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Molecular characterisation of HEV and NoV in mussels from Oued El Maleh estuary in Morocco

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ABSTRACT

Hepatitis E virus (HEV) and Norovirus (NoV) are two enteric viruses responsible for mild or acute gastroenteritis and hepatitis. These viruses are known for their resistance to environmental conditions, and transmitted by the consumption of contaminated water. Shellfish produced close to land can bioaccumulate enteric viruses of human and animal origin, including zoonotic hepatitis E virus that infect both human and swine. Hepatitis E virus (HEV) represents one of the principal causative agents of hepatitis, and norovirus (NoV) is the first causative agent of childhood gastroenteritis in the world, globally causing huge healthcare-associated economic losses. The aim of this study was to evaluate HEV and NoV contamination in samples of mussels (Mytilus edulis Linnaeus, 1758) in coastal area of Mohammedia. Indeed 48 batches of samples of blue mussels (n=576 mussels) were collected from three points of at the mouth of the Oued El Maleh were analyzed for the detection of HEV and NoV using RT-PCR in real time. Overall, one (2%) of these samples tested positive for HEV RNA and thirteen (27%) for NoV. To our knowledge, this is the first notification of the detection of HEV and NoV in mussels collected in the mouth of Oued El Maleh. These findings suggest that a health risk may exist for users of waters in the the coast of Mohammedia and to consumers of shellfish. Monitoring HEV and NoV and similar viruses in shellfish can help prevent viral contamination. Further research is needed to assess the sources and infectivity of HEV in these settings, and to evaluate additional shellfish harvesting areas.

1. INTRODUCTION

Enteric viruses include more than 140 pathogenic viruses associated with waterborne transmission that can infect humans and animals after ingestion and multiplication in the gastrointestinal tract or in the liver [1]. Obligate intracellular parasites, they present diverse structures and belong to different families and genera. They are excreted in the faeces and spread mainly by the faecal-oral route. In humans, they are responsible

for various pathologies, potentially serious, even fatal (acute hepatitis, gastroenteritis, poliomyelitis, meningitis, myocarditis, etc.) [2], [3]. Nororovirus (NoV) and hepatitis E virus (HEV) are among the most worrying viruses in terms of collective poisoning [2]. Norovirus is the main etiological agent of acute gastroenteritis of viral origin in developed countries. This virus is responsible for 67% of foodborne outbreaks and more than 90% of outbreaks that occur in closed communities [4]. Thus 219,000 deaths are attributed to it annually [5], [6].

Noroviruses show remarkable resistance to environmental conditions [7], [8]. They lack a lipid envelope, which makes them less sensitive to physicochemical denaturing agents, and adsorption on suspended matter increases their resistance to disinfectants [9]. They are resistant to various factors such as temperature variations oscillating between -20°C and 60°C, at a pH between 3 and 10, to UV, for which the inactivation is dose and time dependent, and remain stable at chlorine concentrations up to 300 ppm [10]. They thus retain their infectivity in drinking water treated with a chlorine concentration reaching 10 ppm [11]. They are also stable after exposure to quaternary ammonium products, soaps, 70% ethanol, hypochlorite and organic solvents [12].

As for HEV, it is responsible for approximately 20 million infections worldwide according to WHO data, resulting in approximately 3.3 million symptomatic cases of hepatitis E and 56,600 deaths, with significant losses economics associated with health care [13]. A food safety risk may result from HEV contamination of drinking water or irrigation water via animal manure or sewage, leading to concomitant contamination of vegetables, fruits or shellfish [14], [15]. This explains the presence of HEV sequences that have been reported on soft fruits and vegetables, with irrigation water as the suspected source of contamination [16], [17].

Viruses are inert outside the cell of the living host and do not multiply in water or shellfish but their presence in these molluscs is the result of contamination in the surrounding water. Molluscan feeding behavior facilitates the bioaccumulation of pathogens, including viruses [18- 20]. In addition, a long viral persistence has been demonstrated in these marine animals [18], [20], [21]. During epidemic outbreaks of viral diseases, the foods most frequently incriminated are bivalve molluscs [22], [26].

The transmission of viral diseases to humans through the consumption of seafood products has been known since the 1950s [27], and human enteric viruses appear to be the main cause of diseases attributable to shellfish [28] including hepatitis E and noroviruses. Coastal waters, streams, rivers and lakes are routinely contaminated by septic tanks, stormwater, and runoff from farm and animal manure, used in agriculture, and effluents from poorly operated sewage treatment plants or overflows from sewage treatment plants impacted by floods or by the direct influx of untreated wastewater [29].

Genotypes 1 and 2 of HEV are prevalent in developing countries, including countries in Asia, Africa, and Central America [30]. Both genotypes are mainly restricted to humans and are transmitted by the consumption of fecal-contaminated water in areas with poor sanitation [31]. On the other hand, genotypes 3 and 4 have been confirmed as the main causes of zoonotic HEV worldwide; these genotypes represent the main reservoirs found in pigs and wild boars [27], [32], [33]. Consumption of bivalve molluscs may contribute to an increasing risk of zoonotic and foodborne circulation of HEV [34], [35], especially genotype 3 (G3) and genotype 4 (G4), which can infect both humans and other mammals [36].

Shellfish harvesting areas contain variable amounts of viruses which form a part of the microbial plankton. However, other viruses that are excreted by infected people and animals (even when they are asymptomatic) have also been found in these shellfish harvesting areas, especially when these harvesting areas are located

near urban or livestock areas [37], [38].

In this study we will proceed by the molecular technique using RT-PCR in real time and in one step for the detection of HEV and NoV in the mussels of the estuary of Oued El Maleh in Mohammedia, this zone was reported marked by its insalubrities and its pollution by enteric viruses such as EV, AdV, HAV and RVA [39- 41].

2. Materials and methods

2.1 Hydrological and ecological study of the area

The Oued El Maleh estuary of Mohammedia provides important ecosystem services. It is known for its high biological and ecological productivity which makes it a natural reserve sheltering rare species of migratory birds, which finds there a rich and favourable ecosystem, it is also an area for fishing and tourism activities. Many indices have indicated the importance of assessing environmental risks and estimating the anthropogenic contribution to faecal pollution in the area. The Oued El Maleh estuary in Mohammedia (33°42'12.84"N and 7°24'44.04"W) is located on the Atlantic Ocean (Figure 1) it is characterized by its brackish water, the exchange with the ocean is made through the mouth at the time of high tide and base tide. This watercourse is constituted by the morphology of a depression, oriented southeast, bordered by a coastal cliff and a continental dune cord.

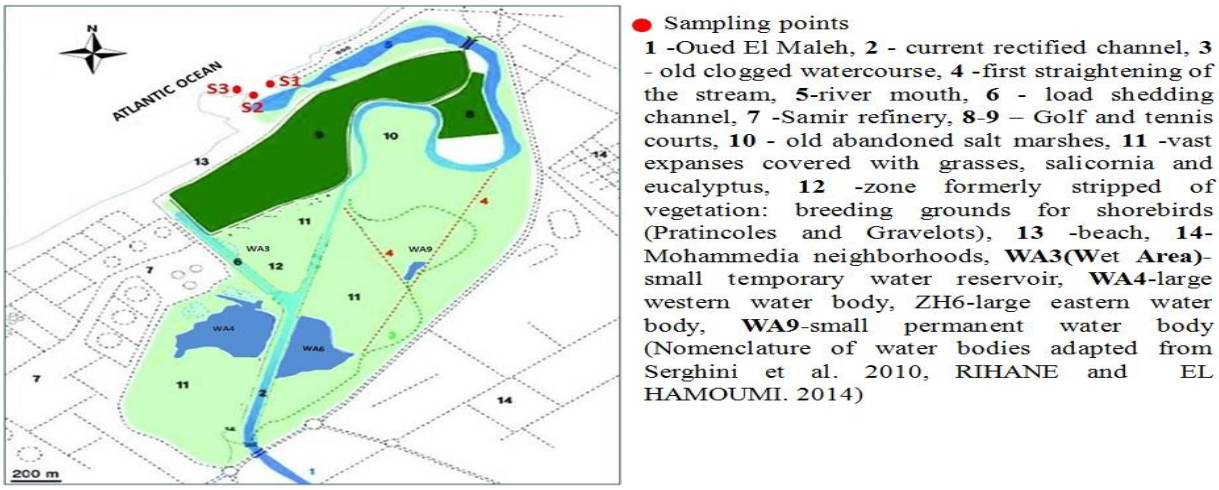

Figure 1: Delimitation of the wetland (RAMSAR) of Mohammedia [41].

The Oued El Maleh crosses the wetland of Mohammedia, this wetland is listed among one of the four Moroccan sites considered by the RAMSAR Convention to be of international interest, which provides a valuable coastal environment for a variety of species of birds, fish, wild plants and animals. It is a very important wintering natural park for migratory birds in Morocco.

This wetland is exposed to grazing, to the discharge of wastewater and the leaching of leachate from the city's former public landfill in its watershed, so various types of pollution can affect the health of the estuarine ecosystem [41], [42]. Polluting and non-polluting human activities have been established in the area, and rapid urbanization has taken place in recent years near this ecosystem and is beginning to damage the natural ecological and biological characteristics.

2.2 Climatological study of the area

The sampling period was marked by irregular rainfall, where the wet period was limited to the months of October, November, January and February with varying heights between 1 mm and 165 mm (Table 1).

Table 1: Average monthly precipitation during the sampling period (July -2018 to July -2019)

Months	Rainfall (mm)
July -18	1
Aug-18	1
Sep-18	2
October-18	70
November-18	165
December-18	3
January-19	20
February-19	41
March-19	0
April-19	0
Mai-19	0
Jun-19	0
July -19	0

2.3 Choice of samples

At the mouth of the Oued El Maleh estuary, on the rocks of the coast of Mohammedia, samples of mussels were taken from 3 sites (S1, S2 and S3) in the direction of the sea currents. The sampling was carried out over a period of one year from 07/02/2018 to 07/03/2019. A total of 576 samples of blue mussels (*Mutilis edulis*) separated into 48 batches of 10 to 12 individuals were transported in sterile and dated plastic boxes and stored at +4°C. Harvesting was done at three separate sites before being mixed, we targeted the adult mussels of the most homogeneous sizes possible (approximately 6 to 8cm) and then, the samples are quickly sent to the laboratory and dissected the same day.

2.4 Dissection

The mussels are washed aseptically and separately with distilled water to remove sand and algae debris and then opened with a knife, starting with the ventral part. Using forceps and a clean scalpel, only the hepatopancreas and the intestine are removed in sterile cryotubes and stored at -80°C for subsequent extraction of total RNA. An aliquot of about 1.5g (about ten to twelve mussels) is needed for the analysis.

2.5 Virus concentration and RNA extraction

2.5.1 Virus concentration

The hepatopancreas and intestines (about 1.5 ± 0.2 g) were dissected, homogenized and diluted with equal volumes of glycine buffer (0.05 M glycine, 0.15 M NaCl, pH 9.0) according to the method described by [41]. The viral concentrate was stored at -80°C until used for viral RNA extraction.

2.5.2 Extraction of total RNA

RNA extraction was performed from the virus concentrate by Trizol (Invitrogen™), according to the manufacturer's instructions, and the qualities of the extracted RNAs were assessed spectrophotometrically at 260 and 280 nm using a NanoDrop 2000/200c spectrophotometer (Thermo Fisher Scientific, USA).

2.6 Molecular characterization of HEV by RT-PCROneStep

The Qiagen OneStep RT-PCR kit (Qiagen®, Hilden, Germany) was used for the HEV amplification. To obtain the standard curve, a stock solution of HEV (10^8 RNA copies/µL) was used. Indeed, 10 µL of each standard are introduced into the last five wells of the plate. In all tests, a positive control (HEV RNA stock solution) and a negative control (RNase-free water) were included. The final volume in each well is 25 µL.

2.6.1 HEV RT-PCR primers and probes

The primers and probe used in real-time RT-PCR, described by [43], target the conserved region in ORF3 and allow the detection of different HEV genotypes. The forward primer (JVHEVF: GGTGGTTTCTGGGGTGAC), reverse primer (JVHEVR: AGGGGTTGGTTGGATGAA) and probe (JVHEVP: TGATTCTCAGCCCTTCGC) sequences. The TaqMan® probe labelled at the 5' end with 6-carboxy fluorescein (FAM) fluorophore and at the 3' end with minor groove binder quencher (MGB). The position of the sequences corresponds to 5261-5330nt of the HEV genome.

2.6.2 Limit of detection of the Qiagen qRT-PCR test

In order to assess the detection limit of Qiagen qRT-PCR, a standard curve was generated using a 10-fold dilution of HEV stock solution (concentration of 10^8 RNA copies/µL), ranging from $4x10^9$ to $4x10^0$ copies/reaction

2.6.3 OneStep RT-PCR amplification for mengovirus

The addition of an external virus such as mengovirus to a shellfish sample has been proposed as a control to assess the extraction efficiency of molecular virus detection methods [44].

2.6.4 Mengovirus RT-PCR primers and probes

The process control was carried out by amplification of the mengovirus gene using the following primers and probes described by [45], (sensMengo110: GCGGGTCCTGCCGAAAGT), and (anti-senseMengo210: GAAGTAACATATAGACAGACGCACA), targeting a non-coding conserved region (5' region) of mengovirus viral RNA and probe (Mengo147: ATCACATTACTGGCCGAAGC), labelled at the 5' end with the fluorophore 6-carboxy fluorescein (FAM), and a quencher at the 3' end by adding a minor groove binder (MGB).

2.6.5 Amplification OneStep qRT-PCR for HEV

The nucleic acid extracts (from mussels) were tested by the Qiagen Onestep RT-PCR kit (Qiagen®, Hilden, Germany). Briefly, at 20µl of mastermix containing: 5µl Qiagen OneStep RT-PCR buffer (1X), 1µL (0.4mM) dNTP mix, 1µL enzyme mix, 2.25µL (900nM) forward primer, 1.25µL (500 nM) reverse primer and 1.125 µL (100nM) probe 5 µL of each extract sample was added undiluted and diluted 1:10 (in RNase and DNase free water). The final reaction volume was 25 µL. The amplification steps were carried out on a real-time thermal cycler, SaCycler-96 (Sacace Biotechnologies). Thermal conditions consisted of initial reverse transcription (RT) (55˚C / 60 min), PCR activation (95˚C / 5 min), denaturation (95˚C / 15 sec), hybridization (60˚C/1 min) and extension (65˚C/1 min). Denaturation, hybridization and extension conditions were repeated for 45 cycles. In all tests, a positive control (mengovirus RNA stock solution) and a negative control (RNase-free water) were included.

2.6.6 Amplification OneStep qRT-PCR for NoV

The real-time RT-PCR for Nov was performed as folloing: The nucleic acid extracts (from mussels) were tested by Techne ® qPCR test Norovirus Genogroups 1 and 2. Briefly, at 20µl of mastermix containing: 10µL of OneStep 2x qRT-PCR MasterMix, 1 µL capsid or RNA-pol primer/probe mix, 1 µL internal extraction

control primer/probe mix, 3 µL RNAse/DNAse free water and 5µL of RNA template into each well. The final reaction volume was 25 µL. The amplification steps were carried out on a real-time thermal cycler, SaCycler-96 (Sacace Biotechnologies). Thermal conditions consisted of initial reverse transcription (RT) (42°C/10mn) followed by 50 cycles of; enzyme activation (95˚C / 2 min), denaturation (95˚C / 10 sec), and data collection (60°C/60 sec).

All samples were analysed in duplicate, and two negative controls (RNase-free water) were included. Five standards 10-fold serial dilution were also amplified.

2.7 Statistical analysis

Spearman's rank correlation analysis was employed to correlate the results of positive samples of rotavirus and HEV pooled by month and pluviometry. All the statistical analyses were performed using the statistical package SPSS Statistics 17.0. The P value above 0.05 was considered as non-significant.

The hypothesis of interest was to see if the environmental parameter, in particular, precipitation, was the cause of the microorganisms presence in shellfish, and if this presence occurred regardless of the impact of anthropogenic activity in the watershed waters used for molluscs production. To verify the existence of a risk factor associated with the presence of microorganisms (HEV and Rotavirus) One-way ANOVA analysis was developed in which the dependent variable result (presence/absence Y=1; Y=0) was placed in relation to independent variables classified as: month (From Julay 2018 to Julay 2019).

3. Results and discussion

Out of a total of 48 shellfish samples tested, only one sample was considered HEV positive, 47 were negative and 3 were above the detection limit (DL) (Table 2). Regarding the positive sample 2W11-18, it gave a positive result when tested undiluted, and showed no amplification when tested diluted. This could be due to a small amount of HEV RNA that could not be detected after sample dilution (less than 10 HEV RNA copies/Tube). Samples 5W07-18, 3W09-18, and 2W05-19 had a Ct value greater than 36.52 when tested undiluted, but no amplification when tested diluted. These samples were not considered positive because we cannot guarantee that their high Ct values were due to the presence of a very low amount of HEV RNA (not detectable in the diluted sample) or to a non-specific reaction, while all blank control samples in the OneStep RT-PCR assays were HEV-negative. As for NoV, by reverse transcription and real-time RT-PCR, NoV and HEV were detected in 27% and 2% of shellfish, respectively, with prevalence of 10/13 (77%) and 4/13 (30.7%) of NoV positive samples for NoV GII and NoV GI. Based on the statistical analysis, the detection rate of NoV was significantly higher than HEV in mussels ($P < 0.05$).

Table 2: RT-PCR detection of HEV and NoV in mussels from l'Oued El Maleh Estuary

Week	HEV	NoV GI	NoV GII	Week	HEV	NoV GI	NoV GII
1W07-18	NA	NA	NA	2W01-19	NA	<LD	+
2W07-18	NA	NA	NA	3W01-19	NA	NA	+
3W07-18	NA	NA	NA	1W02-19	NA	+	NA
4W07-18	NA	NA	NA	2W02-19	NA	NA	+
5W07-18	<LD	NA	NA	3W02-19	NA	+	+

1W08-18	NA	NA	NA	4W02-19	NA	NA	+
2W08-18	NA	NA	NA	2W03-19	NA	NA	NA
3W08-18	NA	NA	NA	3W03-19	NA	NA	NA
4W08-18	NA	NA	NA	1W04-19	NA	NA	NA
1W09-18	NA	NA	NA	2W04-19	NA	NA	NA
3W09-18	<LD	NA	NA	3W04-19	NA	NA	NA
1W10-18	NA	<LD	NA	4W04-19	NA	NA	NA
2W10-18	NA	NA	+	1W05-19	NA	NA	NA
3W10-18	NA	NA	NA	2W05-19	<LD	NA	NA
4W10-18	NA	+	NA	3W05-19	NA	NA	NA
1W11-18	NA	NA	+	4W05-19	NA	NA	NA
2W11-18	+	<LD	+	1W06-19	NA	NA	NA
3W11-18	NA	+	NA	2W06-19	NA	NA	NA
4W11-18	NA	NA	+	3W06-19	NA	NA	NA
1W12-18	NA	NA	NA	4W06-19	NA	NA	NA
2W12-18	NA	NA	+	1W07-19	NA	NA	NA
3W12-18	NA	NA	NA	2W07-19	NA	NA	NA
4W12-18	NA	NA	NA	3W07-19	NA	NA	NA
1W01-19	NA	NA	NA	4W07-19	NA	NA	NA

NA: Not amplified, <LD: Below Detection Limit, + : Positive

Environmental samples may have specificities because of the different types of pollution that may be present. Thus, seawater could contain heavy metals which would limit enzymatic activity during analysis by RT-PCR [46- 48]. The RT-PCR test results showed how important it is to test diluted shellfish samples to avoid underestimating virus-positive samples due to the presence of inhibitors [49- 51]. Similarly, samples should also be tested undiluted to avoid underestimation of samples with low viral concentration. Indeed, the detection of viruses in shellfish is mainly hampered by the presence of PCR inhibitors and a low viral concentration.

The noroviruses were detected in 27% of samples, this result is comparable with those found in the same shellfish samples (Mytilus edulis) showed in another study [41] the presence of rotavirus with a prevalence of 37.5% (18/48), while the presence of a positive case of HEV (2%) indicates that there could also be a

contamination by HEV in the coastal waters of the Mohammedia estuary.

The risk of finding mussels positive for HEV in the year 2019 compared to NoV or RVA was significantly lower. The analysis of the variable month confirms the above data with low risks, statistically significance, and a positive only November, which coincides with the peak of rainfall.

The data analysis showed that the presence of a positive case of HEV which coincides with winter highlighting accordingly that the presence of this organism shows more or less certain seasonality (Tables 2). Based on the statistical analysis, the detection rate of HEV was not significant in the mussels with P=0.568 (P >0.05), in contrary with a study conducted on the same sample on rotaviruses, which showed a highly significant correlation with the precipitation with p = 0.017 (<0.05) [41]. These results are explained by the fact that contrary to other enteric viruses (Rotavirus, Adenovirus, Enterovirus), the presence of HEV is not only linked to the presence of wastewater but rather to the presence of infected humans or contact with infected animals [30], [52]. However, other viruses that are excreted by infected people and animals (even when they are asymptomatic) have also been found from shellfish harvesting areas, especially when these harvesting areas are located near urban or livestock areas [20], [37], [53].

We noticed a correlation between the prevalence of NoV and the cold months, this can be explained by the fact that this virus id causal agent of "Winter vomiting disease", it is characterized by a marked seasonality with a winter peak of acute gastroenteritis (AGE). At the origin of worldwide epidemics called "intestinal flu" or "stomach flu", it is the first virus of gastroenteritis identified in humans [54], [55]. The rather remarkable contamination of batches of shellfish, collected mainly during the winter period, enteric virus concentration of bivalve molluscs preferentially during the winter period [2]. The survival of the virus is favored by low water temperatures and the efficiency of accumulation of enteric viruses by oysters is accentuated in cold periods [56], [57].

In Morocco, few epidemiological studies have been conducted to assess the prevalence of noroviruses. The seasonality of NoVs is a poorly understood phenomenon, but could be related to its ability to survive in the presence of environmental stressors [56].

In our study, all NoV-positive oyster samples were taken during the cold season. Similar studys in Morocco, in particular the work of [58], which was 7% in samples of oysters taken from the Oualidia region between 2016 and 2017, and the work of [59] during the period 2009-2010, the values are significantly different compared to other studies carried out at other sites in different geographical regions: 35% in Tunisia [60], 25.6% in Galicia, Spain [61], 14.2% in Puglia, Italy [62], and 5% in Japan [63]. However, another study [64] reported that detection of NoV in winter is less evident in the Middle East and North Africa than in the other northern hemisphere countries where NoV infections occur mainly during this season [55].

To our knowledge, this is the first study describing the presence of HEV in mussels harvested in the coastal area of Mohammedia, although the presence of other human enteropathogenic viruses, such as EV, AdV, HAV and RVA have already been reported in the region's coastline [39- 41]. Given that hepatitis E was reported in southern Morocco by [65], during a seroprevalence study, which made it possible to highlight the circulation of HEV in the south of the country and to prove that Morocco is endemic for HEV.

HEVs are characterized by their development in generally tropical and subtropical areas with high population density and where contamination of drinking water by faeces is often suspected [66]. HEV is endemic in Morocco and anti-HEV IgG has been detected in 6.1%–10.4% of subjects particularly in the west and the

south of Morocco among healthy adult [3]. In the other hand, Morocco has experienced in recent decades a significant migratory flow of sub-Saharan foreigners from endemic regions, these immigrants are probably carriers of HEV, this remains to be clinically confirmed, but the aquatic environment of the estuary has certainly been exposed to polluted wastewater by human or animal faeces [41].

The hypothesis that molluscs can be indirectly contaminated by both human and animal faeces is also highly probable, given that many homes still use septic tanks in the nearby urban area (Sablettes district, Monica district, etc.) as well as the overflow of the unit networks following the heavy rainfall experienced by the region at the time of sampling (November 2018), to this factor is added the leaching of waste from pig farms, and the faeces of wild boars [20], [41], [66], [67] from the Benslimane region belonging to the same watershed.

4. Conclusion

Although the norovirus and the hepatitis E virus are regularly implicated in foodborne illnesses and even if reliable analytical methods allowing their search in risk matrices have been developed, they do not time subject to any regulatory constraint. Indeed, the detected viral genome does not reflect its infectious nature and its absence is not a reliable indicator of non-contamination. It is therefore important to combine complementary approaches to interpret and assess the viral hazard carried by food.

Shellfish represent the first food vector of faecal contamination because of the direct contact of these species with environmental pollution and the growing demand for this product. Indeed, in areas where infectious HEV and NoV is present, an environmental assessment of the risk threatening human health and the establishment of an alert system accompanied by a restriction on the consumption of shellfish must be systematically maintained by the health authorities, who should alert sanitation utilities and water supply utilities to implement water treatment processes like filtration and disinfection.

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6. Conflict of Interest

The authors declare that there is no conflict of interest

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