Detection of SARS-CoV-2 RNA in bivalve mollusks and marine sediments

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HIGHLIGHTS

- SARS-CoV-2 RNA was detected in 9/12 clam and 3/12 estuarine sediment samples.
- RNA signals disappeared in the PMAxx-triton viability RT-qPCR assay.
- Results indicate a high degree of RNA degradation and a non-infective viral state.
- crAssphage detection prove the presence of human fecal pollution in the studied area.
- This is the first detection of SARS-CoV-2 RNA in the marine environment.

GRAPHICAL ABSTRACT
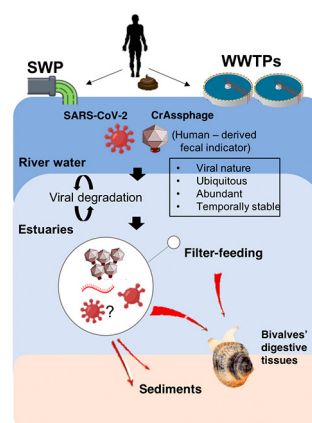
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ABSTRACT

The presence of SARS-CoV-2 in wastewater pose the question of whether this new pandemic virus could be released into watercourses and potentially continue to finally reach coastal waters. In this study, we employed two bivalve molluscan species from the genus *Ruditapes* as sentinel organisms to investigate the presence of SARS-CoV-2 signals in the marine coastal environment. Estuarine sediments from the natural clam banks were also analyzed. Viral RNA was detected by RT-qPCR, targeting IP4, E and N1 genomic regions. Positive samples were also subjected to a PMAxx-triton viability RT-qPCR assay in order to discriminate between intact and altered capsids, obtaining indirect information about the viability of the virus. SARS-CoV-2 RNA traces were detected in 9/12 clam samples by RT-qPCR, from which 4 were positive for two different target regions. Viral quantification ranged from <LoQ to 4.48 Log genomic copies/g of digestive tissue. Regarding the sediment samples, 3/12 were positive by RT-qPCR, but only IP4 region was successfully amplificated. Quantification values for sediment samples ranged from <LoQ to 3.60 Log genomic copies/g of sediment. RNA signals disappeared in the PMAxx-triton viability RT-qPCR assay, indicating non-infectious potential. In addition, the recently discovered human-specific gut associated bacteriophage crAssphage was also quantified as a biomarker for the presence of human-derived wastewater contamination on the study area. CrAssphage was detected in 100% of both types of samples with quantification values ranging from <LoQ to 5.94 Log gc/g digestive tissue and from <LoQ to 4.71 Log gc/g sediment. Statistical analysis also showed that quantification levels for the crAssphage in clams are significantly higher than in sediments. These findings represent the first detection of SARS-CoV-2 RNA in the marine environment, demonstrating that it can reach these habitats and make contact with the marine life.

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1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly spread worldwide to become the most important global health crisis since the 1918 influenza pandemic (Ceylan et al., 2020). Although the evidences indicate a transmission of the virus via infected droplets and aerosols (World Health Organization (WHO), 2020a, b), its presence in the feces of symptomatic and asymptomatic infected patients and consequently in wastewater has drawn attention to the possibility of a fecal-oral route of transmission (Wu et al., 2020). These facts not only have led sanitary authorities worldwide to set-up large-scale wastewater monitoring programs as epidemiological prediction tools (Ahmed et al., 2020; Randazzo et al., 2020; Polo et al., 2020), but also pose the question of whether a wider contamination of the environment with SARS-CoV-2 via wastewater is possible. As other human viruses commonly found in feces, SARS-CoV-2 could be released into watercourses through untreated sewage or poorly treated wastewater effluents. Even if its enveloped nature makes it more prone to degradation under environmental conditions than the typical non-enveloped enteric viruses, assumptions should be made with caution (Wigginton et al., 2015). Recent publications indicate the presence of SARS-CoV-2 in river water receiving untreated human sewage (Guerrero-Latorre et al., 2020; Rimoldi et al., 2020). This suggests that at least part of SARS-CoV-2 virions or their RNA present in sewage effluents could potentially continue the watercourses and be finally released into coastal areas. The potential environmental impacts, including the contact of the virus or its genetic material with aquatic or semi-aquatic organisms, are important questions that must be addressed.

Bivalve mollusks, like clams, mussels or oysters, have a long history as vectors of human viral diseases, such as gastroenteritis and hepatitis A, as a consequence of the fecal contamination of their growing waters (Lees, 2000; Le Guyader et al., 2000; Polo et al., 2015). These marine invertebrates are an integral part of marine ecosystems, and due to their filter-feeding nature, can bioaccumulate and retain enteric viruses for long periods (Polo et al., 2016, 2018). However, bivalves' capacity to filter large volumes of seawater can also be used to investigate signals of human fecal pollution in marine waters. Considering the common detection of SARS-CoV-2 RNA in feces, wastewater and also in river waters, in this work the potential presence of SARS-CoV-2 RNA signals into the estuarine environment was investigated.

2. Material and methods

2.1. Study area

We analyzed 12 estuarine sediment and 12 clam samples (*Ruditapes* sp.) collected between May and July 2020 from two natural clam banks in Galicia, NW of Spain (Fig. 1). *Ruditapes philippinarum* was collected from Bank 1 while *Ruditapes decussatus* was collected from Bank 2. These banks are located in two small estuaries, highly influenced by tides and classified according EU Regulation (EC) No 854/2004. These regulations classify shellfish harvesting areas into A, B or C category on the basis of *E. coli* levels as follows: A (≤230 cfu *E. coli*/100 g shellfish), B (230–4600 cfu *E. coli*/100 g shellfish), C (4,600–46,000 cfu *E. coli*/100 g shellfish).

Bank 1 (43°21′740 N/8°12′220 W) is flooded by the waters of a small river into which the sewage of a camping zone, an urbanization and several populations of the same municipality are discharged. The WWTP-A that discharge into this area has secondary treatment and UV disinfection, and 9000 population equivalents (pe). During the summer, there is a significant increase of the population in this area due to tourism. The current classification of the Bank 1 is provisional "class B" zone, becoming "class C" zone from May to October. Bank 2 (43°19′361 N/8°12′125 W) is a harvesting area flooded by the waters of a small river, into which three municipalities pour their wastewater, one of them without WWTP. This area receives the discharges of a sewage outfall and two WWTPs: WWTP-B with secondary treatment, UV disinfection and microfiltration and 25,000 pe. WWTP-C have only secondary treatment and 4000 pe. The microbiological classification of this Bank 2 fluctuates during the year, being a "class C" zone most of the time. In addition, an outbreak of COVID-19 was reported during the course of this survey in one of the municipalities that pour their treated-wastewater upstream of Bank 2 (Fig. 1).

2.2. Viruses and cell lines

Murine hepatitis virus strain A59 (MHV-A59) was kindly provided by Prof. Stanley Perlman (University of Iowa). NCTC-1469 (CCL-9.1) cells were obtained from the American Type Culture Collection (ATCC®, Manassas, VA, United States). NCTC-1469 cell line was used for propagation of MHV-A59. Briefly, NCTC 1469 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), sodium piruvate (1 mM), 1500 mg/L of sodium bicarbonate, 1% non-essential amino-acids (NEAA) and 10% heat-inactivated horse serum (Lonza-BioWhittaker®, Belgium) using 75 cm^2 cell culture flasks. Cells were incubated at 37 °C and 5% CO_2. Once monolayers were 80% confluent, medium was removed, cells washed twice with PBS (Lonza-BioWhittaker®, Belgium) and MHV-A59 was inoculated at 0.1 multiplicity of infection (MOI) in 5 mL of DMEM without serum. After 1 h of contact, inoculum was removed, replacing it with 10 mL of DMEM supplemented with 2% horse serum and maintained at 37 °C and 5% CO_2. Flasks were then microscopically examined for cytopathic effect that was evident after 20 h as rounding and sloughing, attached and floating syncytial debris. Then, cultures were frozen and thawed two times, centrifuged at 3000 ×g during 5 min to eliminate cell debris, and the supernatant was passed through a Minisart® filter with a pore size of 0.20 μm (Sartorius, Göttingen, Germany). Viral suspensions were kept frozen at −80 °C in 1 mL aliquots.

2.3. Sample processing and virus recovery

2.3.1. Shellfish samples

Processing of clam samples were carried out as previously described (Polo et al., 2015) following the basic principles of the ISO 15216-1:2017. Briefly, for clam samples (30 individuals each), all digestive tissues (DT) were dissected and pooled. Then, portions of 2 g of DT were obtained in triplicate and 10 μL of MHV-A59 stock (equivalent to 2×10^7 genomic copies) was spiked in each portion to be used as viral extraction control (control of process). Tissues were homogenized with one volume (1:1 w/v) in peptone water (0.1%; pH 9), strongly shaken for 1 h at 4 °C, centrifuged at 1000 ×g for 5 min and recovering the supernatant to be used for the viral RNA extraction.

2.3.2. Estuarine sediment samples

For each sediment sample, three replicates of 5 g (wet weight) of sediment were spiked with 10 μL of MHV-A59 stock as a process control and mixed with 45 mL of glycin buffer (0.05 M glycine, 3% beef extract buffer, pH 9.5). Then, samples were strongly shaken for 30 min at 4 °C to detach virions bound to organic material. Samples were then centrifuged at 2000 ×g 10 min and the supernatants (mean volume 49 ± 1 mL) were recovered. Then PEG8000 (final concentration of 8%) and NaCl (final concentration 17.5 g/L) was added to the supernatants and shaken overnight at 4 °C followed by centrifugation for 30 min at 11,000 × g. The resulting viral-containing pellets were eluted in 2 mL of PBS (pH 7.4). These concentrates were then loaded into PD-10 Desalting columns containing Sephadex G-25 Medium (GE Healthcare Bio-Sciences AB, Sweden) previously equilibrated with 25 mL of PBS (7.4 pH). Sample eluates (2 mL) were finally recovered by centrifugation at 1000 ×g for 2 min and used for the viral RNA extraction.

Fig. 1. Map of the estuaries and river catchment in Galicia (NW of Spain) showing the clam banks 1 (B1) and 2 (B2) (red squares), and sampling points (SP1 and SP2). Locations for wastewater treatment plants (WWTP), sewage pump station (SWPS) and non-treated sewage outfalls (SW), a camping area (white triangle) and an urbanization (white square) are indicated. During the summer, there is a significant increase of the population in the area surrounding bank 1 due to tourism. Municipality where the outbreak was reported is marked in yellow.

Table 1
List of primers and probes used.

Virus	Genomic region	Name	Sequence (5′–3′)	Amplicon size (pb)	Reference
SARS-CoV-2	RdRp	nCoV_IP4-Fw	GGTAACTGGTATGATTTCG	107	World Health Organization (WHO), 2020a[a]
		nCoV_IP4-Rv	CTGGTCAAGGTTAATATAGG		
		nCoV_IP4-Pr	Fam-TCATACAAACCACGCCAGG-BHQ1		
	Envelope	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	125	Corman et al., 2020[b]
		E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA		
		E_Sarbeco_P1	Fam-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1		
	Nucleocapsid	nCoV_N1-Fw	GACCCCAAAATCAGCGAAAT	72	World Health Organization (WHO), 2020b[c]
		nCoV_N1 Rv	TCTGGTTACTGCCAGTTGAATCTG		
		nCoV_N1 Pr	Fam-ACCCCGCATTACGTTTGGTGGACC-BHQ1		
MHV-A59	Nucleocapsid	MHV_N-Fw	GCCTCGCCAAAAGAGGACT	66	Kindler et al. (2017)
		MHV_N-Rv	GGGCCTCTCTTTCCAAAACAC		
		MHV_Pr	CAAACAAGCAGTGCCCAGTGCAGC		
CrAssphage	ORF25	CPQ_064F1	TGTATAGATGCTGCTGCAACTGTACTC	148	Stachler et al. (2017)
		CPQ_064R1	CGTTGTTTTCATCTTTATCTTGTCCAT		
		CPQ_064P1	Fam-CTGAAATTGTTCATAAGCAA-MGB		

[a] Set designed by the National Reference Center for Respiratory Viruses, Institut Pasteur, Paris (France).
[b] Set designed by the Institute Charité, Berlin (Germany).
[c] Set designed by the Center for Disease Control and Prevention, Atlanta (USA).

2.4. Viral nucleic acid extraction and amplification

Viral RNA (MHV-A59 and SARS-CoV-2) or DNA (crAssphage) was extracted from 150 μL of clam or sediment supernatants (prepared as described above) by using Nucleospin® RNA/DNA Virus Kit (Macherey-Nagel; Germany) according to manufacturer's instructions, eluted in 50 μL of RNAse free dH$_2$O and analyzed by RT-qPCR and qPCR in the same day.

Viral RNA was detected by RT-qPCR on a Mx3000P qPCR system (Stratagene; USA) instrument using One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio; USA). Primer/probe sets employed for detection of the different viruses are listed in Table 1. MHV-A59 RNA was detected targeting the nucleocapsid gene (N). SARS-CoV-2 RNA detection was carried out with three sets of oligonucleotide primers and probes targeting three different genome regions including the nucleocapsid gene (N1), RdRp gene (IP4) and E gene (E). CrAssphage DNA was detected targeting the ORF25 region. All primer sets and probes were provided by Integrated DNA Technologies (IDT, Leuven, Belgium).

Twist Synthetic SARS-CoV-2 RNA control (Twist Bioscience, CA, USA) was used as a positive control for all target regions. Reaction mix (20 μL) consisted of 6.7 μL of RNA-free H$_2$O, 10 μL of 2× One Step RT-PCR Buffer III, 0.40 μL PrimeScript RT enzyme Mix II, 0.40 μL TaKaRa Ex Taq HS, 1 μL for each set of forward and reverse primers (400 nM) and 0.5 μL for each probe (200 nM). The thermal cycling conditions were as follows:

For MHV-A59 and SARS-CoV-2 N1 and E regions: RT at 50 °C for 15 min, preheating at 95 °C for 3 min and 45 cycles of amplification at 95 °C for 15 s and 58 °C for 30 s. For SARS-CoV-2 IP4 region: RT at 55 °C for 20 min, preheating at 95 °C for 3 min and 45 cycles of amplification at 95 °C for 15 s and 58 °C for 30 s. The thermal cycling profile for crAssphage was 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Each RNA or DNA was analyzed in triplicate and as pure and ten-fold diluted to detect possible presence of PCR inhibitors. Every RT-qPCR assay included negative (nuclease-free water) and positive controls. MHV-A59 recovery rates were calculated and used as quality assurance parameters according to ISO 15216-1:2017. CrAssphage DNA was detected by qPCR using the *Premix Ex Taq* DNA Polymerase (Perfect Real Time) kit (Takara Bio; USA).

SARS-CoV-2 RNA was quantified as genomic copies (gc)/g of DT or g of sediment, plotting the quantification cycles (Cq) to an external standard curve built with 10-fold serial dilutions of a quantified Twist Synthetic SARS-CoV-2 RNA control (Twist Bioscience, CA, USA) or crAssphage DNA control (IDT, Leuven, Belgium). Calibration curves for SARS-CoV-2 RNA were as follow: for N1, $y = -3.446 * Log\ x + 40.00$, amplification efficiency (Eff.) = 95.1%, and correlation coefficient (R^2) = 0.994; for IP4, $y = -3.473 * Log\ x + 41.16$, Eff. = 94.1%, R^2 = 0.998; and for E, $y = -3.498 * Log\ x + 40.93$, Eff. = 93.1%, R^2 = 0.996). The limits of quantification (LoQ) of the overall method for clam samples were 1.44×10^3, 1.26×10^3 and 1.16×10^3 gc/g DT for N1, IP4 and E region, respectively. LoQ for sediment samples were 8.09×10^2, 3.35×10^2 and 3.68×10^2 gc/g DT for N1, IP4 and E region, respectively. Calibration curve for crAssphage was $y = -3.421 * Log\ x + 41.07$, Eff. = 96.8%, R^2 = 0.998. The LoQ of the overall method for the crAssphage was 8.00×10^2 and 2.13×10^2 gc/g DT or /g sediment, respectively.

2.5. PMAxx-triton viability RT-qPCR assay

Propidium Monoazide PMAxx™ is a photo-reactive dye that binds to DNA or RNA with high affinity. Upon photolysis with a blue light, PMAxx™ dye becomes covalently attached to RNA. The RNA-PMAxx bond inhibits the RT-PCR amplification of free-RNA or accessible RNA (RNA that is inside damaged capsids), eliminating the overestimation of viral RNA counts associated to damaged virions. Therefore, quantification cycle (C$_q$) values in PMAxx-RT-qPCR assay will be higher than C$_q$ values in RT-qPCR in the same sample containing encapsidated

viral RNA. PMAxx™ Dye (Biotium, USA) pre-treatment was carried out as previously described (Randazzo et al., 2018). Briefly, an aliquot of 100 μM PMAxx and 0.5% Triton 100-X solution (Fisher-Scientific) was added to 150 μL of clam or sediment sample processed as described above. Samples treated with PMAxx were incubated in the dark at room temperature for 30 min at 150 rpm and immediately exposed to 2 cycles of 15-min photoactivation using a photo-activation system (Led-Active Blue, GenIUL) with a dark incubation of 15 min between photoactivations. Finally, viral RNA was extracted as above. PMAxx positive and negative controls, consisting of clam or sediment samples seeded with thermally inactivated MHV-A59 at 99 °C for 5 min and treated with and without PMAxx respectively, were included in each set of RT-qPCR reactions.

2.6. Statistics

Linear regressions and Spearman's rank correlation coefficients (r) were calculated between viral concentrations using two-tailed 95% confidence intervals. Student's *t*-test was also used to compared both crAssphage DNA and SARS-CoV-2 RNA quantification between shellfish and sediment samples as well as between clam banks. The *p*-values < 0.05 were considered statistically significant. *R software* (v. 3.6.3) and its *IDE Rstudio software* (RStudio Team, Boston, MA; version R v3.6.3) were employed to perform the statistical analysis.

3. Results

Viral quantification from environmental samples implies the inclusion of reliable controls of viral recovery to avoid false negatives and verifying the extent of inhibition in each sample, especially in shellfish samples due to the complicated nature of the matrix. For this purpose, other *Betacoronavirus* (the murine hepatitis virus MHV-A59), was selected as an enveloped surrogate virus with a similar structure to SARS-CoV-2. Samples showed extraction efficiencies ranging from 4.37% to 13.88% in sediment samples and from 4.22% to 19.31% in clam samples, providing enough quantification reliability, reproducibility, and consistency for the subsequent analysis and interpretation.

SARS-CoV-2 RNA was detected in 9/12 clam samples and in 3/12 sediment samples by RT-qPCR. Cq values ranged from 36 to 39.9 depending on the target region and the type of sample. For shellfish samples, Cq values ranged from 36.02 to 39.49 (IP4 region); from 38.02 to 39.81 (E region) and from 37.79 to 39.97 (N1 region). Four out of 9 positive clam samples rendered amplification for two different target regions (two samples simultaneously positive for N1 and E region, one sample for IP4 and N1 region and one sample for IP4 and E region). Quantification values ranged from <LoQ to 4.48 Log gc/g of DT. On the other hand, in sediment samples, only IP4 region was successfully amplificated, showing Cq values ranged from 37.98 to 39.92. Quantification values ranged from <LoQ to 3.60 Log gc/g sediment. Both for clam and sediment positive samples, most of the quantification values were obtained in diluted replicates, indicating the presence of PCR inhibitors. No statistical differences were observed among quantification values among clams and sediment samples or between sampling location. Quantification values for each target region and sample type as well as RNA extraction efficiencies calculated using the murine hepatitis virus MHV-A59 are shown in the Table 2.

PMAxx-triton viability pretreatment combined with RT-qPCR assays were carried out in parallel to evaluate the integrity of the virion capsids. After pretreatment with PMAxx-Triton SARS-CoV-2 RNA signals were removed. These findings indicate the detection of non-encapsidated genomes or, at least, capsid-altered virions, which evidence a non-infectious state of the detected virus.

Regarding crAssphage detection, 100% of both clam and sediment samples rendered positive results. CrAssphage concentrations ranged from <LoQ to 5.94 Log gc/g DT and from <LoQ to 4.71 Log gc/g sediment (Table 2, Fig. 2). Linear regressions and Spearman's rank correlation coefficients (r) calculated between SARS-CoV-2

Table 2
Detection and quantification of SARS-CoV-2 RNA and crAssphage DNA in clam and estuarine sediment samples along the studied period in the natural clam banks 1 (B1) and 2 (B2). Concentrations are expressed as LOG genomic copies (gc) of RNA per g of sediment (Sed) or g of digestive tissue (DT) for each target region (IP4, E and N1). Values for both undiluted RNA (neat) as well as its correspondent 10-fold dilution (diluted) are shown. The extraction efficiency (Eff. %) calculated using the murine hepatitis virus (MHV-A59) for each sample is also shown. –, not detected; <LoQ, below quantification limit.

| Date | Bank | MHV Eff. % | | NA | crAssphage | | SARS-CoV-2 | | | | | |
| | | | | | | | IP4 | | E | | N1 | |
		Sed	Clam		Sed	Clam	Sed	Clam	Sed	Clam	Sed	Clam
06.05.20	B1	4.37	8.29	Neat	–	–	–	–	–	–	–	<LoQ*
				Diluted	3.15	5.94	3.34	–	–	–	–	3.64
	B2	12.39	7.40	Neat	–	5.25	–	–	–	–	–	<LoQ
				Diluted	2.63	5.30	<LoQ	4.48	–	–	–	–
25.05.20	B1	6.32	9.12	Neat	2.26	4.81	3.60	–	–	3.60	–	–
				Diluted	2.54	–	<LoQ	–	–	–	–	3.08
	B2	7.09	11.55	Neat	–	5.02	–	–	–	–	–	–
				Diluted	3.26	5.10	–	–	–	–	–	3.38
03.06.20	B1	6.16	10.87	Neat	–	4.53	–	–	–	–	–	–
				Diluted	3.16	3.54	–	–	–	–	–	–
	B2	13.88	8.78	Neat	–	3.94	–	–	–	–	–	<LoQ
				Diluted	4.71	3.49	–	–	–	3.73*	–	3.59
22.06.20	B1	7.46	7.49	Neat	1.59	4.92	–	–	–	<LoQ*	–	–
				Diluted	2.97	–	–	–	–	–	–	–
	B2	8.96	4.55	neat	4,52	–	–	–	–	–	–	<LoQ
				diluted	4.18	3.43	–	–	–	–	–	3.60
06.07.20	B1	4.78	3.27	Neat	–	3.63	–	–	–	–	–	–
				Diluted	2.63	3.63	–	–	–	–	–	–
	B2	7.10	5.08	Neat	–	–	–	–	–	–	–	–
				Diluted	2.79	3.25	–	–	–	–	–	–
20.07.20	B1	7.49	4.22	Neat	2.06	4.37	–	–	–	<LoQ	–	–
				Diluted	–	4.82	–	–	–	–	–	–
	B2	6.70	19.31	Neat	–	4.34	–	<LoQ	–	<LoQ	–	–
				Diluted	4.34	–	–	–	–	–	–	–

* Sample positive in two independent homogenates.

and crAssphage concentrations using two-tailed 95% confidence intervals showed non-statistical differences. However, Student's t-test carried out between crAssphage quantification in clam and sediment samples showed a very strong statistical difference ($p = 8.64 \times 10^{-5}$). Student's t-test also indicated that quantification values for crAssphage in bank 2 are statistically higher than in bank 1 ($p = 0.03$).

4. Discussion

This study offers important information about the fate of SARS-CoV-2 in the coastal environment and marine organisms, demonstrating that it could reach, under some circumstances, not only river streams but also other environmental compartments like estuarine and marine habitats. Contrarily to Desdouits et al. (2021), which also evaluate the
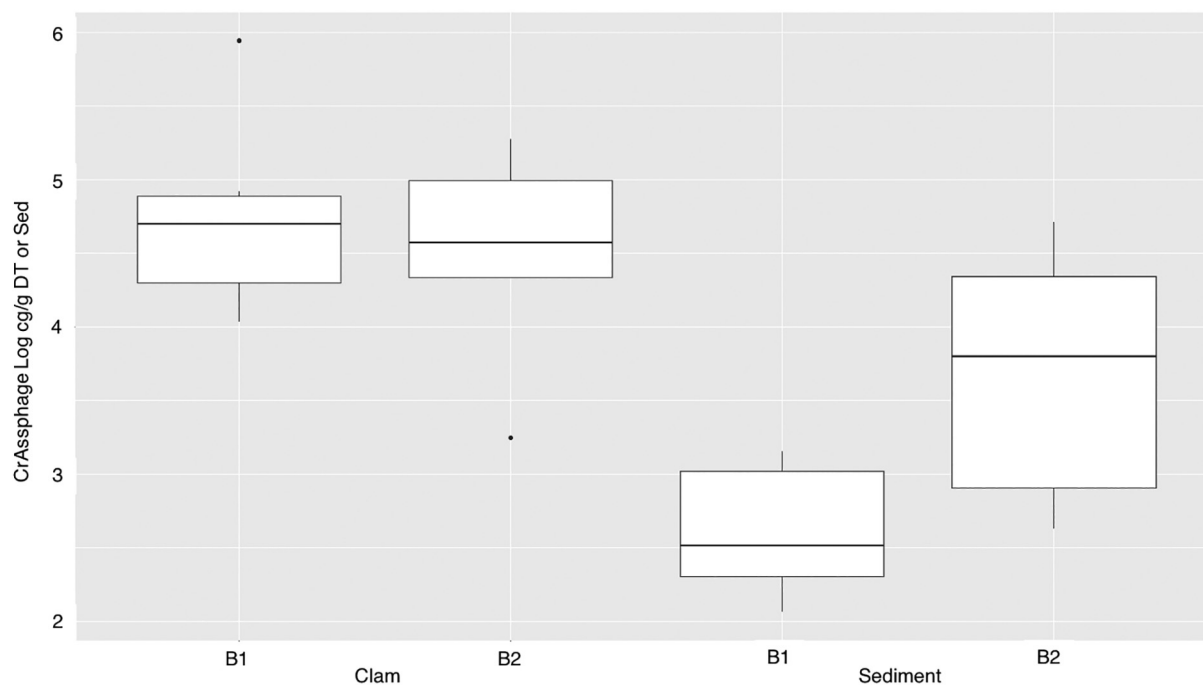

Fig. 2. Boxplot diagrams showing crAssphage quantification in clams and sediment samples in the natural clam bank 1 (B1) and 2 (B2). Results are expressed as Log copies of genome (cg)/g of digestive tissue (DT) or sediment (Sed).

potential seawater and shellfish contamination SARS-CoV-2 in France without detecting any presence of viral RNA, in our study the presence of SARS-CoV-2 RNA in both clam banks of the studied area suggest that at least virus traces were released in the river basins. Although it would be interesting to assess potential correlations between RNA quantification levels and clinical epidemiological trend on the studied area, the relatively short sampling period of environmental samples did not allow to perform this kind of analysis.

Randazzo et al. (2020) already demonstrated that WWTPs with only secondary treatment, can release SARS-CoV-2 RNA in their effluents. Interestingly, surface waters and seawater can contain much higher virus concentrations than treated wastewater, particularly in the case of combined sewer overflows events, where untreated wastewater is discharged into natural water bodies (Phillips et al., 2012; Passerat et al., 2011). Discharges from combined sewer overflows into rivers is very common in many countries, including the European Union and the United States (Butler and Davies, 2004; U.S. Environmental Protection Agency (EPA), 2004) and the presence of SARS-CoV-2 RNA in rivers was also already reported (Guerrero-Latorre et al., 2020; Rimoldi et al., 2020). There is a need to monitor WWTP failures and non-treated discharges that could be at high risk of introducing effluents carrying potentially infective SARS-CoV-2 into natural water systems to reduce the potential risk of virus spillover.

This study may have other implications, since these data may raise concerns about food safety and the risk for the consumers. It is important to note that the mere presence of genetic material of the virus in shellfish does not mean that an infection could take place. To date, no evidences for transmission linked to the ingestion of food exist, and the recent cases associated with frozen food imports reported in China (Han et al., 2021; Liu et al., 2020) are related to the presence of SARS-CoV-2 in the outer food package's surface, which would act as any other fomite (Rose-Martel et al., 2021). The international consensus indicated that it is highly unlikely that the ingestion of SARS-CoV-2 will lead to the disease so shellfish should not be considered a risk or a vector of SARS-CoV-2. In addition, the results obtained here from the PMAxx-triton viability RT-qPCR assay as well as the inconsistent RNA detection for three different target regions indicate that there is a very low risk for public health. Therefore, we state that these data should not be wrongly perceived as a food safety hazard nor, therefore, a basis for unfounded restrictions or disruptions in shellfish market, trade or initiating food recalls.

Our findings indicate a non-infective state of the viral signals in clams or sediments, but do not allow to discriminate between partially degraded virions or completely naked viral RNA. Environmental RNA (eRNA) is thought to degrade fast, however, recent lines of evidence suggest that RNA may persist in the marine environment for much longer than expected (Cristescu, 2019). Previous studies indicate that binding with organic or inorganic compounds or stabilization of RNA molecules in biofilm matrices can provide a prolonged persistence of eRNA and may counterintuitively assist eRNA preservation (Wood et al., 2020). In fact, recent studies showed that SARS-CoV-2 RNA is more stable than the infectious particles in river and seawater (Bivins et al., 2020; Sala-Comorera et al., 2021; Wurtzer et al., 2020). Further studies are guaranteed on the viral RNA persistence in the environment and the true complexity of the "ecology" of viral RNA in aquatic systems.

As stated above, the risk of infection with SARS-CoV-2 from shellfish samples can be considered remote. To date, infectious SARS-CoV-2 has not been detected in untreated or treated wastewater or aquatic environments and, therefore, wastewater does not appear to be a significant transmission route for SARS-CoV-2 (Rimoldi et al., 2020; Westhaus et al., 2021). Data on infective virus survival in wastewater is very limited and do not correlated with RNA concentrations. To our knowledge, only three studies have been addressed the survival of infective SARS-CoV-2 in aquatic environments, including wastewater, river water and seawater (Bivins et al., 2020; de Oliveira et al., 2021; Sala-Comorera et al., 2021). The reported T_{90} values for viable SARS-CoV-2 varied from 1.1 and 3.8 days, depending on the type of water and temperature assayed.

More research on survival of infectious SARS-CoV-2 in wastewater at colder temperatures and in environmental waters is needed to determine if is this time enough for SARS-CoV-2 virions to reach in an infective state the marine environment and to better interpret the potential risks. We agree with Maal-Bared et al. (2020) stating that it is becoming increasingly common to overemphasize the risk resulting from exposure to wastewater and we should avoid it. Nevertheless, the presence of viral RNA in the marine environment should make us adopt a conservative and precautionary stance as we cannot exclude that some viable viral particles could potentially reach the aquatic environment, especially when untreated sewage is directly discharged into coastal areas and in a pandemic scenario where a large part of the population is excreting the virus. In this sense, it has been recently hypothesized in a preprint that the detection of natural SARS-CoV-2 infection in two wild minks in a river environment in Spain could be related with exposure to contaminated wastewaters (Aguiló-Gisbert et al., 2021). Therefore, the impact on marine wildlife health should be also evaluated, especially on aquatic mammals like *Cetacea*, seals and sea otters, which maintain the majority of key receptor binding domains for SARS-CoV-2 (Luan et al., 2020; Mathavarajah et al., 2021).

To prove the presence of human-derived fecal material in both banks of the study area we analyzed both the sediment and clam samples for the presence of crAssphage. CrAssphage (cross-assembly phage) is a bacteriophage, discovered in 2014 by computational analysis of human fecal metagenomes (Dutilh et al., 2014). This bacteriophage, that infects the human gut symbiont *Bacteroides intestinalis*, is highly abundant in the human microbiome on a global scale (Guerin et al., 2018; Shkoporov et al., 2018). Recently, Stachler et al. (2017) developed primers targeting conserved genomic regions to evaluate the abundance of crAssphage as an indicator of human fecal pollution (Stachler et al., 2017) demonstrating its usefulness for impacted urban watersheds (Stachler et al., 2018). The evaluation of crAssphage as a molecular marker for the fecal contamination of the marine environment was also recently reported, although in this case the literature is still very scarce. To our knowledge, only Farkas et al. (2019) reported the frequent presence of crAssphage in marine sediments and mussels and demonstrated that this human-gut associated bacteriophage may be also a suitable fecal pollution indicator in those matrices. In this study, the systematic detection of crAssphage in sediments and clams is reported. Interestingly, statistical analysis also showed that quantification levels for the crAssphage in clams are significantly higher than in sediments. Although further studies are needed, these findings extend those previously reported from Farkas et al. (2019) and suggest not only the great potential of crAssphage as a tool for source tracking of human fecal pollution in shellfish harvesting areas, but also demonstrate the suitability of clams as prospective bio-monitors or sentinel organisms for viral pollution in the marine environment.

Bivalve mollusks are known to efficiently accumulate a variety of anthropogenic contaminants from their surroundings waters and some species like mussels (*Mytilus* spp.) are widely used as a bioindicator for monitoring of coastal water pollution (mussel watch programs) (Beyer et al., 2017). Although monitoring of SARS-CoV-2 in sewage outfalls to avoid contamination of coastal environments constitutes the first choice for control, bivalves could be also used as a surveillance tool, especially in cases of non-point sources or direct discharges of wastewater into small estuaries as well as when sewage sampling is not possible. In this sense, further insights are needed about whether infaunal species, like clams or cockles, could be more prone to bioaccumulate SARS-CoV-2 by its intimate relationship with the sediment since, as previously reported, enveloped viruses tend to associate with particulate matter (Ye et al., 2016). In addition, it was described very recently that heat inactivated SARS-CoV-2 can be bioaccumulated in oysters (Desdouits et al., 2021).

The findings present here can be logically extended to other marine habitats where sewage is directly discharged into natural streams. In this sense, site selection and timing are important factors for catching any viral signal. Small, intricate and closed estuaries with higher water renovation times and impacted by non-treated sewage overflows, similar to this study area, are of special concern, as they are more prone to retain the fecal contamination. The importance of the hydrography and oceanographic conditions like tidal movements and on shore winds in estuaries and habitats highly influenced by these processes and impacted by sewage discharges should be evaluated, as they can allow viruses to come into contact with shellfisheries on numerous occasions, favoring re-contamination events (Robins et al., 2019).

5. Conclusions

To the best of our knowledge, we reported here the first detection of SARS-CoV-2 RNA in a marine organism, demonstrating that SARS-CoV-2 virions, or at least their RNA, may reach coastal waters. The results obtained in the indirect viability assays suggest a non-infectious state of the virus and a high degree of degradation of its nucleic acid, which imply an extremely low risk of acquiring SARS-CoV-2 from shellfish consumption. However, more insights are needed on its occurrence and potential persistence in aquatic systems as well as the potential risks for the marine life.

CRediT authorship contribution statement

David Polo: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Marta Lois:** Investigation, Formal analysis. **María Teresa Fernández-Núñez:** Investigation, Writing – review & editing. **Jesús L. Romalde:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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