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Research Note

Bacterial and Viral Investigations Combined with Determination of Phytoplankton and Algal Biotoxins in Mussels and Water from a Mediterranean Coastal Lagoon (Sardinia, Italy)

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ABSTRACT

Calich Lagoon is a Mediterranean coastal lagoon located along the northwestern coast of Sardinia (Italy). The connection to marine and fresh water determines the high productivity of this coastal lagoon. Despite its great potential and the presence of natural beds of bivalve mollusks (*Mytilus galloprovincialis*), the lagoon has not yet been classified for shellfish production. In this study, through a multidisciplinary approach, the presence of several bacterial pathogens (*Escherichia coli, Salmonella* spp., and *Vibrio* spp.) and viral pathogens (hepatitis A virus and norovirus genogroups I and II) was evaluated from March 2017 to February 2018. In addition, phytoplankton composition in lagoon waters and associated algal biotoxins (paralytic and diarrhetic shellfish poisoning) in mussels were also monitored. The aim of this study was to provide useful data to improve knowledge about their seasonal presence and to assess the potential risk for public health, as well as to provide input for future conservation and management strategies. In mussels, *Salmonella* spp. were found in spring, along with *E. coli,* but *Salmonella* spp. were not found in autumn or winter, even though *E. coli* was detected in these seasons. *Vibrio parahaemolyticus* was found in autumn and winter, but not in spring. Norovirus genogroups I and II were found in winter samples. None of the bacteria were found in summer. Algal biotoxins have never been detected in mussel samples. Among potentially harmful phytoplankton, only *Pseudo-nitzschia* spp. were present, mainly in summer. The results showed that a possible bacterial and viral contamination, together with the presence of potentially toxic microalgae, is a real problem. Therefore, the development of natural resource management strategies is necessary to ensure the good quality of waters and guarantee the protection of consumers.

HIGHLIGHTS

- Multidisciplinary biomonitoring in a Mediterranean coastal lagoon was performed.
- Potentially pathogenic *V. parahaemolyticus* was recovered in low numbers.
- Recovery of norovirus in water and mussels posed an important health significance.
- Occurrence of *V. parahaemolyticus* and norovirus was correlated with that of *E. coli*.
- Coastal lagoons need accurate and multidisciplinary sampling strategies.

Key words: Biotoxins; Mediterranean lagoon; Microbiology; Mussels; Phytoplanktonic community; Virology

Coastal lagoons are productive and diversified ecosystems because of the input of nutrients and organic matter from land and oceans *(18)*. This, together with lagoon shallowness, can result in high productivity *(71)*. Excessive nutrients and organic matter could result in oxygen depletion and algal blooms, but because of their high productivity, coastal lagoons are often exploited for aquacultural activities, including shellfish farming *(22)*. In relation to the increasing intensive development of various forms of aquaculture carried out in Mediterranean coastal lagoons *(31),* multiple factors linked to climate change and variability may affect the occurrence of food safety hazards connected to shellfish consumption *(69)*. Shellfish contamination occurs because of their nature as suspension feeders, which selectively filter small particles of phytoplankton, zooplankton, viruses, bacteria, and inorganic matter from the surrounding water *(25)*. Shellfish-transmitted illness may occur because of naturally occurring pathogens or because of human-generated pathogens before or after shellfish harvesting *(33)*. According to European Commission Regulation 2015/2285 *(28),* the enumeration of

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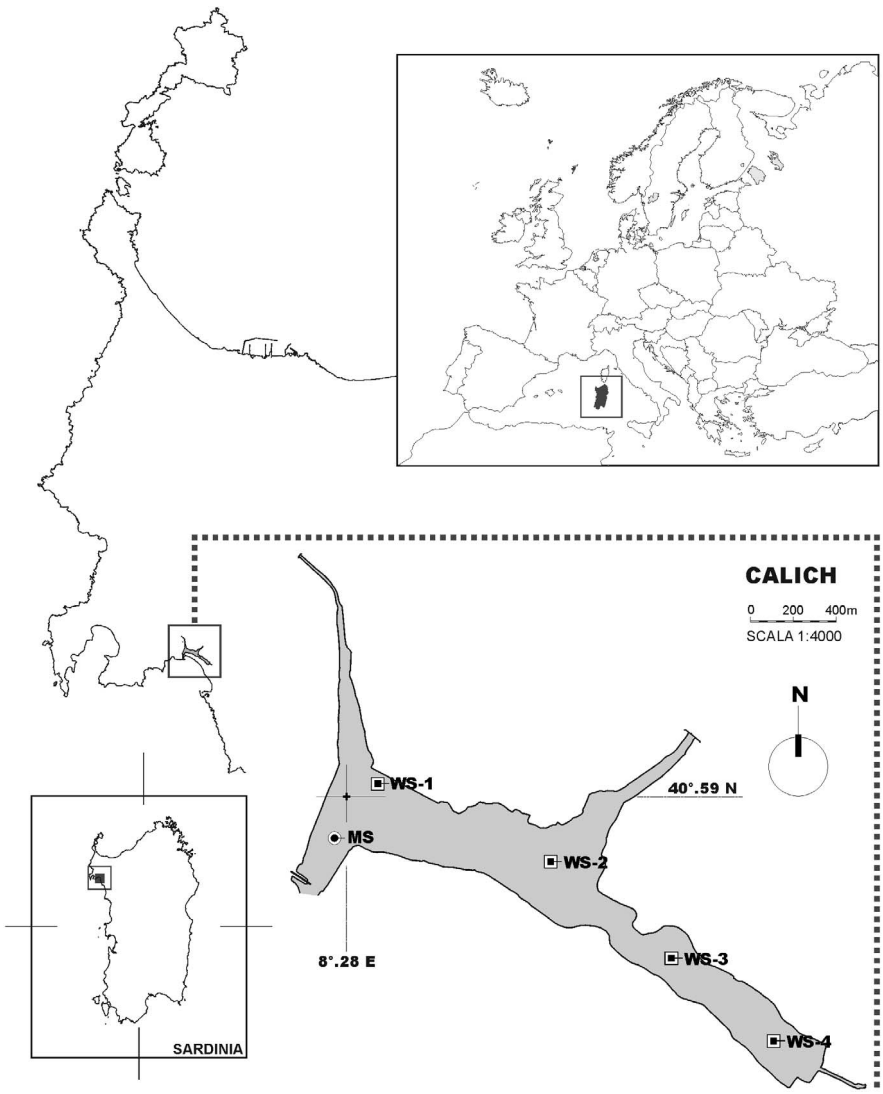

FIGURE 1. *Map showing the study area and the sampling point locations. Black squares (▣) indicate the water sampling points (WSs); the black circle (◉) indicates the mollusk sampling point (MS).*

Escherichia coli as an indicator of fecal contamination is the standard way to assess the level of fecal microorganisms in water and shellfish and, indirectly, to estimate the associated potential risk to human health from all waterborne enteric pathogens *(3)*. In marine environments, *Salmonella* spp. have been detected in coastal waters, mollusks, and other seafood products *(6, 15)*. Although *Salmonella* spp. are among the most common causes of human gastroenteritis *(29),* and despite their presence in marine environments *(15),* the risk of foodborne diseases associated with shellfish consumption is low *(35, 56)*. *Vibrio* spp. are common in aquatic environments, especially in estuarine and marine waters *(68)*. Several authors reported a prevalence of *Vibrio* spp. in seafood samples collected in Italy, ranging from 34 to 100% *(14, 39, 52, 65, 66)*. In Europe, the incidence of foodborne outbreaks associated with the consumption of shellfish contaminated with pathogenic vibrios is increasing, although in many European countries production control plans are being implemented *(5, 19–21, 52)*. Among naturally occurring vibrios, some infections are important, because they include those caused by the three major pathogens: *Vibrio parahaemolyticus, Vibrio cholerae,* and *Vibrio vulnificus (12)*. Diseases caused by *V. cholerae* require quarantine and the obligation to notify the World Health Organization, while diseases caused by *V. vulnificus* are known to cause high mortality rates *(14)*. *V. parahaemolyticus* is responsible for numerous food poisoning cases in some countries, such as the United States and Japan *(14, 67)*. In Europe, only a few outbreaks or sporadic cases caused by *V. parahaemolyticus* were reported in the last decade *(40–42, 44, 47–49, 59, 73)*. Norovirus (NoV) causes self-limiting infections characterized by gastrointestinal symptoms that have an average incubation time of 36 h. It generally lasts for about 48 h and resolves spontaneously without complications. Although viral gastroenteritis has low mortality rates (0.1%), the annual incidence recorded in some countries makes relevant economic and social costs and public health. Hepatitis A virus (HAV), as well as NoV, can contaminate marine and estuarine waters (e.g., through poor wastewater treatment) and infect shellfish. Their consumption, sometimes raw or slightly cooked, make bivalve mollusks a seafood safety problem *(37)*. Although HAV rarely causes death, a serious debilitating disease could incapacitate patients for several months. The contamination of bivalve mollusks with HAV is well documented *(54, 55),* and the main causes of outbreaks worldwide are events of sewage pollution because of sewerage system failures and malfunctioning *(30)*. The

TABLE 1. *Main features of Calich Lagoon*

Parameter	Value
Lagoon area (km^2)	0.87
Catchment area (km^2)	432
Mean depth (m)	1.20
Maximum depth (m)	2.10
No. of inlets	3
Water input ($\times 10^6$ m^3 yr^{-1})	28.6
Residence times (days)	8
No. of outlets	1
Mean salinity (‰)	17.4
Tidal regime	Nanotidal
Type of sea connection	Permanent

presence of potentially toxic harmful algal species constitutes another critical problem for consumers because of the illness derived from production of algal biotoxins, mainly paralytic shellfish poisoning (PSP), diarrheic shellfish poisoning (DSP), and amnesic shellfish poisoning (ASP). A close connection between eutrophication and increased occurrence of harmful algal blooms has been pointed out *(32, 43)*.

Several authors have studied Calich Lagoon with the aim of assessing the population dynamics of natural beds of bivalve mollusks. There are a few studies on the presence of bacteria and biotoxins in shellfish *(4, 23, 63, 64)*. Only the presence of *V. alginolyticus* and low microcystin-LR concentrations have been reported. In contrast, the phytoplanktonic community has been studied *(10, 57, 58)*. In this study, through a multidisciplinary approach, the presence of several bacterial pathogens (*E. coli, Salmonella* spp., and *Vibrio* spp.) and viral pathogens (HAV and NoV genogroups I and II [GI and GII]) was evaluated. In addition, considering that microalgal communities are a fundamental biological component for the trophic status evaluation of aquatic ecosystems and environmental stresses *(60),* phytoplankton composition and major associated biotoxins (PSP, DSP, and lipophilic biotoxins) have been determined. The main objectives of this study were (i) to provide useful data to improve knowledge about their spread and to assess the potential risk for public health and (ii) to implement rational conservation and management strategies of this transitional ecosystem in support of a future request for classification of the lagoon for shellfish production.

MATERIALS AND METHODS

Study site. Calich Lagoon is located along the northwestern coast of Sardinia (Italy) in the middle of the western Mediterranean Sea (Fig. 1). It is part of the Regional Natural Park of Porto Conte. The main morphological features of this coastal lagoon are listed in Table 1. The lagoon is permanently connected to the sea through a natural channel highly modified by human activities, at the output of which is located a tourist harbor, popular in summer. The lagoon receives fresh water from two natural fluvial tributaries (Rio Barca, which drains 70% of the catchment, and Rio Calvia) and one artificial canal (Oruni channel) *(46, 57)*. The catchments are affected by urban, agricultural, and zootechnical activities; consequently, the lagoon is highly eutrophic *(10)*. Natural beds of bivalve mollusks of commercial interest (mainly *Mytilus galloprovincialis* Lamarck) are present close to the entrance of the connecting channel with the sea.

Sampling. The study was conducted from March 2017 to February 2018. Monthly water sampling was carried out to establish the phytoplanktonic community composition and virus analysis, while *M. galloprovincialis* samples were collected seasonally from natural beds for bacterial, viral, and biotoxin investigations. Four water sampling points (WS-1, WS-2, WS-3, and WS-4) representative of different hydrological features, including depth and salinity, were included in the study (Fig. 1 and Table 2). A total of 48 monthly water samples and 4 seasonal shellfish samples from a single mollusk sampling point were examined (Fig. 1). Seasons were considered as follows: winter (December to February), spring (March to May), summer (June to August), and autumn (September to November). Water samples (2.5 L) were taken at a depth of 0.5 m from the surface, using 1.5 L in clean polyethylene bottles for phytoplankton analysis and 1 L in sterile glass bottles for virus analysis. Depending on the *M. galloprovincialis* size, 30 to 60 individuals of each sample were randomly selected for analysis. All samples were shipped refrigerated to the laboratories of the Veterinary Public Health Institute of Sardinia in insulated boxes. Analysis started within 24 h of sampling and was carried out as soon as possible, based on the timing established by the procedures adopted.

Phytoplankton analyses. An aliquot of the water sample (1 L) was fixed in situ with Lugol's iodine solution for quantitative analyses of phytoplankton. The remaining 0.5 L was used for the observation of live microalgae. Samples were kept cool and preserved during transporting to the laboratory, where they were examined using Utermöhl's method *(70)* in accordance with European Union reference method UNI EN 15204:2006 ("Water

TABLE 2. *Geographical location and values of main hydrological variables of water sampling points*

	Water sampling points[a]:			
	WS-1	WS-2	WS-3	WS-4
Longitude	40°36′04″N	40°35′51″N	40°35′31″N	40°36′04″N
Latitude	008°17′24″E	008°17′40″E	008°18′34″E	008°17′24″E
Min.–max. depth (m)	1.10–1.35	1.30–1.45	1.40–1.60	1.05–1.35
Temp (°C)[b]	17.76	19.64	18.68	18.29
Salinity (ppt)[b]	24.38	19.84	18.75	19.75
pH[b]	8.68	8.86	8.61	8.85
Dissolved oxygen (%)[b]	125	114	118.2	121.9

[a] WS, water sampling point; min., minimum; max., maximum.
[b] Temperature, salinity, pH, and dissolved oxygen were detected in March 2017, concurrent with the first sampling *(1)*.

Quality—Guidance Standard on the Enumeration of Phytoplankton Using Inverted Microscopy (Utermöhl Technique)"). Under an inverted microscope Olympus IX 73 (Olympus, Shinjuku, Tokyo, Japan), 5-mL fixed subsamples were observed at magnifications of 200× and 400× for microalgal identification and count. Phytoplanktonic abundance was expressed as the number of cells per liter.

Toxin analyses. The presence of PSP, DSP, and lipophilic biotoxins was determined. PSP was analyzed starting from shellfish tissue, following the AOAC International 959.08 method *(2)*. A quantity of 100 g of each sample was homogenized and extracted with 100 mL of 0.1 N HCl. The pH value settled around 3.0, and the mix was boiled for 5 min. After cooling to room temperature, the pH was adjusted again, adding 0.5 N HCl or 0.1 N NaOH. The mix was brought to a 200-mL volume with distilled water and centrifuged at 1,660 × *g* for 5 min (Sigma 6-16K, Osterode am Harz, Germany). Then, 1 mL of supernatant was injected intraperitoneally into three Swiss mice with a body weight of 19 to 21 g. The mouse behavior was observed after injection, and the potential lethal time was recorded. The test was considered positive when the mice died within a specific time. The death time was used to quantify the level of toxin present. According to European Commission Regulation 15/2011 *(27),* for the research of lipophilic toxins, the mouse bioassay test was used instead of the liquid chromatography–mass spectrometry method. For the lipid extract, 100 g of mussels was mixed with 300 mL of acetone (VWR Chemicals, Fontenay-sous-Bois, France) in an Ultra-Turrax T25 basic (Sigma, Saint Louis, MO), filtered, and reextracted with 200 mL of acetone. The acetone supernatant was mixed and evaporated with a Rotavapor (Büchi R-200/205, Labortechnik AG, Switzerland). The volume of the aqueous extract was adjusted to 100 mL with distilled water, transferred into a separatory funnel, and then 100 mL of diethyl ether (Sigma) was added for the liquid-liquid partition. The aqueous layer was collected into the evaporation flask, while the organic layer was collected into a clean glass container. The aqueous phase was transferred in a separatory funnel. The step was repeated twice. The three organic fractions were combined in the evaporation flask and evaporated to dryness using a rotary evaporator. The solvent evaporated, and the resultant residue was suspended in aqueous solution of 1% Tween 60 (Sigma). Next, 1 mL of extract was injected intraperitoneally into three albino Swiss-strain mice weighing between 19 and 21 g. The death of two of three injected mice within a 24-h observation period was evaluated positively for the presence of lipophilic toxins.

***E. coli*.** All bivalve samples were examined using the three-tube most-probable-number (MPN) method for enumeration of *E. coli* in accordance with the European Union reference method ISO 16649-3:2015 (International Organization for Standardization, Geneva). Then, 10 mL of meat and liquor mixed together were added to a flask containing 90 mL of physiological solution, resulting in a final 1:10 dilution. Aliquots of 10 mL of the initial suspension (1/10) were transferred to each of five tubes of mineral modified glutamate medium double strength (Oxoid, Basingstoke, UK). Furthermore, aliquots of 1 mL of the 1:10 homogenate were transferred to each of the five tubes of mineral modified glutamate medium single strength (Oxoid). Finally, 1 mL of the further dilutions (10-2, 10-3, etc.) was transferred to each of the five tubes of mineral modified glutamate medium single strength. All tubes were incubated aerobically at 37 ± 1°C for 24 h. Each incubated tube that changed color from purple to yellow, showing the presence of acid, was assumed positive. Subcultures from these tubes were plated on chromogenic tryptone bile glucuronide (TBX) agar (Oxoid) to obtain isolated colonies and confirm β-glucuronidase activity *(24)*. The TBX plates were incubated aerobically at 44 ± 1°C for 24 h. At the end of incubation, the plates were examined for the presence of colonies showing any shade of dark or light blue or blue-green, indicating the presence of presumptive β-glucuronidase–positive *E. coli (24)*. By counting the number of positive tubes that have given rise to the presence of blue or blue-green colonies on TBX agar, the level of *E. coli* per 100 g of meat and liquor was estimated using the MPN table, generated with the MPN calculator referenced in ISO 7218:2007/Amd 1:2013 and included in the method ISO 16649-3:2015. The enumeration of *E. coli* in bivalve molluscan shellfish by the MPN technique has limits of detection of fewer than 18 *E. coli* cells for the combination 000; 1,700 *E. coli* cells for the combination 533, and more than 18,000 *E. coli* cells for the combination 555.

***Salmonella* spp.** All samples were analyzed for *Salmonella* spp. detection according to the ISO 6579-1:2017. Briefly, 25 g of each sample was added to 225 mL of buffered peptone water and incubated at 37 ± 1°C for 24 h. Following incubation, 100 µL of the buffered peptone water enrichment was inoculated in 10 mL of Rappaport-Vassiliadis soya peptone enrichment broth and incubated at 41.5 ± 1°C for 24 h. At the same time, 1 mL of the buffered peptone water enrichment was transferred to 10 mL of Muller-Kaufmann tetrathionate-novobiocin broth amended with iodine and novobiocin and incubated at 37 ± 1°C for 24 h. Following incubation, the two broths were subcultured onto the surface of one xylose lysine desoxycholate agar plate and on chromID Salmonella agar (bioMérieux, Marcy l'Etoile, France) to obtain well-isolated colonies and incubated at 37 ± 1°C for 24 h. After incubation, plates were examined for characteristic *Salmonella*-like colonies. The suspected colonies (with black centers and a reddish, light transparent zone on xylose lysine desoxycholate agar and purple with jagged edges on SM2) were inoculated in nutrient agar at 37 ± 1°C for 24 h. Subsequently, for the biochemical confirmation triple sugar iron agar (Difco, BD, Franklin Lakes, NJ) and Vitek 2 compact (bioMérieux) were used, while the anti-*Salmonella* A-67 + Vi omnivalent serum (Sifin, Berlin, DE) was used for serological confirmation.

***Vibrio* spp.** Samples were analyzed for *V. parahaemolyticus* detection following ISO/TS 21872-1:2017, using thiosulfate–citrate–bile salts–sucrose agar (Oxoid) and triphenyltetrazolium chloride soya tryptone agar (Oxoid) as isolation media. Typical colonies were screened by an oxidase and catalase test, Gram staining, and sugar fermentation with triple sugar iron saline agar (Difco). Biochemical identification was carried out by Vitek 2 compact (bioMérieux). All isolates identified as *V. parahaemolyticus* by biochemical tests were submitted to biomolecular confirmation by PCR. Identification of presumptive *V. parahaemolyticus* isolates was performed by means of PCR targeting the *toxR* gene *(36)* (Table 3). The detection of the genes associated with enteropathogenicity (*tdh* and *trh*) of *V. parahaemolyticus* was performed according to Bej et al. *(11)*.

Viral analyses. Determination of HAV and NoV GI and GII was carried out according to the ISO/TS 15216-2:2013, a qualitative standard method also used for bottled water for human consumption. From water samples, an aliquot of 500 to 1,000 mL was filtered (47-mm membrane and 0.45-µm pore size), treated with Tris–glycine–beef extract buffer, and centrifuged at 4,000 × *g* for 20 min (Thermo Fisher Scientific, Waltham, MA). The digestive glands (hepatopancreas) collected from 10 mollusk

TABLE 3. *Primer and probe sequences used for the investigation of* Vibrio parahaemolyticus, *hepatitis A virus, norovirus GI, and norovirus GII in Calich Lagoon*[a]

Pathogen	Primer and probe	Sequence	Target
V. parahaemolyticus	*toxR* (F)	GTC TTC TGA CGC AAT CGT TG	*toxR*
	toxR (R)	ATA CGA GTG GTT GCT GTC ATG	*toxR*
	tdh (F)	CCA TCT GTC CCT TTT CCT GC	*tdh*
	tdh (R)	CCA AAT ACA TTT TAC TTG G	*tdh*
	trh (F)	GGC TCA AAA TGG TTA AGC G	*trh*
	trh (R)	CAT TTC CGC TCT CAT ATG C	*trh*
HAV	HAV68 (F)	TCA CCG CCG TTT GCC TAG	HAV
	HAV240 (R)	GGA GAG CCC TGG AAG AAA G	HAV
	HAV150 (probe)	CCT GAA CCT GCA GGA ATT AA	HAV
NoV GI	QNIF4 (F)	CGC TGG ATG CGN TTC CAT	NoV GI
	NV1LCR (R)	CCT TAG ACG CCA TCA TCA TTT AC	NoV GI
	NVGG1p (probe)	TGG ACA GGA GAY CGC RAT CT	NoV GI
NoV GII	QNIF2 (F)	ATG TTC AGR TGG ATG AGR TTC TCW	NoV GII
	COG2R (R)	GATCG ACG CCA TCT TCA TTC ACA	NoV GII
	QNIFs (probe)	AGC ACG TGG GAG GGC GAT CG	NoV GII

[a] F, forward; R, reverse; HAV, hepatitis A virus; NoV GI, norovirus genogroup I; NoV GII, norovirus genogroup II.

samples were excised, pooled, and blended by using Ultra-Turrax T25 basic (Sigma). Viral RNA was extracted using the Nuclisens Magnetic Extraction Kit—MiniMag (bioMérieux). The reverse transcription PCR one-step method was performed with a 7500 Fast Real Time System (Applied Biosystems, Foster City, CA) for the detection of HAV and NoV GI and GII. All amplification reactions were carried out using specific primers, probes, and the Ultrasense one-step quantitative reverse transcription PCR system (Invitrogen, Carlsbad, CA). The cycling conditions were as follows: reverse transcription for 60 min at 55°C, followed by 5 min at 95°C, and 45 cycles of 15 s at 95°C, 1 min at 60°C, and 1 min at 65°C. Each run included extracted RNA from samples, NoV GI, NoV GII, HAV, and mengovirus as positive reverse transcription PCR controls and negative controls. The primers and the probes are reported in Table 3.

RESULTS

Phytoplankton community and biotoxins. Small variations were observed in phytoplanktonic composition and abundance among WSs, with few and sporadic exceptions. Mean calculation was reported for phytoplanktonic monthly abundance from the four WSs in a narrow range and then was used as a representative value for the lagoon. A total of 63 taxa were observed during the study, distributed in 10 classes (Table 4). Moreover, organisms too difficult to classify because of their small size (<5 μm) were included in the undetermined category, distinguished as ultraplankton and phytoflagellates. *Bacillariophyceae* was the most diversified class, with 28 taxa, 11 of which were identified to the species level and distributed in 22 genera. *Dinophyceae* included 14 taxa, 9 of which were classified to the species level. Other groups, including *Chlorophyceae, Chrysophyceae, Cryptophyceae, Cyanophyceae, Coccolithophyceae, Euglenophyceae, Pyramomimonaphyceae,* and *Rhaphydophyceae,* contributed fewer species. The highest abundance of cells was observed between May and July 2017, when total density exceeded 100×10^6 cells per L several times, while the lowest cell numbers were observed between December 2017 and February 2018. The highest densities were always determined by small cells, mainly *Cyclotella* sp. (belonging to *Bacillariophyceae*), *Cryptophyceae,* and ultraplankton. Regardless, small cells (≤20 μm) were more abundant than larger ones. Although the *Dinophyceae* did not contribute more than 14% to the total density (except for January 2018, when they reached 31%), some species, for example, *Peridinium quinquecorne* Abé and *Kryptoperidinium foliaceum* (F. Stein) Lindemann, were recurrent in all WSs and in several months during the study period, reaching high abundances (Fig. 2). Among potentially toxic harmful algal species, three species of *Pseudo-nitzschia* (genera causative of ASP) were observed (Tables 4 and 5). Although they were present in autumn and winter, they reached high densities in summer only (maximum of 1.4×10^6 cells per L in August). No microalgae producers of PSP and DSP have been found, and no PSP and lipophilic toxin (including DSP) presence was reported (Table 5).

Bacteria. The results of bacterial analyses conducted in water and mollusk samples are summarized in Tables 5 and 6. *E. coli* was found in three of the four seasonal mollusk samples. *E. coli* was absent in summer, while in spring, its enumeration met the European law limit of 230 MPN/100 g *(26)*. The highest counts were found in autumn and winter (270 and 330 MPN/100 g, respectively). *Salmonella* spp. were only present in spring. *V. parahaemolyticus* was detected in autumn and winter samples. Potentially pathogenic *V. parahaemolyticus* carrying *toxR* and *trh* genes was recovered in low numbers.

Viruses. HAV was never found. NoV GII was detected only in mussels collected in winter. Both water samples and mussels were positive for NoV GI in winter (Tables 5 and 6). NoV GI was detected in January 2018 in mussels and in water samples collected in WS-3 and WS-4. In February 2018, it was detected in water samples from WS-1, WS-2, and WS-4.

TABLE 4. *List and monthly presence and absence of phytoplankton taxa detected in Calich Lagoon from March 2017 to February 2018*[a]

	17 Mar.	17 Apr.	17 May	17 June	17 July	17 Aug.	17 Sep.	17 Oct.	17 Nov.	17 Dec.	18 Jan.	18 Feb.	Frequency of appearance (%)	Maximal abundance (10^3 cells/L)	Maximal relative contribution (%)
Bacillariophyceae															
Achnanthes sp.	+[b]	+	−	−	−	−	−	−	−	−	−	+	12.5	1	<1
Amphiprora sp.	+	−	−	−	+	−	−	−	−	−	−	−	6.3	6	<1
Amphora spp.	−	+	+	+	+	+	+	−	−	+	−	+	31.3	633	30.20
Aulacoseira sp.	−	−	−	−	−	+	−	−	−	−	−	+	4.2	6	3.69
Cerataulina pelagica	−	−	−	−	−	+	−	−	−	−	−	−	8.3	25	<1
Chaetoceros minimus	−	−	−	−	−	+	−	−	−	−	−	−	6.3	63	<1
Chaetoceros sp.	−	−	−	+	−	+	+	−	−	+	−	+	22.9	80	14.75
Chaetoceros subtilis	+	+	−	−	−	−	−	−	−	−	+	+	16.7	65	5.27
Chaetoceros tenuissimus	−	−	−	−	−	+	−	+	+	−	+	−	20.8	39,484	92.54
Cocconeis spp.	+	+	+	+	−	+	+	−	−	−	+	+	31.3	48	4.51
Cyclotella sp.	+	+	+	+	+	+	+	+	+	+	−	−	62.5	94,763	97.10
Cylindrotheca closterium	+	+	+	+	+	+	+	+	+	−	−	+	54.2	725	4.75
Detonula pumila	−	−	−	−	−	+	+	−	−	−	−	−	14.6	35	<1
Diploneis sp.	−	−	−	−	−	−	−	−	−	−	−	+	2.1	1	<1
Fragilaria sp.	−	−	−	−	−	−	−	−	−	−	−	+	4.2	15	8.60
Leptocylindrus danicus	−	−	−	−	−	−	+	−	−	−	−	−	2.1	4	<1
Leptocylindrus minimus	−	−	−	−	−	−	+	−	−	−	−	−	6.3	92	<1
Licmophora sp.	−	−	+	−	−	−	−	−	−	−	−	−	2.1	0	<1
Navicula spp.	+	+	+	+	+	+	+	+	+	+	−	+	41.7	59	15.88
Nitzschia longissima	−	−	−	−	−	−	−	−	−	−	−	+	2.1	1	<1
Nitzschia spp.	+	+	−	−	+	+	−	+	+	+	+	+	56.3	54,467	76.65
Pseudo-nitzschia spp.	−	−	−	+	−	+	+	−	−	+	+	−	25.0	1,384	1.63
Rhizosolenia setigera	−	−	−	+	+	+	+	−	+	−	−	−	31.3	504	4.76
Skeletonema costatum	−	−	−	−	−	+	+	+	−	+	−	−	16.7	469	25.82
Skeletonema sp.	+	−	+	−	+	−	−	−	−	−	+	+	18.8	130	6.91
Surirella sp.	−	−	−	−	−	−	−	−	−	−	−	+	2.1	4	2.26
Synedra sp.	−	+	−	−	−	+	−	−	−	−	−	−	6.3	2	<1
Thalassiosira sp.	−	−	+	+	−	+	−	−	+	+	+	+	29.2	70	18.44
Dinophyceae															
Akashiwo sanguinea	−	−	−	−	−	+	−	−	−	−	−	−	2.1	2	<1
Gonyaulax sp.	−	−	−	−	−	+	−	−	−	−	−	−	4.2	4	<1
Gymnodinium spp.	+	+	+	+	+	+	−	+	+	−	−	−	25.0	808	22.99
Gyrodinium spp.	+	+	+	+	−	+	+	+	+	−	−	+	41.7	174	21.87
Heterocapsa rotundata	−	−	−	+	+	−	−	−	−	−	+	−	18.8	741	65.98
Kryptoperidinium foliaceum	−	−	+	+	+	−	+	+	−	−	+	+	45.8	231	4.21
Minuscola bipes	−	−	−	−	−	−	−	+	−	−	−	−	4.2	6	<1
Oxyrrhis marina	−	−	+	+	−	+	+	−	+	−	−	−	20.8	340	4.97
Peridinium quinquecorne	−	+	+	+	+	+	+	+	+	+	−	−	52.1	83	4.94
Polykrikos shwartzii	−	−	−	+	−	−	−	−	−	−	−	−	2.1	2	<1

TABLE 4. *Continued*

	17 Mar.	17 Apr.	17 May	17 June	17 July	17 Aug.	17 Sep.	17 Oct.	17 Nov.	17 Dec.	18 Jan.	18 Feb.	Frequency of appearance (%)	Maximal abundance (10^3 cells/L)	Maximal relative contribution (%)
Prorocentrum triestinum	−	−	−	−	−	+	+	+	+	+	−	−	29.2	26	4.94
Protoperidinium divergens	−	−	+	−	−	−	−	−	−	−	−	−	2.1	$<1 \times 10^3$	<1
Protoperidinium sp.	−	−	−	+	−	+	+	+	−	−	−	−	16.7	16	<1
Scrippsiella sp.	−	−	+	−	+	+	+	+	+	+	−	+	43.8	574	4.94
Chlorophyceae															
Ankistrosdesmus sp.	+	−	−	−	−	−	+	−	−	−	−	−	6.3	48	<1
Ankyra sp.	−	+	−	−	−	−	−	−	−	+	−	−	4.2	47	4.42
Coelastrum sp.	−	−	−	−	−	−	+	−	−	−	−	−	2.1	207	2.06
Pediastrum sp.	−	−	−	−	−	−	+	−	−	−	−	−	2.1	69	<1
Tetraselmis sp.	+	+	−	−	+	−	−	−	+	−	−	−	22.9	72,754	51.11
Chrysophyceae															
Calycomonas sp.	−	+	−	−	−	−	−	−	−	−	−	−	2.1	8	1.47
Kephyrion sp.	−	−	−	−	−	−	+	+	−	−	−	−	4.2	1,796	17.87
Cryptophyceae															
UND *Cryptophyceae*	+	+	+	+	+	+	+	+	+	+	+	+	87.5	5,436	83.68
Cyanophyceae															
Anabaena sp.	−	−	−	−	−	−	+	−	−	−	−	−	2.1	259	2.58
Aphanizomenon sp.	−	−	−	−	−	−	+	−	−	−	−	+	4.2	3,791	37.71
Spirulina sp.	−	−	+	−	−	−	−	−	−	−	−	−	6.3	934	1.13
UND *Cyanophyceae*	−	−	−	−	−	−	−	−	−	−	−	+	2.1	130	30.25
Coccolithophyceae															
Emiliania huxleyi	+	−	−	−	−	−	−	−	−	−	−	−	4.2	106	3.15
Halopappus sp.	−	−	−	−	−	+	+	−	−	−	−	−	10.4	871	<1
Syracosphaera pulchra	−	−	−	−	−	−	−	+	−	−	−	−	2.1	2	<1
Euglenophyceae															
Euglena sp.	−	−	−	−	+	−	−	+	−	−	−	−	6.3	755	6.69
Eutreptiella sp.	−	−	+	+	+	+	+	+	+	−	−	−	43.8	191	6.52
Pyramomimonaphyceae															
Pyramimonas sp.	−	−	+	+	−	+	+	−	−	−	+	−	16.7	1,251	1.25
Rhaphydophyceae															
UND *Rhaphydophyceae*	−	−	−	−	−	+	−	−	−	−	−	−	2.1	2	<1
UND															
Phytoflagellates (<5 μm)	+	+	+	−	+	+	−	−	−	−	+	+	47.9	28,229	32.93
Ultraplankton	+	+	+	+	−	+	−	−	−	+	−	+	52.1	100,429	77.87

[a] The last three columns show the frequency of appearance (%), the maximal abundance (10^3 cells per L), and the maximal relative contribution (%) for each taxa during the study.
[b] +, presence; −, absence; UND, undetermined.

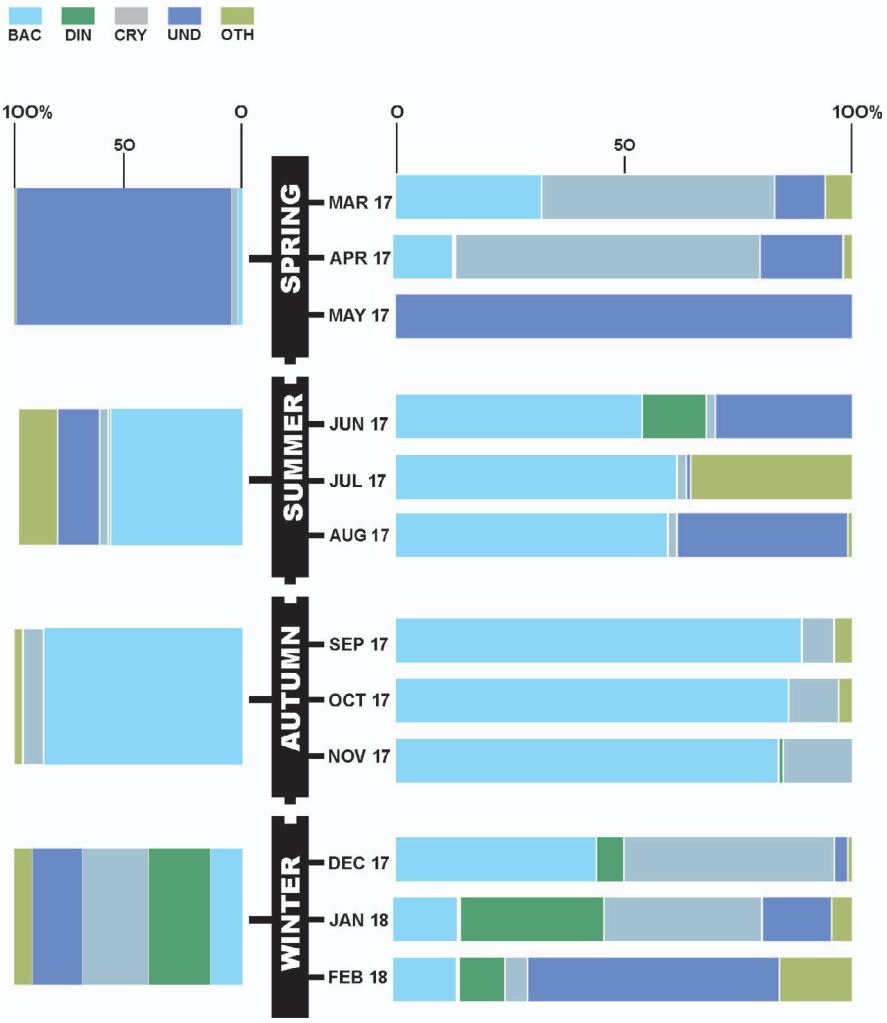

FIGURE 2. *Monthly (right) and seasonal (left) contribution (%) of phytoplankton classes to the total abundance. Seasonal contribution is calculated as the average monthly abundance. BAC,* Bacillariophyceae; *DIN,* Dinophyceae; *CRY,* Cryptophyceae; *UND, undetermined; OTH, other classes (sum of* Chlorophyceae, Chrysophyceae, Cyanophyceae, Coccolithophyceae, Euglenophyceae, Pyramomimonaphyceae, *and* Rhaphydophyceae*).*

DISCUSSION

Several studies have been carried out in Calich Lagoon covering a lot of topics, from shellfish farming *(13, 17, 50, 51)* to fish fauna *(16)* and from ecotoxicology *(34)* to the ecology of planktonic components *(57, 61)*. A notable number of these published results indicated that Calich Lagoon is a productive system; therefore, it is used for fishing and fish farming. Nevertheless, it could be better exploited by implementing different forms of aquaculture. On this basis, this study aimed to update the scientific framework through an integrated approach considering how biotic factors (viral, bacterial, and phytoplanktonic) influence the water quality of the lagoon and the potential risk for public health connected to food resources (natural or cultured). The water quality and the state of health of Calich Lagoon have been influenced by variable factors *(1)*. Aquaculture does not seem to have been a major source of eutrophication as happened in other lagoons, such as in Tau Lagoon in France *(22)*.

In shellfish samples collected in spring, *Salmonella* spp. and *E. coli* were found. Previous studies have shown that the

TABLE 5. *Detection of viral pathogens and harmful phytoplanktonic species in monthly water samples from March 2017 to February 2018 in Calich Lagoon*[a]

	17 Mar.	17 Apr.	17 May	17 June	17 July	17 Aug.	17 Sep.	17 Oct.	17 Nov.	17 Dec.	18 Jan.	18 Feb.
Viral pathogens												
Norovirus GI	–	–	–	–	–	–	–	–	–	–	+	+
Norovirus GII	–	–	–	–	–	–	–	–	–	–	–	–
Hepatitis A	–	–	–	–	–	–	–	–	–	–	–	–
Phytoplankton												
HAS-PSP	–	–	–	–	–	–	–	–	–	–	–	–
HAS-DSP	–	–	–	–	–	–	–	–	–	–	–	–
HAS-ASP	–	–	–	+	–	+	+	–	–	+	+	–

[a] +, presence; –, absence; HAS-PSP, harmful algal species causing paralytic shellfish poisoning; HAS-DSP, harmful algal species causing diarrheic shellfish poisoning; HAS-ASP, harmful algal species causing amnesic shellfish poisoning.

TABLE 6. *Detection of bacterial and viral pathogens and biotoxins in seasonal mollusk samples from March 2017 to February 2018 in Calich Lagoon*[a]

	Spring	Summer	Autumn	Winter
Bacterial pathogens				
Escherichia coli	130 MPN	<180 MPN	270 MPN	330 MPN
Salmonella spp.	+	−	−	−
Vibrio parahaemolyticus	−	−	+	+
Viral pathogens				
Norovirus GI	−	−	−	+
Norovirus GII	−	−	−	+
Hepatitis A	−	−	−	−
Biotoxins				
PSP	−	−	−	−
DSP	−	−	−	−

[a] +, presence; –, absence; PSP, paralytic shellfish poisoning; DSP, diarrheic shellfish poisoning.

presence of *Salmonella* spp. is strongly related to the bivalve species considered, the harvesting areas, and chiefly the sampling season *(62)*. Although the *E. coli* count did not exceed the acceptable limit of 230 MPN/100 g, its presence constituted a potential risk to consumer health. As reported by previous authors *(7),* recovering *E. coli* in winter samples is not surprising. Sardinian seawater had stable salinity, temperature, dissolved oxygen, and pH during the year, without significant variations *(7)*. The presence of other bacterial and viral pathogens was detected in Calich Lagoon in autumn 2017 (*V. parahaemolyticus*) and winter 2018 (*V. parahaemolyticus,* NoV GI, and NoV GII) and was positively correlated with the presence of *E. coli* above the legal limits. Several studies *(14, 66)* have documented that in Sardinian harvesting areas, the presence of potential pathogenic *V. parahaemolyticus trh*$^+$ strains is low. Pathogenic *V. parahaemolyticus* isolates might survive in winter or be the first strains to appear in the aquatic environment and gradually become substituted as the water warms. Regarding viruses, as reported *(39, 66),* HAV has never been found in samples collected in Sardinia, while the prevalence of NoV ranged between 25 and 62% *(7, 39, 67)*. According to previous studies *(7),* the NoV prevalence in Sardinian mollusks showed high seasonal variability *(7)*. The prevalence of NoV levels varied markedly by season, with annual peak occurring in winter. The seasonality of NoV would not seem related to the temperature of the water *(7)*. The recovery of NoV GI and GII posed an important health significance, in relation to the ability of such viruses to withstand the environment for longer periods in the presence of favorable conditions *(39)*. Neither viral nor bacterial pathogens were found in mollusks collected in summer. However, elevate phytoplanktonic abundance was detected in the same period in water samples, with *Bacillariophyceae* as the dominant group (60%). The interaction between the mussel population and microalgae as a food source (*Bacillariophyceae* in particular) is known to favor the growth and the production of bivalves *(45, 53, 72)*. Special attention was given to phytoplankton causative of human syndromes, because a relevant number of potentially toxic harmful algal species have been observed in the past years along the Sardinian coasts *(8)*. Moreover, PSP- and DSP-positive events have frequently occurred in transitional and marine Sardinian areas since 2002, with an expansion of their distribution along the coasts *(6, 8, 9, 38)*. *Pseudo-nitzschia* was the only potentially toxic taxa documented in this study, confirming that it is the most important one in terms of distribution in Sardinia, as it is elsewhere, although the presence of ASP has never been reported. *Pseudo-nitzschia* spp. were not distinguished and counted together; hence, their toxicity has not been investigated.

In conclusion, Calich Lagoon should become an important ecosystem for regional economy because it is productive, attractive to tourists, and a source of biodiversity. More data are necessary for understanding the health-related problems connected with the consumption of seafood in such a complex habitat. Water conditions can suddenly and quickly vary because of weather conditions or environmental changes. This vulnerability imposes on the development of accurate and multidisciplinary sampling strategies that consider possible bacterial and viral contamination in seafood and seawater, together with the presence of potentially toxic harmful algal species. In the present study, different pathogens were detected in all seasons except for summer, which was characterized by high phytoplankton densities. These findings represent a first report of the presence of many bacteria and viruses (*E. coli, Salmonella* spp., *V. parahaemolyticus,* NoV GI, and NoV GII) and of *Pseudo-nitzschia* spp. in Calich Lagoon. Particular attention should be paid to the implementation of intensive monitoring programs to improve knowledge about their spread and to assess the potential risk for public health. The results of our study should be useful baseline data to support a future request for classification of this lagoon for shellfish production.

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