 *Table 2 : List and characteristics of primers (F and R) and probes (P) used for Rt-qPCR analyses. From Corman*  
 275 *et al. (2020). E: envelope protein gene; RdRp: RNA-dependent RNA polymerase gene; N or N1 (in wastewater):*  
 276 *nucleocapsid protein gene.*

Medium	Gene	Oligonucleotide	Sequence	Final concentration
Mussels	RdRp	RdRp_SARsR-F	GTG-ARA-TGG-TCA-TGT-GTG-GCG-G	600 nM
		RdRp_SARsR-R	CAR-ATG-TTA-AAS-ACA-CTA-TTA-GCA-TA	800 nM
		RdRp_SARsR-P2	CAG-GTG-GAA-CCT-CAT-CAG-GAG-ATG-C	100 nM
	E	E_Sarbeco_F	ACA-GGT-ACG-TTA-ATA-GTT-AAT-AGC-GT	400 nM
		E_Sarbeco_R	ATA-TTG-CAG-CAG-TAC-GCA-CAC-A	400 nM
		E_Sarbeco_P1	ACA-CTA-GCC-ATC-CTT-ACT-GCG-CTT-CG	200 nM
	N	N_Sarbeco_F	CAC-ATT-GGC-ACC-CGC-AAT-C	600 nM
		N_Sarbeco_R	GAG-GAA-CGA-GAA-GAG-GCT-TG	800 nM
		N_Sarbeco_P	ACT-TCC-TCA-AGG-AAC-AAC-ATT-GCC-A	200 nM
Water	RdRp	nCoV_IP4-14059Fw	GGT-AAC-TGG-TAT-GAT-TTC-G	400 nM
		nCoV_IP4-14146Rv	CTG-GTC-AAG-GTT-AAT-ATA-GG	400 nM
		nCoV_IP4-14084P	TCA-TAC-AAA-CCA-CGC-CAG-G	200 nM
	N1	2019-nCoV_N1-F	GAC-CCC-AAA-ATC-AGC-GAA-AT	400 nM
		2019-nCoV_N1-R	TCT-GGT-TAC-TGC-CAG-TTG-AAT-CTG	400 nM
		2019-nCoV_N1-P	ACC-CCG-CAT-TAC-GTT-TGG-TGG-ACC	200 nM

277

278

### 279 3. Results and discussion

#### 280 3.1. Detection of SARS-CoV-2 genomes in raw wastewaters

281 Obepine group has performed the wastewater analyses on the two WWTPs studied, and  
 282 summarized Figure 1A (Réseau Obepine, 2021). Table 3 contains the concentrations of the  
 283 three targeted SARS-CoV-2 genes in raw wastewaters. These data were averaged over the  
 284 week for caging exposure to treated wastewater, or over the duration of exposure during  
 285 laboratory exposures to raw wastewater. The contamination profiles of untreated wastewater  
 286 by SARS-CoV-2 from Reims and the center Seine WWTPs in 2020 were remarkably similar,  
 287 and wastewater from both sites exhibited concentrations of comparable values (Table 3).  
 288 During spring exposures of zebra mussels to treated wastewater at the output of WWTPs,  
 289 water contamination by the SARS-CoV-2 genome was very high (almost 500,000 copies/L  
 290 for E gene), but dropped considerably until it reached its lowest values at the end of these  
 291 exposures (Figure 1A or <DL, Table 3). On the other hand, the exposures to treated  
 292 wastewater carried out at the end of 2020 corresponded to a period when the index was  
 293 quite high (between 50 and 150, Figure 1). During this second caging exposure, genome  
 294 concentrations of SARS-CoV-2 in raw water remained stable (between 38,000 and 91,000  
 295 copies/L for E gene, Table 3). This range of values was also found within exposures carried

296 out in the laboratory after half of September 2020. Indeed, a notable increase in  
 297 concentrations between the two experiments carried out in September 2020 was observed  
 298 (Table 3).

299

300 *Table 3 : Concentrations (gene copies/L) of the SARS-CoV-2 genome in raw wastewater from WWTPs in Reims*  
 301 *and center Seine, averaged over the week or over the duration of exposure. The concentrations under the various*  
 302 *dilution conditions are estimates. Data are expressed as mean ± standard deviation (SD). The concentration*  
 303 *estimate for the dilution conditions were obtained with respect to the 100% condition. E: envelope protein gene;*  
 304 *RdRp: RNA-dependent RNA polymerase gene; N1: nucleocapsid protein gene; NA: not analysed; DL: detection*

Experiment	Condition / Week	RNA concentration in raw wastewater (average over the week or over the duration of the experiment)					
		<i>RdRp gene (copies/L)</i>		<i>E gene (copies/L)</i>		<i>N1 gene (copies/L)</i>	
		Mean	± SD	Mean	± SD	Mean	± SD
Spring caging (April-May 2020)	Reims WWTP (W15)	NA	± NA	489525	± 411855	NA	± NA
	Reims WWTP (W16)	NA	± NA	464844	± 426420	NA	± NA
	Reims WWTP (W17)	NA	± NA	21891	± 17883	NA	± NA
	Reims WWTP (W18)	398	± 406	< DL	± < DL	NA	± NA
	Reims WWTP (W19)	390	± 202	20634	±	NA	± NA
	center Seine WWTP (W16)	NA	± NA	223704	± 16364	NA	± NA
	center Seine WWTP (W17)	NA	± NA	NA	± NA	NA	± NA
	center Seine WWTP (W18)	NA	± NA	44178	±	NA	± NA
	center Seine WWTP (W19)	NA	± NA	2045	±	NA	± NA
Autumn caging (September- October- November 2020)	Reims WWTP (W39)	39276	± 23004	80507	± 39936	141385	± 189176
	Reims WWTP (W40)	14983	± 12229	71795	± 50457	31469	± 19527
	Reims WWTP (W41)	12110	± 6586	77802	± 55418	32343	± 15197
	Reims WWTP (W42)	5110	± 2640	37909	± 22827	19212	± 15800
	Reims WWTP (W43)	8169	± 5701	41674	± 19787	21552	± 7286
	Reims WWTP (W44)	11287	± 2161	59840	± 19573	36114	± 21821
	Reims WWTP (W45)	12417	± 8961	73853	± 29035	42215	± 16489
	Reims WWTP (W46)	8388	± 2969	40722	± 18186	46769	± 39254
	Reims WWTP (W47)	9156	± 3901	79050	± 78280	27520	± 19756
	Reims WWTP (W48)	3814	± 4826	91106	± 124838	15137	± 11540
1st laboratory exposure (August 2020)	100 % raw wastewater	6173	± 2942	21400	± 12297	24157	± 25522
	33 % raw wastewater	2037	± 971	7062	± 4058	7972	± 8422
	10 % raw wastewater	617	± 294	2140	± 1230	2416	± 2552
2nd laboratory exposure (September 2020)	100 % raw wastewater	4244	± 421	17237	± 8801	30611	± 18872
	33 % raw wastewater	1401	± 139	5688	± 2904	10102	± 6228
	10 % raw wastewater	424	± 42	1724	± 880	3061	± 1887
3rd laboratory exposure (September 2020)	100 % raw wastewater	32230	± 19355	73026	± 41874	57763	± 33155
	33 % raw wastewater	10636	± 6387	24098	± 13818	19062	± 10941
	10 % raw wastewater	3223	± 1935	7303	± 4187	5776	± 3316
4th laboratory exposure (November 2020)	100 % raw wastewater	9913	± 4086	113242	± 120853	26232	± 18650
	33 % raw wastewater	3271	± 1348	37370	± 39882	8657	± 6154
	10 % raw wastewater	991	± 409	11324	± 12085	2623	± 1865

305 *limit.*

306

307 *3.2. Detection of SARS-CoV-2 genome in digestive tissues of zebra mussels exposed to*  
 308 *treated wastewaters*

309 First, no detection of SARS-CoV-2 RNA was observed for negative controls, either for the  
 310 0% raw wastewaters condition (100% spring water) or for the NTCs during PCRs. These

311 negative controls, combined with the systematic detection of SARS-CoV-2 RNA for the  
312 positive controls, allow the justification of the following results.

313 The number of pools of digestive tissues from mussels caged in potentially contaminated  
314 wastewater as well as the number of pools with detection of the SARS-CoV-2 genome (at  
315 least one of the three genes tested) are shown on Figure 1B and on Figure 2A. Table 4  
316 described the detections of the SARS-CoV-2 genome in zebra mussel samples.

317 The first objective of our study was whether the SARS-CoV-2 genome could be detected by  
318 the zebra mussel caged at the exit of the WWTP. RNA of SARS-CoV-2 was found in  
319 digestive glands of mussels caged at the exit of both center Seine and Reims WWTPs (Table  
320 4, Figure 2A). These detections covered, for each season, the entire exposure period (from  
321 April 14 to May 07, 2020 during spring caging, and from September 18 to November 20,  
322 2020 during autumn caging, Table 4). Since the concentration values of the various SARS-  
323 CoV-2 genes were obtained in raw wastewater, the connection with their detection in caged  
324 mussels exposed to treated wastewater must be considered with caution. During the first  
325 caging campaign, corresponding to a decreasing phase of the raw wastewater index (Figure  
326 1A), 10% of the exposed mussel pools showed positivity to the SARS-CoV-2 genomes in  
327 their digestive tissues (Figure 2A). Surprisingly, when only the results from the center Seine  
328 WWTP were considered, this percentage raised to 21%, compared to 5% at the outlet of  
329 Reims WWTP. In Reims, the two positive samples were reported in week 16, corresponding  
330 to very high concentrations of viral genomes in raw wastewater (464,844 copies of E gene  
331 per liter), but also in week 18, during which however the concentrations in the wastewater  
332 were below the detection limit (Table 3). For the center Seine WWTP, even with less data,  
333 the same observation was made, namely that the detection of SARS-CoV-2 genome in  
334 mussels mainly occurred during weeks 18 and 19 when the concentrations found in the raw  
335 wastewater were much lower (44,178 and 2,045 copies of E gene per liter respectively,  
336 Table 3). The detection of the SARS-CoV-2 genomes in digestive tissues of zebra mussels  
337 was therefore possible even with small amount present in the aquatic environment, and this  
338 detection lasted several days.

339 The second experiment was performed when the concentration of the SARS-CoV-2 genome  
340 in raw wastewater increased until a plateau (Figure 1A), and showed 18% of positivity to the  
341 virus genome in mussels (only for the Reims WWTP, Figure 2A). Looking more closely,  
342 genome positivity for SARS-CoV-2 in mussels was mainly observed during weeks when the  
343 concentration in the water was quite high (about 70,000 copies of E gene per liter, weeks 40,  
344 45, 47, especially for the E gene, Table 3).

345 Several other studies have assessed the presence of SARS-CoV-2 genomes upstream and  
346 downstream of WWTPs. Most of them observed presence of the viral genomes in raw  
347 wastewater from urban WWTP (Balboa et al., 2020; Randazzo et al., 2020; Rimoldi et al.,  
348 2020). All these studies have reported the absence of SARS-CoV-2 genome in treated  
349 wastewaters after secondary  $\pm$  tertiary treatments. Wurtzer et al. (2020b) and Westhaus et al.  
350 (2021) have nonetheless detected SARS-CoV-2 genomes after WWTP in France and  
351 Germany, respectively. Correlatively, Guerrero-Latorre et al. (2020) and Rimoldi et al. (2020)  
352 have reported presence of SARS-CoV-2 genome in rivers not linked to water treatment  
353 plant. There are therefore still gray areas as to the fate of this virus within hydrosystems.

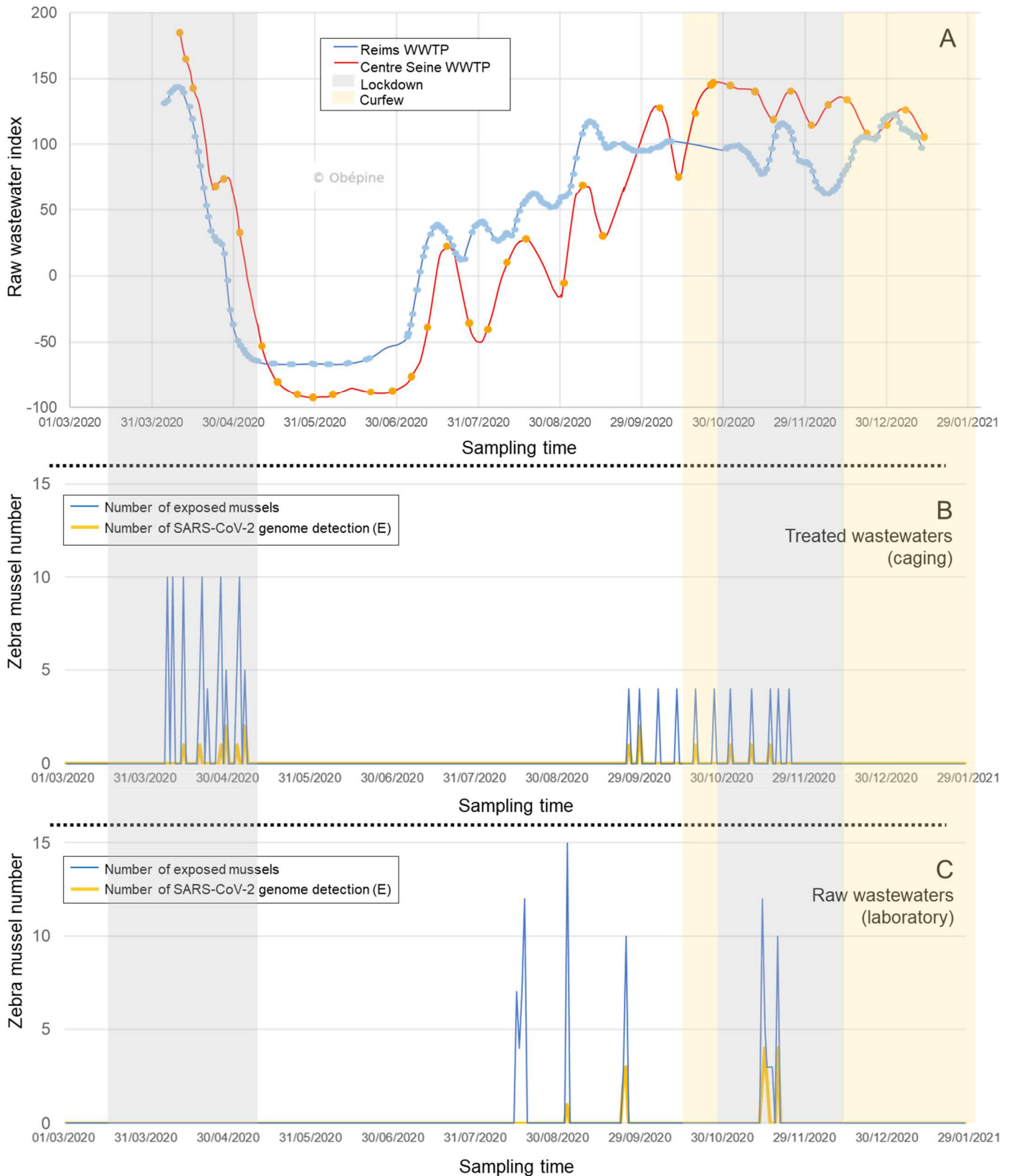
354 Among the three genes used to detect SARS-CoV-2 genome, only the envelope (E) and of  
355 the nucleocapsid (N) genes were detected in mussels (Table 4). Even with maximum  
356 concentration of the samples, no detection of the RNA-dependent RNA polymerase (RdRp)  
357 gene was reported, contrary to N gene detected only with the maximum concentration (Table  
358 4). The same conclusion can be made with regard to the concentrations of the three SARS-  
359 CoV-2 genes in untreated wastewater. In fact, the concentrations for the E gene were  
360 approximately 6 times higher than those of the RdRp gene and 2 times higher than those of  
361 the N gene (Table 3). These differences may be due to the various PCR efficiencies for  
362 these genes but also to the non-homogeneous fragmentation of viral genomes inside our  
363 biological matrix (Wurtzer et al., 2021). These discrepancies had already been revealed by  
364 other studies, for analyses of the SARS-CoV-2 genome in wastewater or in sludges. Several  
365 genes can be targeted by RT-qPCR to study the presence of the SARS-CoV-2 genome,  
366 based on the genes of envelope, nucleocapsid, ORF1ab, or even the RNA-dependent RNA



367 polymerase (Kitajima et al., 2020). However, to date, there is no harmonization of procedures  
368 or standardization of the detection of SARS-CoV-2 genome and variations of results  
369 according to these assays were reported (Farkas et al., 2020). Corman et al. (2020) reported  
370 that the RdRp gene had a lower detection limit than the N and E genes. However, other  
371 studies observed different results, synthesized by Nalla et al. (2020). These authors have  
372 tested seven RT-qPCR assays linked to SARS-CoV-2 and concluded that N2 set and E gene  
373 are the most sensitive (Kitajima et al., 2020; Nalla et al., 2020), while Shirato et al. (2020)  
374 reported that only the RT-qPCR assays carried on the nucleocapsid gene worked for them.  
375 Rimoldi et al. (2020) evaluated the presence of 3 genes of the SARS-CoV-2 (Orf1ab, N, E) in  
376 different aquatic environments (WWTPs and rivers). Only one of the sites showed positivity  
377 to the SARS-CoV-2 genome with all 3 genes detected, and this is the only site where the E  
378 and N genes were both detected. Desdouits et al. (2021) used Corman's E set for the  
379 envelope gene and IP4 set for RdRp gene, and these two genes were expressed in tissues  
380 lysates of *Crassostrea gigas* after controlled exposure to heat-inactivated SARS-CoV-2. In  
381 our study, the viral genome positivity of the digestive gland samples was mainly linked to the  
382 E gene, and a few of these samples also had positivity via the N gene (Table 4). Despite the  
383 lack of harmonization on the methods used, it would have been interesting to use other  
384 specific genes of SARS-CoV-2 (Orf1ab, RdRp IP4 set, other regions of N or E genes, etc.) to  
385 potentially improve its detection within digestive glands of zebra mussels. Our experiments  
386 were performed shortly before the discovery of the SARS-CoV-2 variants (late 2020, early  
387 2021). These mutations, depending on the site where they occur, can greatly influence the  
388 sensitivity of the primers selected for the detection of the SARS-CoV-2 genome in organisms  
389 and wastewaters (Khan and Cheung, 2020; Vogels et al., 2021). The lack of sensitivity of the  
390 RdRp primer could be linked to a mutation at the level of the binding site of this primer. It is  
391 now therefore necessary to perform multiple assays during RT-qPCR with different primers  
392 to prevent this risk (Peñarrubia et al., 2020; Vogels et al., 2021).

393 Few studies had reported detection of SARS-CoV-2 genome in treated wastewaters. In our  
394 study, detection of the SARS-CoV-2 genome in the digestive glands of zebra mussels

395 exposed at the WWTP outlet was observed. The use of a filter feeder and sessile species  
396 could explain this difference. The detection of SARS-CoV-2 genomes directly in wastewater  
397 was often represented by a value at a time point as well as on a volume of water which is not  
398 fully representative of the water mass. Correlatively, zebra mussels, because of their sessility  
399 and their filtration capacity, allow a more extensive characterization of the pollution of their  
400 environment (Kraak et al., 1991; Palos Ladeiro et al., 2014). In fact, these organisms can  
401 bioaccumulate biological and chemical pollutants for several days or even weeks, allowing  
402 pollution to be monitored over time, and filter a significant volume of water that is better  
403 representative of the mass of water (Bervoets et al., 2005; Palos Ladeiro et al., 2018;  
404 Wiesner et al., 2001). Concerning the SARS-CoV-2 genome, oysters have already proven  
405 their effectiveness by accumulating this virus during laboratory exposures (Desdouits et al.,  
406 2021). Also in the marine environment, the *Ruditapes* genus had shown its efficiency in  
407 accumulating the SARS-CoV-2 genome in their digestive tissues (Polo et al., 2021). The  
408 authors of this study concluded that mollusc bivalves can be used as biomonitoring tools for  
409 various anthropogenic contaminants, including the SARS-CoV-2 virus. During our study,  
410 zebra mussels were useful to detect SARS-CoV-2 genome in both untreated and treated  
411 wastewaters, even if the concentrations in wastewater was under the detection limit (1,000  
412 copies/L). All these characteristics make such bivalve, and particularly zebra mussels, good  
413 indicators for the detection of SARS-CoV-2 genomes in such environments. These  
414 organisms can potentially support or even improve the sensitivity of the direct detection of  
415 the viral genome in water samples.



416

417 *Figure 1 : Detection of E gene of SARS-CoV-2 in raw wastewater (A) and in pool of digestive glands of zebra*  
 418 *mussels (B and C) from the March 1<sup>st</sup> 2020 to January, 29<sup>th</sup> 2021.*

419 *A: raw wastewater index of SARS-CoV-2 genome (gene E) from Reims (blue) and center Seine WWTPs*

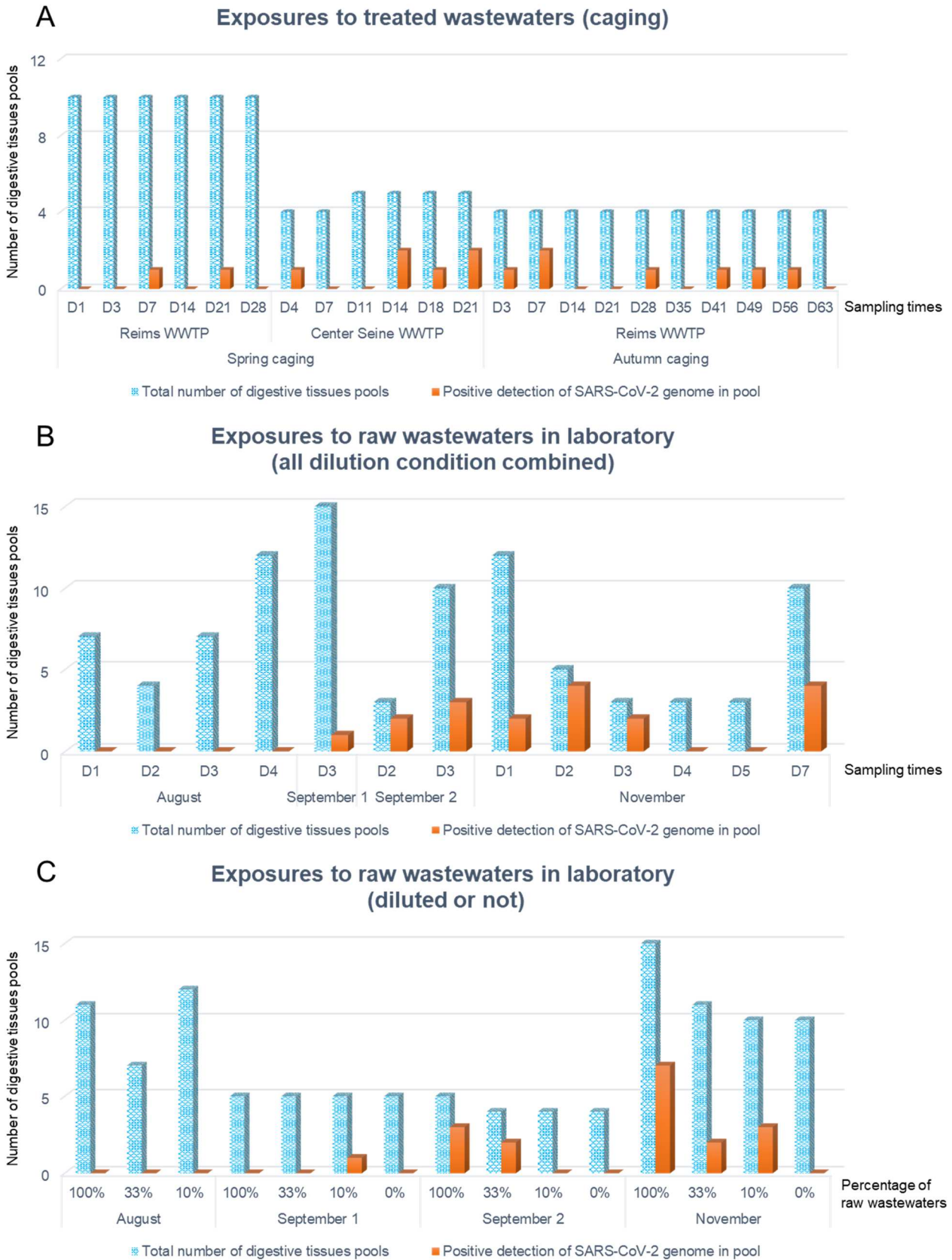
420 *(orange), according to OBEPINE group. Data are represented as a trend index based on RT-qPCR quantification*

421 on the E gene of the SARS-CoV-2 genome and assessed with a digital model of Kalman filter type (Forward-  
422 Backward). Confinement and curfew periods for Reims city were indicated by different colors.  
423 B: number of pools of digestive glands of zebra mussels caged at the exit of Reims and center Seine WWTPs  
424 (blue curve) and number of pools with detection of at least one SARS-CoV-2 gene (orange curve).  
425 C: number of pools of digestive glands of zebra mussels exposed in the laboratory to raw wastewater (blue curve)  
426 and number of pools with detection of at least one SARS-CoV-2 gene (orange curve).

427

428 To further support the use of this sentinel species as an indicator of the presence of the  
429 SARS-CoV-2 genome in environmental waters, improvements on the extraction and  
430 detection of the SARS-CoV-2 genome in the digestive tissues of zebra mussels are required.  
431 Indeed, to improve detection of SARS-CoV-2 genome inside this complex biological matrix,  
432 the PCR cycle number has been increased to 50. Of the total positive detection data on E  
433 gene (Table 4), 73% had Cq lower than 42.75, but a few were higher (all Cq were comprised  
434 between 35.64 to 46.32). These high values underlined the limits of detection or extraction of  
435 this viral genetic material, and particularly in the biological matrix used here. Contrary to the  
436 quantification of SARS-CoV-2 genome in water, a pre-concentration step to concentrate the  
437 viral genome before analyses is not necessary (Kitajima et al., 2020). Moreover, the number  
438 of digestive glands per pool was not elevated (3). Several modifications can be considered to  
439 improve the viral extraction, such an addition of a purification step to limit as much as  
440 possible the enzymatic inhibitors which could be found in the biological matrix, preventing the  
441 good progress of the detection. In parallel, new experiments could be performed to improve  
442 the sensitivity of the detection of SARS-CoV-2 genome in mussels but also to characterize  
443 the bioaccumulation pattern in the tissues of *D. polymorpha*. These experiments must be  
444 performed in the laboratory in controlled conditions, to observe (or not) a dose-dependent  
445 accumulation relationship, and using untreated wastewater with higher levels of SARS-CoV-  
446 2 genomes than in treated water.

447



448

449 *Figure 2 : Total number of digestive tissues pool exposed (blue) and number of positive detections of SARS-CoV-*  
 450 *2 genome in pools (orange) according to exposure, exposure condition and sampling times. A: Results obtained*

451 *after exposure to treated wastewaters (spring and autumn) on the zebra mussels caged after Reims and center*  
452 *Seine WWTPs according to sampling times. B: Results obtained after exposure to raw wastewaters (August,*  
453 *September 1 and 2 and November exposures) from Reims WWTP according to sampling times (all dilution*  
454 *conditions combined). C: Results obtained after exposure to raw wastewaters (August, early September*  
455 *(September 1), September end (September 2) and November exposures) from Reims WWTP according to*  
456 *experiment and dilution conditions.*

457

### 458 *3.3. Zebra mussels as biological indicators of water contamination by the SARS-CoV-2* 459 *genome*

460 The second aim of this study was to assess the interest of using zebra mussels as  
461 bioindicator of water contamination by the SARS-CoV-2 genome. Controlled laboratory  
462 exposures were therefore put in place to address the questions raised during exposure to  
463 treated wastewater. Also, to maintain the natural contamination by SARS-CoV-2 in the  
464 receiving aquatic environment, mussels were exposed to raw wastewater from Reims  
465 WWTP.

466 Regarding the experiments performed in the laboratory, the genome detection of SARS-CoV-  
467 2 genome was higher when the mussels were directly submitted to raw wastewater (100%)  
468 compared to the diluted wastewaters (33 or 10%, Figure 2C). Indeed, the most concentrated  
469 condition (100% raw wastewater) resulted in a positivity of 28% of the samples (10/32),  
470 greater than 15% (4/27) and 13% (4/31), caused respectively by conditions 33% and 10% of  
471 raw wastewater ratios. Zebra mussels were therefore useful to detect the SARS-CoV-2  
472 genome in accordance with its presence in wastewaters. Furthermore, there was a similarity  
473 between the growing phase of the COVID-19 pandemic after summer 2020, confirmed by an  
474 increase of the raw wastewater index between July and October (Figure 1A), and the  
475 detection of the SARS-CoV-2 genome in mussels (Figure 2B). Indeed, when exposed to raw  
476 wastewaters in August, none of the 15 pools of exposed mussel digestive gland had the  
477 genome of this virus, and this number increased with time. During the first exposure in early  
478 September 2020, 7% (1/15) of the pools exhibited positivity for the SARS-CoV-2 genome, to  
479 increase to 38% (5/13) at the end of September, almost identical to the values found in  
480 November (33%, 12/36, Figure 2B). This result was in accordance with the sudden increase

481 in the concentration of the SARS-CoV-2 genome in raw wastewater between early and late  
482 September (Table 3). This increase was all the more important for the E gene and also  
483 continued after September. For illustration, the concentrations of these genes in the raw  
484 wastewater were lower than those of the 33% diluted wastewater (Table 3). This originally  
485 suggested that the zebra mussel can be used as indicator of the SARS-CoV-2 genome  
486 detection in proportion to the contamination load present in freshwater environment and  
487 contributes to emphasize the uses of zebra mussels as sentinel species for SARS-CoV-2  
488 contamination of wastewaters. Desdouits et al. (2021) and Polo et al. (2021) demonstrated  
489 the accumulation of anthropogenic virus by bivalves in several coastal sites including SARS-  
490 CoV-2 virus within digestive tissue. Contrary to Polo et al. (2021), Desdouits et al. (2021) but  
491 did not report the presence of SARS-CoV-2 genome in the field (in water or in bivalve  
492 molluscs).

493 Nonetheless, even if the laboratory experiments allowed to expose zebra mussels to higher  
494 SARS-CoV-2 genome contamination, the experimental plan used in our study (short  
495 exposure due to the possible toxicity of raw wastewaters) only allowed the qualitative  
496 detection of the presence of SARS-CoV-2 genome in organisms but did not allow the  
497 genome quantification. Indeed, the possible toxicity of raw wastewater, causing an advanced  
498 dissection of organisms exposed to the most concentrated raw sewage conditions (18% of  
499 samples during the exposure at the end of September and 26% of samples during the last  
500 exposure, in November), did not allow mussels to be exposed any longer. To dispense with  
501 the toxicity of raw wastewater, a longer exposure of the mussels (from 14 to 21 days) in the  
502 laboratory to a non-infectious SARS-CoV-2 or to low pathogenic CoV strains could improve  
503 characterization of virus accumulation within mussels (Desdouits et al., 2021; Wurtzer et al.,  
504 2020b).

505 Thanks to our results, the use of this bivalve as a bioindicator and possible matrix to follow  
506 the presence of SARS-CoV-2 genome in water is conceivable, whether at the level of  
507 treatment plants, but also at the level of freshwater (rivers, etc.) or in countries whose water  
508 treatment structures are still underdeveloped. As announced by several recent studies, the

509 bivalve taxon represents a complementarity, even a more than plausible alternative for the  
 510 detection of viruses in the environment (Capizzi-Banas et al., 2021; Desdouits et al., 2021;  
 511 La Rosa et al., 2021; Polo et al., 2021). Various fields of application can therefore be  
 512 envisaged, such as environmental biomonitoring for health purposes.

513

514 *Table 4: Presence (+) or absence (-) of SARS-CoV-2 RNA in digestive glands of zebra mussels according to*  
 515 *tested genes and exposure conditions. The samples presented in this table are positive for at least one of the*  
 516 *three genes tested. E: envelope protein gene; RdRp: RNA-dependent RNA polymerase gene; N: nucleocapsid*  
 517 *protein gene.*

Experiment	Date	Exposure time	Exposure condition	RdRp gene detection	E gene detection	N gene detection
Spring caging	April 14, 2020	D7	Reims WWTP	-	+	-
Spring caging	April 20, 2020	D4	SIAAP WWTP	-	+	-
Spring caging	April 28, 2020	D21	Reims WWTP	-	+	-
Spring caging	April 30, 2019	D14	SIAAP WWTP	-	+	-
Spring caging	April 30, 2020	D14	SIAAP WWTP	-	+	-
Spring caging	May 04, 2020	D18	SIAAP WWTP	-	+	-
Spring caging	May 07, 2020	D21	SIAAP WWTP	-	+	-
Spring caging	May 07, 2020	D21	SIAAP WWTP	-	+	-
2nd laboratory exp.	Sept. 05, 2020	D3	10% raw wastewater	-	+	-
3rd laboratory exp.	Sept. 26, 2020	D2	100% raw wastewater	-	+	+
3rd laboratory exp.	Sept. 26, 2020	D2	100% raw wastewater	-	+	-
3rd laboratory exp.	Sept. 27, 2020	D3	100% raw wastewater	-	+	-
3rd laboratory exp.	Sept. 27, 2020	D3	33% raw wastewater	-	+	+
3rd laboratory exp.	Sept. 27, 2020	D3	33% raw wastewater	-	+	+
Autumn caging	Sept. 28, 2020	D3	Reims WWTP	-	+	+
Autumn caging	Oct. 02, 2020	D7	Reims WWTP	-	+	-
Autumn caging	Oct. 02, 2020	D7	Reims WWTP	-	+	-
Autumn caging	Oct. 23, 2020	D28	Reims WWTP	-	+	-
Autumn caging	Nov. 05, 2019	D41	Reims WWTP	-	+	-
Autumn caging	Nov. 13, 2020	D49	Reims WWTP	-	+	-
Autumn caging	Nov. 20, 2020	D56	Reims WWTP	-	+	-
4th laboratory exp.	Nov. 17, 2020	D1	100% raw wastewater	-	+	+
4th laboratory exp.	Nov. 17, 2020	D1	33% raw wastewater	-	+	-
4th laboratory exp.	Nov. 18, 2020	D2	100% raw wastewater	-	+	-
4th laboratory exp.	Nov. 18, 2020	D2	100% raw wastewater	-	+	-
4th laboratory exp.	Nov. 18, 2020	D2	100% raw wastewater	-	+	-
4th laboratory exp.	Nov. 18, 2020	D2	100% raw wastewater	-	+	-
4th laboratory exp.	Nov. 19, 2020	D3	100% raw wastewater	-	+	+
4th laboratory exp.	Nov. 19, 2020	D3	100% raw wastewater	-	+	-
4th laboratory exp.	Nov. 23, 2020	D7	33% raw wastewater	-	+	-
4th laboratory exp.	Nov. 23, 2020	D7	10% raw wastewater	-	+	-
4th laboratory exp.	Nov. 23, 2020	D7	10% raw wastewater	-	+	-
4th laboratory exp.	Nov. 23, 2020	D7	10% raw wastewater	-	+	-

518

519

#### 520 4. Conclusion



521 Out of a total of 666 mussels exposed to water potentially contaminated by the SARS-CoV-2  
522 genome, i.e. 222 pools of digestive glands, 33 pools showed positivity to the genome of this  
523 virus, representing almost 7%. This detection was observed during the two major  
524 epidemiological phases in France and both with raw wastewater and treated wastewater.  
525 Moreover, SARS-CoV-2 genomes was detected in *D. polymorpha* as well at the outlet of the  
526 Reims wastewater treatment plant as that of center Seine one. This corroborated the results  
527 in untreated wastewater but also brought a novelty with the resilience of the genetic material  
528 of the virus after treatment of these waters. This detection is proportional to the  
529 contamination in the wastewaters and can allow a temporal and spatial monitoring. The  
530 zebra mussel therefore appears to be an attractive candidate for detecting the presence of  
531 the SARS-CoV-2 genome in raw and treated wastewaters, but also in other hydrosystems.  
532 The detection of the genome of other enteric viruses could be relevant using this sentinel  
533 species.

534

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539

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