One-year monitoring of potentially pathogenic microorganisms in the waters and sediments of the Lesina and Varano lagoons (South-East Italy)

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Abstract

In this study, two Mediterranean coastal lagoons (Lesina and Varano) of southern Italy, located in the north of the Apulia region, were investigated for the presence of Shiga toxin *Escherichia coli* (STEC) and potentially enteropathogenic *Vibrio* species in parallel with norovirus (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This study aimed to evaluate the presence of potentially pathogenic bacteria and viruses in the water and sediments of these ecosystems. From March 2022 to February 2023, a total of 98 samples were collected: 49 water samples and 49 sediment samples. STEC strains were isolated in three samples (3.1%), of which one (2%) was water (*stx1* and *stx2* positive) and two (4.1%) were sediment (both *stx2* positive) samples. *Vibrio* spp. were detected in twenty samples (20.4%), of which nine were water (18.4%) and eleven were sediment (22.4%) samples. The species detected included *V. parahaemolyticus*, *V. cholerae,* and *V. vulnificus*. NoV was detected in 25 (25.5%) samples, while none of the water or sediment samples were positive for HAV, HEV, and SARS-CoV-2. The results of this study provide an overview of the presence of potentially pathogenic microorganisms in areas influenced by anthropogenic pressure. Monitoring the circulation of these pathogens could be useful to evaluate the water flowing into the lagoons, in particular discharge waters (*i.e.,* urban, agricultural, and livestock runoff), considering the presence of fish and shellfish farms in these sites.

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Introduction

Mediterranean lagoons are coastal marine environments located between land and sea, generally separated from it by coastal barriers made of sand, shingles, or, less frequently, rocks, and connected to it through channels or inlets. Exchanges with the sea are limited, and continental inputs derive from freshwater, groundwater, and discharge water from various human activities (*e.g.*, urban, agricultural, recreational) (D'Adamo *et al.*, 2014; Caroppo *et al.*, 2018). Due to their position, lagoons have peculiar functional and structural features (Caroppo *et al.*, 2018). They provide high biological diversity and numerous ecosystem services such as fishing, aquaculture, and recreational and cultural activities (Pérez-Ruzafa *et al.*, 2011). Such ecosystems are characterized by physiographic and ecological properties that are difficult to find in other aquatic environments (Renzi *et. al.*, 2019).

In this study, two lagoons in southern Italy (north Apulia region), Lesina and Varano, categorized as transitional water according to the Water Framework Directive (European Parliament and Council of the European Union, 2000 - 2000/60/EC), were investigated. These lagoons have relevant biological and economic value. The lagoons of Lesina and Varano are part of the Gargano National Park and are recognized as Sites of Community Importance (IT9110015 and IT91100001, respectively) according to the Birds and Habitats Directive (Council of the European Communities, 1992 - 92/43/EEC; European Parliament and Council of the European Union, 2009 - 2009/147/EC). These areas are also designated as both

Special Protection Areas and Special Areas of Conservation (IT9110037). These two lagoons represent one of the most important areas at national and international levels for aquatic avifauna and the quality of the environment. In these areas, several bird species, including some rare ones, stop and spend the winter. These sites contain numerous priority habitats such as dune sclerophyllous scrubs and juniper dunes, pine forests, and Mediterranean salt steppes (Natura 2000, Italian Ministry of Environment and Energy Security). These ecosystems provide a significant income source for the local economy through fishing, tourism, and aquaculture (Fabbrocini *et al.*, 2017; Caroppo *et al.*, 2018). Shiga toxin-producing *Escherichia coli* (STEC) are known to cause severe illnesses such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) infections, thus representing a public health issue. STEC are zoonotic bacteria, especially associated with the consumption of contaminated food and water (European Food Safety Authority BIOHAZ Panel, 2020). Ruminants, particularly cattle, are the primary reservoirs of STEC. They shed these pathogens through their feces in farm environments, contaminating irrigation water or other surface water and vegetables (Bell *et al*., 2021). *Vibrio* species are natural inhabitants of aquatic environments (freshwater, estuarine and marine environments). The genus comprises more than 100 confirmed species, some of which are associated with human infections. *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio alginolyticus* are the most common pathogenic species. *Vibrio* spp. are responsible for infections in humans due to the consumption of contaminated water or raw or undercooked seafood or skin wound exposure to contaminated salt or brackish water (Baker-Austin *et al.*, 2018). Noroviruses (NoVs) are positive-sense, non-enveloped ssRNA viruses, that belong to the *Caliciviridae* family causing outbreaks or sporadic cases of gastroenteritis and are classified genetically into six genogroups, GI to GVI (Green, 2013). Hepatitis A virus (HAV) is a non-enveloped positive-stranded RNA virus belonging to the family *Picornaviridae*, genus *Hepatovirus*. HAV is responsible for a common form of viral hepatitis. Human HAV strains can be grouped genetically into three genotypes (I, II and III) and seven subgenotypes (IA, IB, IC, IIA, IIB, IIIA and IIIB). Subgenotypes IA, IB and IIIA are responsible for a considerable number of HAV cases annually worldwide (Robertson *et al.*, 1992; Pérez-Sautu *et al.*, 2011). NoV and HAV are transmitted by the fecal-oral route; infected people shed the viruses through feces and so high viral loads can be found in sewage (Bonadonna *et al.*, 2019). Hepatitis E virus (HEV) is a single-stranded RNA virus belonging to the *Hepeviridae* family, subfamily *Orthohepevirinae*, responsible for acute and less frequently, chronic hepatitis in humans (Purdy *et al.*, 2022). Five genotypes (HEV-1, 2, 3, 4 and 7) are known to infect humans (Johne *et al.*, 2014). The virus can also be transmitted through the fecal-oral route and thus detected in urban sewage and water environments (Takuissu *et al.*, 2022). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a positive-sense single-stranded RNA virus belonging to the family *Coronaviridae*. This virus is the causative agent of an acute respiratory disease that has rapidly spread worldwide, resulting in the SARS-CoV-2 pandemic emergency. Sars-CoV-2 RNA is commonly detected in the feces of COVID-19 patients like other human viruses and could reach wastewater if sewage is not properly treated (Polo *et al.*, 2021). Numerous watercourses flow into the Lesina and Varano lagoons and may impact these ecosystems. Treated wastewater of the municipalities surrounding the basins, wastewater originating from fish and livestock farms, and agricultural runoff are discharged into the lagoons. Considering the important activities of Lesina and Varano lagoons, combined with the scarcity of prevalence data on STEC and potentially enteropathogenic *Vibrio* species in parallel with NoV, HAV, HEV, and SARS-CoV-2, this study aimed to provide data on the presence of human potentially pathogenic bacteria and viruses in the water and sediments of these ecosystems.

Materials and Methods

Geographic area and sampling sites

The lagoons of Lesina and Varano are located in the north of the Apulia region (southeast Italy) (Figure 1). The Lesina lagoon is long and narrow, it extends parallel to the coast for about 20 km. The lagoon has an area of approximately 51.36 km^2 and an average depth of around 1.5 m (Solazzi *et al.*, 1972). Water exchanges with the Adriatic Sea are carried out through two artificial channels: Acquarotta on the western side and Schiapparo on the eastern side. The lagoon receives numerous inputs in the southern zone. The most important inputs are from the Sannicandro Sewage canal, the Idrovora drainage canal, the rivers Lauro and Zannella, and the Elce stream that collect water, both raw and treated, from the catchment area in which there are fish farming, agriculture, and urban activities (Caroppo *et al.*, 2018).

The Varano lagoon represents the largest brackish system in southern Italy; it has a trapezoidal shape and covers an area of 65 km^2, with a perimeter of 33 km and an average depth of 4 m (Figure 1). The lagoon is connected to the sea on the western and eastern sides through two artificial tidal channels, Capoiale and Varano, respectively (Fabbrocini *et al.*, 2017). The lagoon receives several freshwater inputs from underground springs and wastewater mainly from agricultural activities. In the southeastern part, Sant'Antonino and San Francesco channels flow into the lagoon draining two urban wastewater treatment plants (Scirocco *et al.*, 2018). In the Lesina lagoon, water and sediment samples were taken from Cammarata Channel, the final effluent from the wastewater treatment plant serving the town of Lesina and Poggio Imperiale [station 1 (ST1)], and at the mouth of the water channel [station 2 (ST2)] (Figure 2). The sampling sites of the Varano lagoon were three: ST1 and ST2 from the final effluent from San Francesco channel, the wastewater treatment plant serving the town of Cagnano Varano, and the mouth of the water channel [station 3 (ST3)]. One water and one sediment samples were collected from each site for both lagoons.

In the sampling period, the average water temperature varied from 10°C (February and March) to 29°C (July and August), while the average environmental temperature ranged from 13°C (February and March) to 30°C (July and August). These two parameters were almost identical for the two lagoons.

Sample collection and analysis

From March 2022 to February 2023, monthly samplings were conducted at the stations of the Lesina and Varano lagoons indicated above (Figure 2). In each station, water samples were collected by immersing glass bottles in the study area, and at the same time, sediment samples were picked up from the top layer of the ground. A total of 98 samples were analyzed, comprising 49 water samples and 49 sediment samples. 44 samples were from the Lesina lagoon, while 54 were from the Varano lagoon. The samples were transferred to the laboratories of the *Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata* at refrigeration temperature (4°C) and analyzed within 24 hours.

The water and sediment samples were investigated for the presence of STEC and potentially enteropathogenic *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* according to the standards ISO/TS 13136 (2012) and ISO 21872-1 (2017), respectively.

V. cholera isolates were subjected to further molecular characterization for the detection of virulence genes (*hlyA*, *ctxA*, *tcpA*, *tcpI*, *ompU*, *zot*, *stn/sto*) according to Rivera *et al.* (2001). Virological analyses were performed for the detection of NoV, HAV, HEV, and SARS-CoV-2. NoV and HAV were detected according to the standard ISO 15216-2:2019; HEV was investigated according to the protocols of La Bella *et al.* (2021). Sars-CoV-2 was detected by droplet digital polymerase chain reaction according to previously reported protocols (Mancusi *et al.*, 2022). Before molecular analyses, 500 mL of each water sample and 5 g of sediment sample were artificially contaminated with 10 µL of mengovirus process control and concentrated according to the protocols of the World Health Organization (2003) (WHO/V&B/03.03) and Farkas *et al.* (2017), respectively. Viral nucleic acids of 1 mL aliquot water and sediment concentrate were extracted using the NucliSens MiniMag extraction system (bioMerieux, Paris, France) following the manufacturer's instructions. The eluted RNA (100 µL) was immediately analyzed or stocked at -80°C until use.

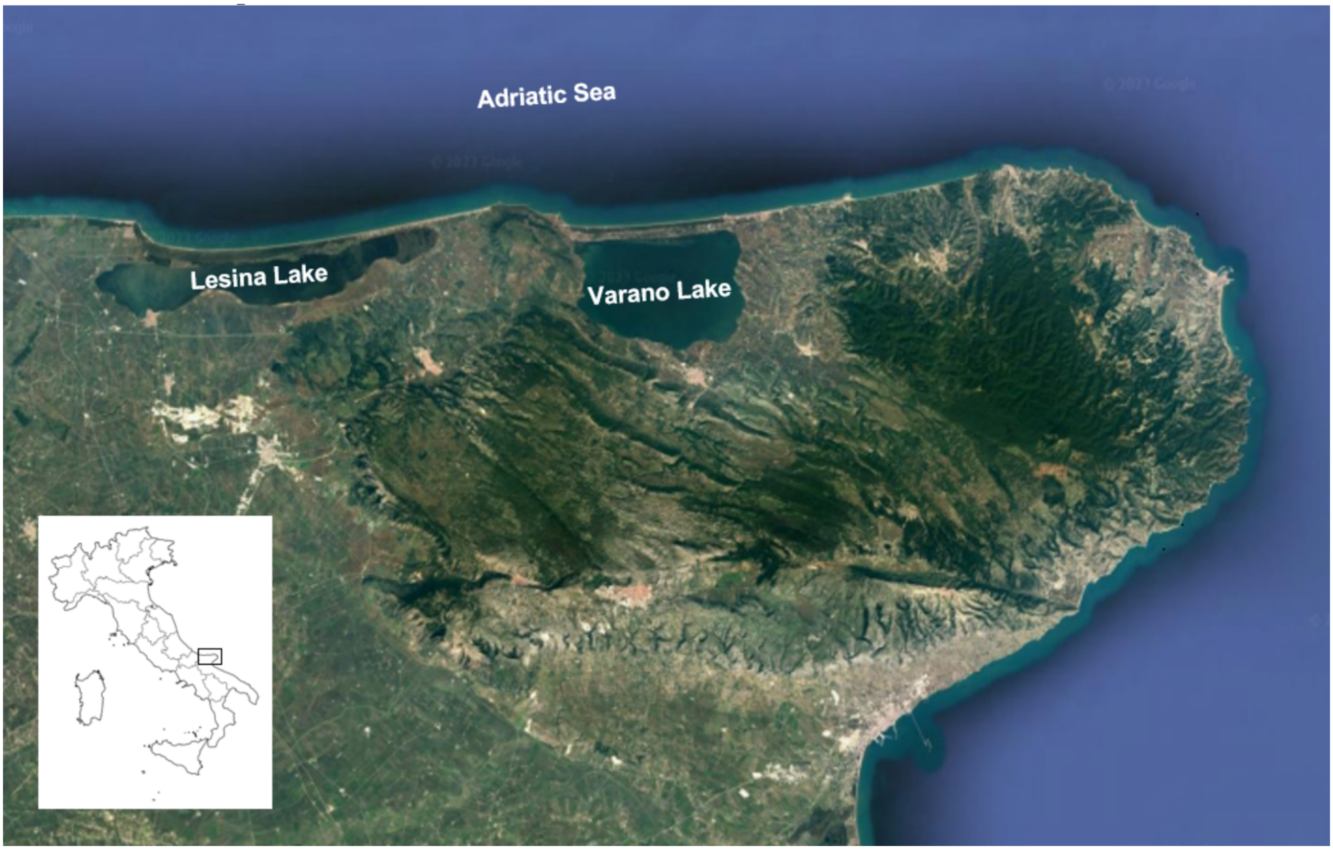

Figure 1. Map showing the study area. Reproduced from: Google Earth 10.43.0.2© 2023 Google LLC.

Figure 2. Map showing the sampling point locations. Reproduced from: Google Earth 10.43.0.2© 2023 Google LLC.

Results

The presence of STEC strains was detected in three samples (3.1%; 3/98), of which one (2%, 1/49) was water and two (4.1%; 2/49) were sediment samples. Out of the three positive samples, one tested positive for both the *stx1* and *stx2* genes and two for the *stx2* gene only. No sample contained "top five" serogroup-associated genes. STEC strains were recovered from the ST2 (water sample) of the Lesina lagoon (*stx1* and *stx2* positive strain), the ST2 (sediment sample), and ST3 (sediment sample) of the Varano lagoon (*stx2* positive strains). Of the 98 samples analyzed, the presence of *Vibrio* spp. was detected in 20 samples (20.4%; 20/98), of which 9 of water (18.4%; 9/49) and 11 of sediment (22.4%; 11/49). Species detected included *V. parahaemolyticus* (4 isolates), *V. cholerae* (14 isolates), and *V. vulnificus* (2 isolates). *V. parahaemolyticus* strains were negative for the presence of *tdh* and *trh* genes. None of the *V. cholerae* isolates carried out *hlyA*, *ctxA*, *tcpA*, *tcpI*, *ompU*, *zot*, *stn/sto* genes. *Vibrio* was detected in ST1 (1 *V. cholerae* isolate from water, 1 *V. parahaemolyticus* and 1 *V. cholerae* isolate from sediment samples) of the Lesina lagoon, ST2 (5 *V. cholerae* isolates from water, 2 *V. cholerae*, 2 *V. parahaemolyticus*, and 1 *V. vulnificus* isolates from sediment samples) of the Lesina lagoon, ST1 (1 *V. cholerae* isolate from water sample) of the Varano lagoon, ST2 (1 *V. cholerae* and 1 *V. parahaemolyticus* isolate from sediment samples) of the Varano lagoon, ST3 (1 *V. cholerae* from water samples, 2 *V. cholerae* and 1 *V. vulnificus* isolates from sediment samples) of the Varano lagoon. NoV was detected in 25/98 (25.5%) samples, with 15 GII-positive and 10 GI+GII-positive samples. NoV GII-positive samples were collected from ST1, ST2 and ST3 of the Varano lagoon (water and sediments), and from ST3 (sediments) of the Lesina lagoon. The samples that contained both genogroups GI and GII were taken from ST1 (sediments), ST2 (water and sediments), ST3 (water and sediments) of the Varano lagoon, and ST2 (sediments) of the Lesina lagoon. HAV, HEV and SARS-CoV-2 were not detected in water or sediment samples. The results are summarized in Table 1.

Discussion and Conclusions

In this study, the Lesina and Varano lagoons were taken into consideration to evaluate the circulation and spread of STEC in the environment and potentially enteropathogenic *Vibrio* species in parallel with NoV, HAV, HEV, and SARS-CoV-2, since they can affect the water quality.

STEC infections were the fourth most commonly reported zoonoses in humans in the European Union in 2022 (European Food Safety Authority and European Center for Disease Prevention and Control, 2023). In the Apulia region (Southern Italy), the mean annual reporting rate of HUS cases was among the highest rates observed in Italy (Loconsole *et al*., 2020).

Among animals, STEC-positive samples were detected in cattle (41.5%), followed by goats and sheep (1.3%) (European Food Safety Authority and European Center for Disease Prevention and Control, 2023). Cattle and small ruminants are recognized to be important reservoirs of STEC strains, especially cattle that are considered natural reservoirs. Colonized animals are responsible for the shedding of the pathogen in the farm environment. Improper management practices may contribute to the contamination of surface water and groundwater (Persad *et al.*, 2014). The detection of STEC strains in 3.1% of the analyzed samples may probably derive from the runoff of livestock farms present in the adjacent areas of the lagoons. According to the European Union regulations (European Parliament and Council of the European Union, 2004 - 853/2004/EC), the Lesina and Varano lagoons are classified as class A areas for shellfish farming. Further studies are needed to understand the role of STEC in waters, considering that effluents receiving treated wastewater are often used for agricultural purposes. The detection of *Vibrio* species (20.4%) confirms the ubiquity of these microorganisms in aquatic environments. *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were previously detected in fresh and coastal waters in Italy (Torresi *et al.*, 2018; Bonadonna *et al.*, 2019). During the one-year monitoring period, *Vibrio* isolates were detected monthly, thus no correlation between *Vibrio* spp. and seasonality was observed. *Vibrio* species are common in warmer waters, around 18°C and are particularly sensitive to seawater temperature and salinity. Climatic changes, in particular the rise of seawater temperature and altered salinity coastal areas, contribute to a greater spread of *Vibrio* species in warmer and less saline waters (Trinanes and Martinez-Urtaza, 2021) and consequently to the infections caused by pathogenic species (Baker-Austin *et al*., 2018). These bacteria have been demonstrated to cause a wide range of infections, due to the ingestion of raw or undercooked seafood or from exposure to contaminated water. *V. cholerae* is the causative agent of epidemic cholera; *V. parahaemolyticus* and *V. vulnificus* are responsible for several clinical manifestations ranging from mild gastroenteritis to severe wound infections and septicemia, depending on host susceptibility (Baker-Austin *et al.*, 2018). Although the *Vibrio* species detected in this

Table 1. Number of positive samples and site of sampling.

Site	Type of sample	STEC	*Vibrio* spp.	NoV GI+GII	NoV GII	HAV	HEV	Sars-CoV-2
ST1-Varano	water	-	1	-	2	-	-	-
ST1-Varano	sediment	-	-	2	4	-	-	-
ST2-Varano	water	-	-	3	2	-	-	-
ST2-Varano	sediment	1	2	2	2	-	-	-
ST3-Varano	water	-	1	1	2	-	-	-
ST3-Varano	sediment	1	3	1	2	-	-	-
ST1-Lesina	water	-	1	-	-	-	-	-
ST1-Lesina	sediment	-	2	-	-	-	-	-
ST2-Lesina	water	1	5	-	-	-	-	-
ST2-Lesina	sediment	-	5	1	1	-	-	-

ST, station; STEC, Shiga toxin *Escherichia coli;* NoV, norovirus; GI, genogroup I; GII, genogroup II; HAV, hepatitis A virus; HEV, hepatitis E virus; Sars-CoV-2, severe acute respiratory syndrome coronavirus 2.

study were not pathogenic, *Vibrios* should be monitored to highlight important changes in virulence capabilities. Indeed, *Vibrio* spp., through horizontal gene transfer among strains and other species, can acquire virulence factors that contribute to their pathogenicity (Brumfield *et al*., 2021).

The presence of NoVs was well documented in environmental studies dealing with water matrices such as wastewater, surface water, and recreational water (La Rosa *et al*., 2017; Bonadonna and La Rosa, 2019; Cioffi *et al*., 2020). In this study, NoVs were detected both in the water and sediment of the Varano lagoon and only in the sediment of the Lesina lagoon, confirming NoV circulation in environmental waters. The majority of positive samples was collected in the period March-June and September, unlike other studies in which a significantly higher prevalence was observed in the cold months (Farkas *et al*., 2017 Iaconelli *et al.*, 2017). There is a wide variability of prevalence found in several studies conducted in Italy and Europe in marine, riverine, and estuarine receiving waters. Bazzoni *et al*. (2019) detected only one sample positive for NoV GII in the waters of Calich lagoon in Sardinia, while Bonadonna *et al.* (2019) detected NoV in 5/20 marine water samples (25%; four GII-positive and one GI-positive samples). In North Wales (UK), other authors found the 10% (NoV GI) and 13% (NoV GII) of the water samples and the 18% (NoV GI) and 8% (No VGII) of the sediment samples positive for NoVs (Farkas *et al.*, 2017). The NoV found in samples of the Lesina and Varano lagoons might pose an important health significance considering the presence of an important cultivation of bivalves which could be contaminated.

HAV and HEV RNA were not detected in the samples collected in this study. The absence of HAV and HEV in the analyzed samples is in accordance with the low prevalence of these viruses among human cases in the Apulia region. In fact, few sporadic cases were notified during the one-monitoring study in the Apulia region: five cases of HAV and one of HEV infections in 2022 and three cases of HAV and zero of HEV in the first semester of 2023 (Bollettino SEIEVA, 2023a, 2023b). Probably, the concentrations of these viruses were too low to be detected and wastewater treatment completely removed viruses, or the cases of infections did not occur in the geographic area considered.

SARS-CoV-2 was not detected in the analyzed samples. This finding suggests that SARS-CoV-2 did not reach the waters of the Lesina and Varano lagoons at significant levels. This could be explained by considering the circulation and dilution effects of the viral particles in water and the efficiency of the wastewater treatment systems.

To the best of our knowledge, this is the first study that investigates the simultaneous presence of STEC, *Vibrio* spp., NoV, HAV, HEV, and Sars-CoV-2 in the waters and sediments of the Lesina and Varano coastal lagoons. The results of this study provide an overview of the presence of potentially pathogenic microorganisms in areas influenced by anthropogenic pressure. Monitoring the circulation of these pathogens could be useful to evaluate the water flowing into the lagoons, in particular discharge waters (*i.e*., urban, agricultural, and livestock runoff), considering the presence of fish and shellfish farms in these sites and the possible agricultural water use. In addition, the results reported here demonstrate the usefulness of molecular testing for microbiological investigations. Further studies could provide more information about the zoonotic and anthroponotic sources of contamination.

In conclusion, environmental studies on surface waters (*i.e.*, treated urban, agricultural, and industrial wastewater) can provide extensive insight into the ecology of the coastal lagoons to better understand the potential contamination routes and implement management actions. The detection of pathogenic microorganisms could represent a threat to human health and more data are necessary to understand the risk related to the consumption of seafood, often eaten locally raw or undercooked. Particular attention should be given to constant monitoring of sanitary conditions to improve knowledge about the spread of pathogenic microorganisms and to evaluate the potential risk to public health. From a One Health perspective, the results of this study could be useful for assessing the risk and management of possible bacterial and viral contamination in seafood reared in this geographical area.

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