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Longitudinal Study on Seasonal Variation of Marine Biotoxins and Related Harmful Algae in Bivalve Mollusks Bred in Sardinia (Italy, W Mediterranean Sea) from 2015 to 2020 and Assessment of Potential Public Health Risks

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Abstract: Annual and interannual dynamics of shellfish toxins and associated harmful algal species (HAS) were analyzed from 2015 to 2020 in Tortolì Lagoon (Sardinia, west Mediterranean Sea). Analysis of seasonal occurrence of different harmful algae, such as *Dinophysis* spp., *Prorocentrum* spp., *Pseudo-nitzschia* spp. and *Alexandrium minutum*, was performed. The species *Dinophysis acuminata* and *Dinophysis sacculus* were responsible for the accumulation of lipophilic toxins belonging to the okadaic acid group (OAs) and pectenotoxins2 (PTX2) in bivalve mollusks. The highest HAS detection was recorded in the winter months; in particular, *Dinophysis* spp. was mostly present in January–February. Out of 1090 analyzed mollusk samples, 39 were non-compliant, exceeding the legal limits (160 µg OA eq/kg e.p.) reported in Regulation 853/2004 of the European Commission. A statistical analysis related to the presence of OA and PTX2 in mollusks with various environmental parameters (pH, water temperature, dissolved oxygen, algal density) was implemented, proving a clear winter seasonality. The present study highlights the necessity to better understand the different factors able to influence the production and accumulation of toxins in bivalve mollusks bred in an important Sardinian production area. The contribution of this research is important not only from an environmental and productive point of view but also from the view of implementing management in order to mitigate any harm to human health.

Keywords: bivalve mollusks; okadaic acid; pectenotoxins; potentially toxic algae; public health

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1. Introduction

Harmful algal blooms (HABs) negatively affect aquatic systems, not only considering ecological impacts but also considering the economic aspects for fisheries and tourism in several coastal areas. About 300 harmful algal species (HAS) can generate negative events [1], 100 of which produce natural toxins that could be noxious to humans and animals [2].

The term "HAB" was initially coined to describe a toxic event able to impact humans but has recently evolved to also include negative ecological impacts [3]. Sometimes,

this event creates only an esthetic annoyance (e.g., discoloration of water or red tides by Dinoflagellates); in the worst cases, toxins eventually produced by HAS may be accumulated by filter-feeding organisms such as bivalve mollusks. Phenomena of human intoxication by aerosols such as those produced by *Ostreopsis ovata* have also been reported [4]. At present, three main groups of marine biotoxins are regulated by EC Regulation No. 853/2004 [5,6]: domoic acid (DA), causing amnesic shellfish poisoning (ASP); saxitoxin (STX) and derivates, responsible for paralytic shellfish poisoning (PSP); and the lipophilic toxins (LTs) group, including okadaic acid (OA) and its derivates, *Dinophysis* toxins (DTXs) and associated esters, responsible for diarrhetic shellfish poisoning (DSP), and pectenotoxins (PTXs), yessotoxins (YTXs), gymnodimine (GYMs), spirolides, pinnatoxins, portimine and azaspiracid (AZAs) [7–9]. PSP is a potentially fatal syndrome that occurs when shellfish consumers are exposed to neurotoxins known as STX and derivates [10] produced by several dinoflagellates belonging to the genus *Alexandrium*, such as *Alexandrium pacificum*, *Alexandrium tamarense*, *Alexandrium catenella*, *Alexandrium acatenella* and *Alexandrium minutum*. *A. minutum* is the most widespread toxic species in the western Mediterranean Sea [11]. Other algae producing PSP are *Gymnodinium catenatum* and *Pyrodinium bahamense* [12]. Paralytic shellfish toxins (PSTs) are water-soluble tetrahydropurine toxins. There are more than 50 toxins that are chemically close to one another [13,14]. They are heat-stable and are not destroyed by cooking. They act on mammalian cells by blocking the voltage-gated sodium channels, leading to symptoms including a tingling sensation of the lips, mouth and tongue, numbness of the extremities, headache, dizziness, nausea, vomiting, diarrhea and, in severe cases, death by asphyxiation [15].

ASP is intoxication with neurologic effects on humans due to the presence of DA, a heat-stable neuroexcitatory amino acid. The first report of ASP human intoxication was reported in 1987 in Canada due to the consumption of mussels, giving rise to different symptoms such as vomiting, abdominal pain, diarrhea, neurological symptoms and memory loss, and, in extreme cases, leading to death [16]. The causative agents were identified to be several species of *Pseudo-nitzschia* [17], a cosmopolitan planktonic ocean genus, with 52 described species, 26 of which are known to be potential DA producers [18]. Finally, DSP is human intoxication encountered worldwide due to the consumption of contaminated shellfish with toxins produced by some dinoflagellate species belonging to the genus *Dinophysis* [19], such as *Dinophysis fortii*, *Dinophysis caudata*, *Dinophysis sacculus*, *Dinophysis acuminata* and *Dinophysis acuta*, and some other species of the genus *Prorocentrum* [20] such as *Prorocentrum lima* and *Prorocentrum mexicanum*. DTXs are inhibitors of serine/threonine protein phosphatases and cause gastrointestinal symptoms in humans [21]. PTXs also belong to LTs, but that are not diarrheagenic after oral administration and do not exhibit the same mechanism of action of the OA group of toxins [15]. They show hepatotoxicity in mice following intraperitoneal injection and cytotoxicity in several mammalian cells with tumorigenic properties; however, they have low toxicity via oral administration [22]. Despite their global distribution, different species of *Dinophysis* generally form a small fraction of the plankton but can cause toxic episodes at a very low cell density. This fact is probably attributable to the lipophilic nature of the toxins [23] (Table 1). Among the factors able to influence the physiological processes of HAS, the variation in seasonal temperature represents an important factor to investigate [20]. The seasonal variation in toxin accumulation in bivalve mollusks is determined by the appearance of toxic algae and can follow different season trends depending on causative toxic algae as well as the climate seasonal variations in different periods of the year [24].

Algal blooms have been known since ancient times [25]. Algae of the genus *Alexandrium* were reported in Italy in the 1990s along the coasts of Emilia Romagna and in the Adriatic Sea in conjunction with the accumulation of PSP toxins in mussels. Blooms of algae belonging to the *Alexandrium* genus have also been reported in the Gulf of Naples and Salerno, in the northern Tyrrhenian Sea and in the Adriatic Sea [26]. Blooms of *A. minutum* and *A. catenella* were constantly described in Sardinia from 2002 to 2012 and,

more recently, in 2018, causing a ban on the harvesting and marketing of bivalve mollusks that had accumulated PSP-type toxins in their tissues [27–29].

Table 1. Syndromes due to marine biotoxins, toxins, algal species and symptoms in humans and animal organisms.

Syndromes	Toxins	Algal Species	Intoxication Diseases
Amnesic shellfish poisoning	Domoic acid	*Pseudo-nitzschia* spp.	Vomiting, abdominal pain, diarrhea, neurological symptoms, memory loss, and, in extreme cases, leading to death
Paralytic shellfish poisoning	Saxitoxin and derivates	*Alexandrium* spp. *Gymnodinium catenatum* *Pyrodinium bahamense*	Tingling sensation of the lips, mouth and tongue, numbness of the extremities, headache, dizziness, nausea, vomiting, diarrhea and, in severe cases, death by asphyxiation
Diarrhetic shellfish poisoning	Okadaic acid, its derivates and Dinophysis toxins	*Dinophysis* spp .*Prorocentrum* spp.	Gastrointestinal symptoms in humans
Lipophilic toxins group syndromes	Pectenotoxins	*Dinophysis* spp.	Hepatotoxicity in mice following intraperitoneal injection and cytotoxicity in several mammalian cells with tumorigenic properties; however, they have low toxicity via oral administration
	Yessotoxins	*Goniaulax spinifera* *Protoceratium reticulatum*	No human cases of human intoxication have been reported. Neurological symptoms in mice following intraperitoneal injection
	Gymnodimine	*Alexandrium ostenfeldii* *Karenia selliformis*	Neurological symptoms
	Spirolides	*Alexandrium ostenfeldii*	Neurological symptoms
	Pinnatoxins	*Vulcanodinuim rugosum*	Neurological symptoms
	Portimine	*Vulcanodinuim rugosum*	Portimine has very low acute toxicity to mice but is highly cytotoxic to cultured cells
	Azaspiracid	*Azadinium* spp.	Neurological symptoms in mice following intraperitoneal injection, also in mice, after oral exposures may cause tumors in different organs

Alexandrium blooms have also been reported in Sicily [30]. Since 1989, it is well known that some species of *Dinophysis* responsible for the production of OAs are an important part of the Adriatic phytoplankton: in the same year, DSP intoxication was associated with the presence of *D. fortii*, *D. tripos* and *D. caudata* [26]. The toxicity of Adriatic mussels due to liposoluble toxins is frequent, particularly in autumn and summer. In 2010, an extensive algal bloom from the Gulf of Trieste to the coasts of Emilia Romagna due to the genus *Dinophysis* was described [4].

In Sardinia, the first reported cases of positivity for OAs in bivalve mollusks sustained by blooms of the genus *Dinophysis* occurred in the Cagliari area in 2002–2003. More recently, again in the Cagliari area and in the Tortolì and Orosei production areas, algal blooms of *D. acuminata* were described from 2015 to 2020 [31–33].

Massive *Ostreopsis ovata* algal blooms affecting human health were reported in the Gulf of Genoa in 2005–2006 [34].

In order to minimize the risk of shellfish poisoning via the consumption of contaminated seafood, the European Union (EU) requires the member states to carry out official monitoring programs both for toxin-producing phytoplankton in water and mollusks that are bred [35]. According to Regulation (EC) No. 853/2004 [6], bivalve mollusks must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits: (a) PSP: 800 µg STX eq/kg e.p.; (b) ASP: 20 mg DA eq/kg e.p.; (c) for OA DTX and PTX together: 160 µg OA eq/kg e.p.; (d) AZA: 160 µg AZA eq/kg e.p.; (e) as regards YTX: 3.75 mg YTX eq/kg e.p. according to Regulation (EC) No 786/2013, amending Annex III to Regulation (EC) No 853/2004 of the European Parliament and of the Council as regards the permitted limits of YTX in live bivalve mollusks [36].

The Sardinian production of mollusks plays an important economic role. The annual amount of shellfish production is about to 13,000 tons [37]. The breeding of shellfish represents the first rise in aquaculture in terms of the quantities produced and the number of farms. Mollusks bred in Sardinia are consumed by local populations and are marketed in several Italian cities and European countries: their contamination could pose severe large-scale risks to seafood consumers.

The purpose of the present study was to investigate the seasonal composition and abundance of HAS and related toxins (PSP, ASP and DSP) over a 6-year monitoring program (2015–2020) carried out in several species of bivalve mollusks (i.e., *Ruditapes decussatus*, *Mytilus galloprovincialis*, *Crassostrea gigas*) bred in Tortolì Lagoon, one of the most important Italian shellfish farming areas [38].

2. Materials and Methods

2.1. Study Site

Tortolì Lagoon (Figure 1) is located in the central-eastern coast of Sardinia (39°56′43″ N, 9°40′37″ E) and is a transitional ecosystem formed in a coastal area of alluvial origin. The depth of the lagoon is low (mean 1.5 m) and consists of sandy material of average particle size. It is classified as a "euryhaline zone" with a salinity of 30–40 psu [39]. It has a saline nature due to its proximity to the sea and is significantly influenced by the contribution of freshwater from two tributaries. These rivers provide uncontrolled fresh water, especially in periods of flooding (e.g., on the occasion of intense and prolonged rainfall because of their torrential character). On the contrary, the supply of fresh water is almost zero during the summer. The climate of the area is classified as "inland Mediterranean" [39] and is characterized by mild and relatively rainy winters and dry and hot summers.

Figure 1. Study area and location of the sampling sites for harmful algal species and bivalve mollusks, from 2015 to 2020. 1, 2, 3, 4 and 1 V indicate sampling points (stations) in Tortolì Lagoon.

2.2. Sampling

The study was conducted over six years, from January 2015 to March 2020. A total of 542 water samples and 1090 mollusks samples (Table 2) were collected. Water and bivalve mollusks were collected at the same time every two weeks, in agreement with the Sardinian Regional Monitoring Programme [40]. No data about dinoflagellate cysts were collected from this area during the study.

Table 2. Number of total water and mollusk samples analyzed from 2015 to March 2020.

Year	2015	2016	2017	2018	2019	2020
Water samples	88	84	111	110	119	30
Mollusk samples	134	197	218	202	271	68

2.2.1. Water Samples

Each 1-L water sample was taken in clean polyethylene (PE) bottles at a depth of 0.5 m from the water surface and immediately fixed with Lugol's iodine solution, in order to prevent algal degradation. Water samples were analyzed according to Utermöhl's method [41], in accordance with the EU reference method UNI EN ISO 15204:2006 [42]. The cell count was performed on settling chambers of a volume of 25 mL under an inverted microscope at 200 magnification (Olympus I73). Microalgal abundance was expressed as the number of cells per liter (cells/L) with a detection limit of 120 cells/L, for a subsample of 25 mL and a level of significance of 0.05 (EN 15204:2006) [42].

2.2.2. Shellfish Samples

Shellfish samples were collected from five sampling points: 1, 2 and 3 (*Mytilus galloprovincialis*), 4 (*Crassostrea gigas*) and 1 V (*Ruditapes decussatus*). Shellfish collection was performed with different methods according to the rearing system adopted for each species. Mussels were harvested by hand, cutting the vertical ropes ("droppers") and catching the mussels in bins. Oysters were harvested by picking the floating bags (pouches) used for oyster culture. The harvesting of clams was conducted manually or using rakes with a collection basket at ca. 1 m depth on the sandy bottom. Overall, 1090 bivalve mollusks (773 mussels, 162 oysters and 155 clams) were analyzed during the six years of study.

2.3. Determination of Biotoxins

The determination of PSTs in shellfish samples was performed by mouse bioassay (MBA).

The PSTs were extracted from shellfish tissue in accordance with the AOAC 2005 Official Method (959.08) [43]. After the homogenization (SUMBEAM model 4153–50) of 200 g of each sample, 100 g was preserved for any further confirmatory analysis, and the other 100 g was extracted in 100 mL of 0.1 N HCl. The pH was adjusted to approximately 3 and the mixture was then boiled for 5 min. After cooling the mixture to 25 °C, the pH was again adjusted by the addition of either 5 N HCl or 0.1 N NaOH. Water was added to make up a final volume of 200 mL.

After 5 min of centrifugation (3000 rpm, 1660 RCF), 1 mL of the translucent supernatant was injected intraperitoneally into three Swiss mice (body weight, 19–21 g, about 25 days old, obtained from Envigo RMS, Udine-Italy). The symptoms (in terms of mouse behavior post-injection) were observed, and the time of death was recorded. The test was considered positive when the mice died within a specific time period, and the level of toxins present was determined at the time of death. The preserved aliquots of the MBA-positive samples were analyzed by liquid chromatography with fluorescence detection (LC-FLD) for confirmation and characterization of the PSTs, in accordance with the AOAC 2005 Official Method 2005.06 [44]. Since PSTs do not exhibit natural UV absorption or fluorescence, they must be oxidized into fluorescent derivatives before detection by FLD.

This process involves oxidation of the toxins into iminopurine derivatives before their separation and determination by LC-FLD (precolumn oxidation). The method consisted of the following steps: (i) duplicate acid extraction of shellfish homogenates with 1% acetic acid solution (the first extraction with heating); (ii) clean-up by solid-phase extraction (SPE) using a Supelclean LC-18 SPE 3 mL cartridge (Supelco, Sigma-Aldrich Srl, Milan, Italy); (iii) addition of an aliquot of the C18-cleaned extract to an SPE-COOH ion-exchange 3 mL cartridge (Bakerbond, J.T. Baker, VWR, Milan, Italy) for sequential elution to obtain three individual fractions (fractions 1–3); and (iv) oxidation of the PSTs (from aliquots of the C18- and SPE–COOH–processed extracts) with periodate or peroxide oxidants depending on the type of toxin (N-hydroxylated or non-N-hydroxylated). The LC analysis was carried out on an Agilent system (UHPLC Infinity 1290 II, Agilent Technologies, Milan, Italy) equipped with a binary pump (1290 High Speed), an autosampler, a column oven and the 1260 Series Fluorescence Detector Spectra (Agilent Technologies). Agilent ChemStation software OpenLAB (Agilent Technologies) was used to perform the data acquisition and peak integration (accessed on 10 April 2020). Separation of the PSTs was performed using an Inertsil C18 reversed-phase column 15 × 4.6 mm i.d., 5 mm (GL Sciences, CPS Analitica for chemistry, Milan, Italy) equipped with a Security Guard C18 column (Phenomenex, Bologna, Italy). The column was maintained in the column oven at 30 °C. The detection wavelengths were set at 340 nm for excitation and 395 nm for emission. The mobile phase gradient used to elute the PST oxidation products consisted of two mobile phases (A 1/4 0.1 M ammonium formate, pH 6; and B 1/4 0.1 M ammonium formate in 10% methanol, pH 6) under the following conditions: 0–5% B in the first 5 min, 5–70% B for the next 8 min, 70% B for the next 2 min, 70% B for the next 4 min and 100% A for 5 min before the next injection. The flow rate was 1 mL/min. The injection volumes were 25 and 50 mL for the oxidation products of the peroxide and periodate reactions, respectively. The concentrations of each toxin or epimeric pair (GTX1þ4, GTX2þ3, C1þ2, dcGTX2þ3 and C3þ4) were quantified with linear calibration curves, achieved using certified PST reference standards (provided by the National Research Council Canada, Halifax, Nova Scotia). To calculate the total sample toxicity in terms of STX e.q., the toxicity equivalence factor (TEF) was applied for each toxin. For the epimeric pairs, the highest TEF was used so that the overestimated toxicity would always be obtained [44].

The LTs were investigated with a liquid chromatography-tandem mass spectrometry approach (LC-MS/MS) in accordance with the Regulation (EC) 15/2011 [45], where a suite was composed of OA; DTXs; PTXs; YTXs; and AZAs. The extraction procedure was carried out in accordance with AESAN EU-RL-MB Lipophilic toxins Version 5: 2015 [46].

Mussel tissue homogenates (2.00 ± 0.05 g) were added to 9 mL 100% methanol. Each sample was homogenized via vortex mixing for 3 min; afterwards, it was centrifuged, and the supernatant was transferred into a 20 mL volumetric flask. The extraction of the residual tissue pellet was repeated with another 9 mL of methanol.

The supernatant was combined with the first extract, and then the final extract was brought to a volume of 20 mL with methanol. The methanolic extract was filtered through a dry methanol-compatible syringe filter and finally injected into an LC-MS/MS instrument for the determination of AZAs, PTXs and YTXs.

The determination and quantification of the total OA-DTX content were performed using alkaline hydrolysis before LC-MS/MS analysis. The hydrolysis consisted of adding NaOH 2.5 M to an aliquot of methanolic extract, homogenizing in a vortex for 0.5 min and heating the mixture at 76 °C for 40 min. Then, after cooling to room temperature, the mixture was neutralized with HCl 2.5 M and homogenized in the vortex for 0.5 min. The final step was the filtration before the injection into the LC-MS/MS instrument.

The analysis was performed by an ultra high performance liquid chromatography system using a Thermo Scientific Accela 1250 coupled to a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer in 2015 and a Perkin Elmer Flexar F-15 coupled to an ABSCIEX QTRAP 4500 in later years. All systems were equipped with an electrospray ionization (ESI) source.

The quantification limit of this analytical method for the OA toxin group was 60 µg OA eq/kg e.p.

DA was identified by RP-HPLC using UV detection in accordance with the Standard Operating Procedure AESAN, 2008 [47]. An aliquot (4.0 ± 0.1 g) of mussel tissue previously homogenized was placed into a graduate centrifuge tube, combined with 16 mL of extraction solvent (methanol/water 50:50) and homogenized extensively (3 min 10,000 rpm). The extract was centrifuged at 3000× *g* for 10 min. A portion of the supernatant was filtered through a centrifugal filter (0.5 mL Millipore 10 K) and centrifuged to 9000 g for 30 min.

Determination of the DA content in samples was performed on an Agilent 1290 instrument. The chromatographic separation was carried out on a reversed-phase column (Zorbax Eclipse Plus C18 RRHD 2.1 × 50 mm ×1.8 µm, Agilent) using isocratic conditions (water/acetonitrile (90:10 *v*/*v*) with 0.1% of trifluoroacetic acid).

2.4. Statistical Analysis

The concentrations of OA and PTX2 were analyzed using the following generalized linear model (GLM):

$$y = \mu + S{+}Y + date + T{+}P + S{+}DO + e \quad (1)$$

where y is the biotoxin concentration expressed as µg eq./g; µ is the overall mean; S is the effect of the species of bivalve mollusks (3 levels: mussel, clam, oyster); Y is the effect of the years (6 levels: 2015, 2016, 2017, 2018, 2019, 2020); date was the random effect of the date of sampling; T, P, S and DO are the main physicochemical parameters: temperature, pH, salinity and dissolved oxygen, respectively; e is the random residual term. Generalized mixed linear model analysis was performed using SAS PROC GLIMMIX (SAS Inc, Cary, NC, USA, 2011). Due to the markedly skewed distribution of these two toxins, the GLM was carried out assuming a lognormal distribution. The two random effects (i.e., date and residual) were assumed to be normally distributed with their parameters $(0,I\sigma_d^2)$ and $(0,I\sigma_e^2)$, where I is an identity matrix and σ_d^2 and σ_e^2 are the variance components associated with date and residual terms, respectively. The contribution of the date variance to the total variance was computed as: $r_d = \sigma_d^2/(\sigma_d^2 + \sigma_e^2)$.

Differences were declared significant when *p*-values from the ANOVA test were lower than 0.05. When species or year was found to be significant (i.e., *p*-value for ANOVA test lower than 0.05), means were separated by the Tukey honestly significant difference test with significance declared at $p < 0.05$. R software (version 3.6.3) (accessed on 9 December 2020) [48] was used for statistical analyses and graphic representations. Results were expressed as mean value ± standard deviation (SD) and maximum value. Spearman correlation analysis was also performed.

3. Results

3.1. Harmful Algae

A total of nine HAS (Figure 2 and Table 3), almost all belonging to the class of *Dinophyceae*, were found during the study. The seasonality influenced their presence: in general, HAS detection was more frequent in the winter months (especially in February), and in October. Some exceptions were observed: in June 2016 (Figure 2b), there was the highest suite of different HAS (*Pseudo-nitzschia* spp., *D. acuminata*, *D. caudata*, *D. sacculus*, *P. mexicanum*, *P. lima*). The lowest HAS detection was recorded in 2019 (Figure 2e).

Figure 2. Temporal dynamics of the occurrence of harmful algal species in mollusk production areas of Tortolì Lagoon from 2015 to March 2020. Letters (**a–f**) refer to years 2015→2020. (**a**): year 2015; (**b**): year 2016; (**c**): year 2017; (**d**): year 2018; (**e**): year 2019; (**f**): year 2020.

Table 3. Frequency of potentially toxic taxa expressed as a percentage found in shellfish farming waters in the Sardinia region, years 2015–2020.

	% Frequency of Taxa in the Years					
Taxa	**2015**	**2016**	**2017**	**2018**	**2019**	**2020 ***
Alexandrium minutum	ND	1.10%	ND	ND	4.20%	ND
Dinophysis acuminata	79%	76%	47.70%	33.60%	33.60%	73%
Dinophysis caudata	4.50%	ND	ND	ND	ND	ND
Dinophysis rotundata	4.50%	9.50%	ND	ND	1.60%	6%
Dinophysis sacculus	42%	23.80%	16.20%	13.60%	5.80%	3%
Dinophysis spp.	ND	1.10%	ND	ND	ND	ND
Prorocentrum lima	5.60%	10.70%	8.10%	3.60%	0.80%	6.60%
Prorocentrum mexicanum	6.80%	2.30%	1.80%	0.90%	0.80%	ND
Pseudo-nitzschia spp.	69%	86%	79%	70%	39%	6%

* 2020 sampling refers to January to March; ND = not detected.

Among the HAS detected (Table 3), *Pseudo-nitzschia* species and *D. acuminata* were the most present either in frequency (respectively, from 2016 to 2019, and in 2015 and 2020) or in cell abundance. *Pseudo nitzschia* spp. were present every month in all six years included in the study, always with the highest abundances, especially in January and March (values >1 $\times$ 10^6 cells/L). The highest abundance was detected in October 2015 (22 $\times$ 10^6 cells/L). *Pseudo-nitzschia* spp. were mostly present in March. Conversely, they were less abundant in April (only 2.2% of the total).

D. acuminata was the most present among the dinoflagellates, with a peak detection of 79% of total HAS detected in 2015 and a maximum value of abundance of 13 $\times$ 10^5 cells/L. It was present in 52% of the samples, mostly in January (8.6%), and less so in November (2%). *D. sacculus* was found in 17.9% of the analyzed samples, particularly in February (3.8%). *D. acuminata* and *D. sacculus* were found every year, in all months, with very few exceptions. The highest abundances of *Dinophysis* species were reported when the water temperature was between 10 and 13.2 °C (January to March), and between 20 and 12.2 °C (October–December).

The percentages of *Prorocentracea* (*P. lima* and *P. mexicanum*) ranged between 10.7% of total HAS in 2016 of *P. lima* and 6.8% of total HAS of *P. mexicanum* in 2015. The prevalence of *P. lima* was quite low. It was mostly detected from April to September, showing its maximum cellular abundance in 2016 in June and July (respectively, 2.8 $\times$ 10^3 cells/L and 3.2 $\times$ 10^3 cells/L). It was never found in January, November and December. *P. mexicanum* was detected principally in summer, with the highest frequency of detection in August 2015, with a peak abundance of 600 cells/L.

Among the HAS producing PSTs, *A. minutum* was the only one detected, in only four samples, collected in November 2018 and from April to June 2019.

3.2. Toxins

Biotoxin concentrations are shown in Table 4. OA was detected every year: the concentration was over the legal limits (160 µg OA eq/kg e.p.) in 39 samples (Table 4).

The majority of the non-compliances were detected in February and March. A single positive sample was reported in April and December 2015. The sample analyzed in December presented the highest OA value (1244 µg OA eq/kg e.p.). In 2019 and 2020, positive samples were recorded in January. The maximum concentration of PTX2 was 173 µg eq/kg e.p. in February 2019 in an oyster sample.

Values of PTX2, OA and some environmental parameters in samples collected in 2019 and 2020, with the contemporary presence of OA and PTX2, are shown in Table 5. In one oyster sample in 2019, the single value of PTX2 was over the legal limit of 160 µg OA eq/kg e.p. In two samples of oysters, in December 2019 and February 2020, the PTX2 values were crucial for non-compliance of the sample.

As shown in Figure 3, we noticed that in the winter months, there is an exponential growth of this biotoxin, with the peak almost always reached in February 2015 (1092 µg OA eq/kg e.p.), 2018 (174 µg OA eq/kg e.p.), 2019 (453 µg OA eq/kg e.p.) and 2020 (309 µg OA eq/kg e.p.). A short and similar persistence phase was observed in March 2018 and 2019, while in 2016, the concentrations of OA found in the tissues of the bivalves showed a fluctuating trend, reaching a further peak in March (299 µg OA eq/kg e.p.).

The PTX2 toxin was identified only in 2019 and in 2020 in the first two months of each year, in concomitance with the lowest water temperature (Figure 4).

Table 4. Distribution of non-compliance referring to OA and PTX2 between the years 2015 and 2020.

Year	Number of Non-Compliant Samples	Matrix	Months	OA Values (µg eq OA/kg p.e)	PTX2 Values (µg eq OA/kg p.e)
2015	8	*Mytilus galloprovincialis*	February	192 ± 34	ND
				1092	ND
				188 ± 33	ND
				301	ND
			March	326	ND
				181 ± 32	ND
			April		
210 ± 37			December		
1244	6	*Mytilus galloprovincialis*	March	208 ± 84	ND
				204 ± 83	ND
				277 ± 108	ND
				299	ND
				190 ± 78	ND
				200 ± 82	ND
				167 ± 70	ND
2017	3	*Mytilus galloprovincialis*	February	165 ± 70	ND
				165 ± 70	ND
2018	2	*Mytilus galloprovincialis*	February	174 ± 70	ND
				161 ± 50	ND
2019	10	*Mytilus galloprovincialis*	March	161 ± 68	ND
		Crassostrea gigas	February	251 ± 82	173
			March	214 ± 86	58
			December	121 ± 54	56
		Ruditapes decussatus	January	201 ± 66	ND
			February	453	48
				402	47
				232 ± 70	ND
				208 ± 62	ND
			March	162 ± 68	ND
2020	10	*Crassostrea gigas*	February	203 ± 83	ND
				177 ± 73	ND
				207 ± 84	52
				309	103
				168 ± 70	82
				126 ± 55	55
		Ruditapes decussatus	January	259 ± 102	ND
			February	237 ± 94	ND
				308	ND
				234 ± 93	ND

ND = not detected.

Table 5. Values of PTX, OA and parameters of water in samples with concurrent presence of PTX2 and OA.

SamplingDate	Sampling Station	Shellfish Product	PTX2 (µg OA/kg e.p.)	OA (µg OA/kg e.p.)	OA + PTX2 (µg OA/kg e.p.)	pH (Unit of pH)	Temperature (°C)
11 February 2019	4	*Crassostrea gigas*	173 ± 65	251 ± 82	424	6.85	12.1
14 February 2019	4	*Crassostrea gigas*	41	117	158 ± 67 *	6.64	9.4
18 March 2019	4	*Crassostrea gigas*	58	214 ± 86	272 ± 106	7.03	14.7
16 December 2019	4	*Crassostrea gigas*	56	121 ± 54	177 ± 73	6.85	12.2
04 February 2019	1 V	*Ruditapes decussatus*	48	453	501	8.70	13.6
11 February 2019	1 V	*Ruditapes decussatus*	47	402	449	8.80	12.1
02 February 2020	4	*Crassostrea gigas*	55	126 ± 53	181 ± 75	7.30	12.6
12 February 2020	4	*Crassostrea gigas*	52	207 ± 84	259 ± 102	6.70	12.7
17 February 2020	4	*Crassostrea gigas*	103	309	412	7.07	8.0
24 February 2020	4	*Crassostrea gigas*	82	168 ± 70	250 ± 99	7.30	13.5

* value under the legal limit.

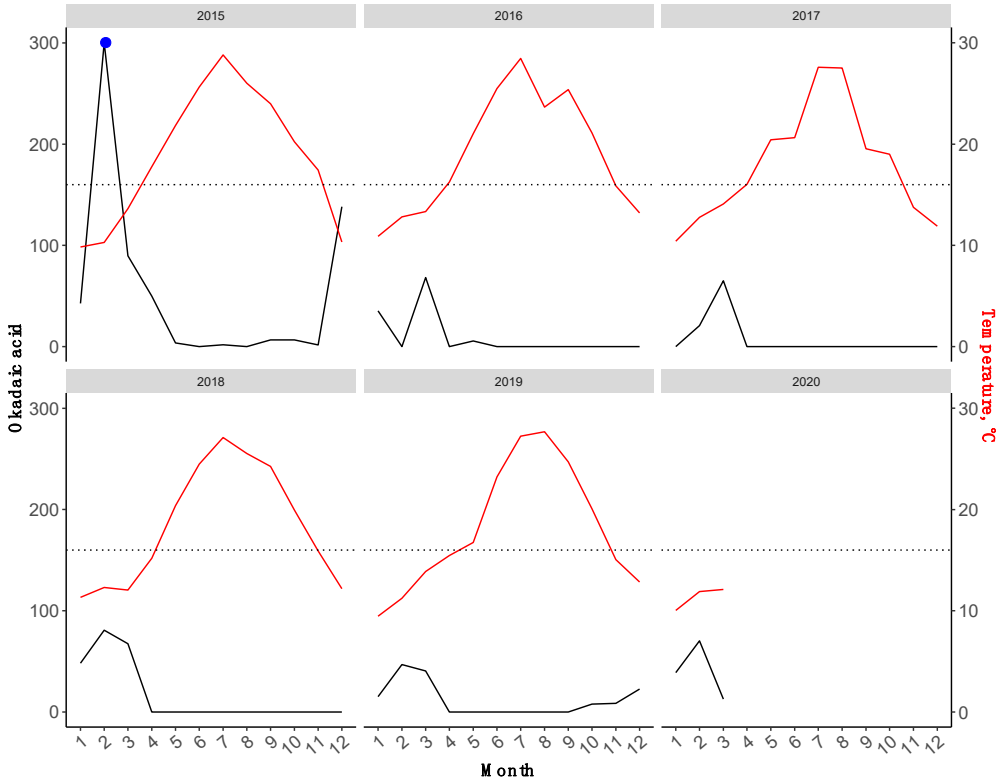

Figure 3. The annual trend of okadaic acid according to the temperature. Each blue dot indicates a value exceeding 160 µg eq OA/kg e.p.

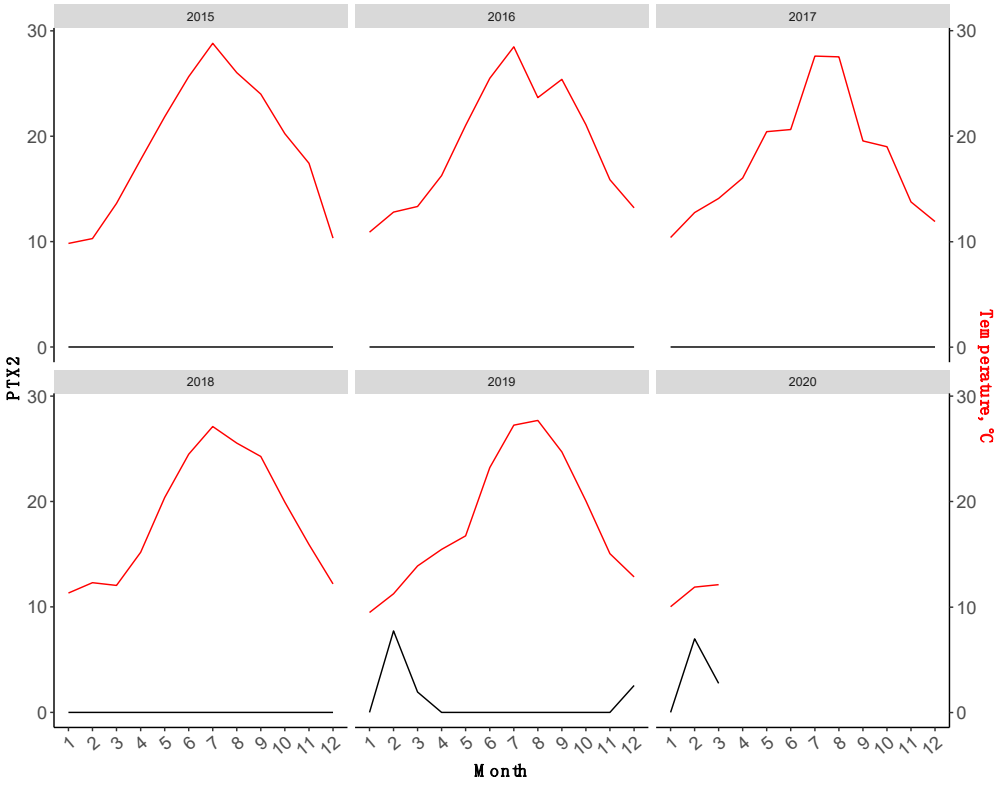

Figure 4. The annual trend of pectenotoxins2 for each analyzed year, and the trend and concentration of pectenotoxins2 in the tissues of the bivalve mollusks under study, in relation to the water temperature of the period.

In the data collected over the 6-year study period, the presence of DA was never detected, despite the high frequency of detection and the high concentrations of *Pseudo-nitzschia* spp. PSTs were never detected.

3.3. Statistical Results

Statistical analysis and Spearman's correlations among different parameters (in particular, OA, PTX2 and environmental parameters) were performed. Results are expressed as the mean value ± standard deviation (SD) and the maximum value (Table 6). Due to the very high number of samples with zero levels of PTX2, the GLM was carried out only for OA concentrations. Regarding the latter, no significant differences were observed among species (mussels, clams, oysters) or years (Table 7). The only significant effects ($p < 0.05$) were temperature and salinity. The random effect of the sampling date accounted for 43% of the variability, suggesting a quite strong impact of seasonal variations on the OA concentrations. The matrix of Spearman's correlations is reported in Table 8, which shows the results expressed as correlation coefficients and their *p*-values. OA and PTX2 levels were lowly (0.24), but strongly significantly ($p < 0.001$), correlated. Temperature showed negative correlations with OA (−0.33), PTX2 (−0.09) and algal abundance (−0.14), suggesting that when the temperatures decrease (i.e., winter months), the amount of algae and toxins significantly increases ($p < 0.001$). This could be explained by the presence of HAS in farmed water, where they may determine the intake of OA by bivalve mollusks reared in that area. The HAS presence is influenced by different factors, such as the water temperature and nutrients. The relation between the water temperature and biological activity determines an increase in the growth rate, in correspondence with the optimal growth temperature. With a water temperature below or under the optimal range (that is, specific for every algal species), the growth rate decreases [49]. The correlation between PTX2 and algal abundance was non-significant, probably because of the high number of samples with no PTX2 concentrations. Correlations between salinity and OA, PTX2 and algal abundance were never significant. pH showed weak, but significant, correlations with OA (0.12) and PTX2 (0.08), but a non-significant correlation with algal abundance. Correlations between oxygen and OA and algal abundance were significant, whereas the correlation between oxygen and PTX was not significant. The positive correlations between oxygen and OA and oxygen and algal abundance suggest that when the concentration of oxygen in the water increases, there is an increment in the OA and algal abundances [50].

Table 6. Concentrations of OA and PTX2 determined in bivalve mollusks from Tortolì Lagoon during 2015 to 2020.

		OA (µg OA/kg e.p.)			PTX2 (µg OA/kg e.p.)		
Year	**Species**	**Mean**	**SD**	**Max**	**Mean**	**SD**	**Max**
2015	*Mytilus galloprovincialis*	47.6	159	1244	0	0	0
2016	*Mytilus galloprovincialis*	16.3	48.5	299	0	0	0
2016	*Ruditapes decussatus*	3.11	16.9	125	0	0	0
2017	*Mytilus galloprovincialis*	10.2	36.3	167	0	0	0
2017	*Crassostrea gigas*	2.66	19.4	141	0	0	0
2017	*Ruditapes decussatus*	0	0	0	0	0	0
2018	*Mytilus galloprovincialis*	23.7	48	174	0	0	0
2018	*Crassostrea gigas*	9.27	30.7	141	0	0	0
2018	*Ruditapes decussatus*	5.03	27.6	151	0	0	0
2019	*Mytilus galloprovincialis*	4.48	23.6	161	0	0	0
2019	*Crassostrea gigas*	11.9	47.1	251	5.56	25	173
2019	*Ruditapes decussatus*	55.2	102	453	1.94	9.5	48
2020	*Mytilus galloprovincialis*	2.88	13.9	72	0	0	0
2020	*Crassostrea gigas*	120	97.3	309	22.5	37.1	103
2020	*Ruditapes decussatus*	132	105	308	0	0	0

SD = standard deviation. Max = highest value recorded.

Table 7. Results of the generalized linear model applied to OA levels assuming a lognormal distribution.

Effect	*p*-Value
Species	0.85
Year	0.55
Temperature	0.03
pH	0.61
Oxygen	0.04
Salinity	0.78

Table 8. Spearman correlation matrix with correlation coefficients and their *p*-value.

Correlation/ *p*−Value	OA	PTX2	Algal Abundance	Temperature	pH	Oxygen	Salinity
OA		0.24	0.13	−0.33	0.12	0.28	−0.03
PTX2	$p < 0.001$		−0.02	−0.09	0.08	−0.03	−0.04
Algal abundance	$p < 0.001$	NS		−0.14	−0.05	0.26	0.00
Temperature	$p < 0.001$	$p < 0.001$	$p < 0.001$		−0.28	−0.26	0.32
pH	$p < 0.001$	$p < 0.001$	NS	$p < 0.001$		0.04	−0.65
Oxygen	$p < 0.001$	NS	$p < 0.001$	$p < 0.001$	NS		−0.04
Salinity	NS	NS	NS	$p < 0.001$	$p < 0.001$	NS	

NS = not significant.

4. Discussion

The results of this 6-year study performed in Tortolì Lagoon highlight the presence of HAS during the entire monitoring. The percentage of non-compliant samples was 3.5% and concerned mainly the presence of DSP toxins, while ASP and PSP toxins were never detected. The OA toxin group was largely predominant among LTs. It was found with more frequency beyond the regulation limits in mussels (1.8%), followed by clams (0.9%) and oysters (0.82%). During 2019 and 2020, the presence of PTX2 was noticed several times. Already in the past, positive shellfish to the OA toxin group were detected in Sardinia, related to the presence of a few *Dinophysis* species (mainly *D. acuminata* and *D. sacculus*), which resulted in the closure of farms [31,32]. A high degree of seasonality in the accumulation of OA in bivalve mollusks clearly emerged in this study. Values of OA above the legal limits were mainly reported in winter (February and March) when the water temperature averaged 11 °C and the salinity was 8 psu. The highest OA value in mussels was described in December 2015 (1244 µg eq OA/kg p.e). Statistical analysis confirmed that cellular abundances and water temperature resulted in being extremely correlated; in addition, significant correlations between OA and oxygen, and between PTXs and water temperature were observed. Previously, Turkish and Greek studies highlighted the same seasonal trend in other parts of the Mediterranean Sea [51,52].

From 2015 to 2019, all non-compliant shellfish samples for OA were mussels. Between 2019 and 2020, several non-compliances in OA values were observed in other shellfish species including oysters and clams, with the levels of OA in oyster and clams showing an associated co-occurrence of PTX2. Between 2019 and 2020, several non-compliances in OA values (often in association with the presence of PTX2) were detected. Several factors may explain why mussels were much more frequently positive for OA. Firstly, mussels are reared in three different production areas of the lagoon and in much larger numbers. In addition, mussels may be considered better bioaccumulators than oysters. Some studies have reported that the oyster *C. gigas* can react to contamination by closing its valve to reduce tissue contamination [53]. Despite this, in Tortolì Lagoon, in 2019 and 2020, there was a greater accumulation of OA in oysters and clams. A possible explanation could be due to the sea current and the location of each farming point [54,55]. In fact, mussel breeding sites are located near the outlet of the sea, while oysters and clams are located in

areas more protected from sea currents. OA was present in mussels at the same time as it was present in clams and oysters, but with the contamination level below the legal limit. In general, the temporal relation between the presence of *Dinophysis* spp. and bivalve mollusk toxicity has not always been clear. As suggested by Reguera et al., 2012 [56], several factors can determine the production of toxins by microalgae, such as some environmental conditions and the presence of different strains, toxic or non-toxic, of the same species. As a matter of fact, the presence of *Dinophysis* species in the water column is not always linked to the appearance of LTs in shellfish. Strains of the same species may produce OA or PTXs, or both [57], or may not produce toxins. According to Godhe et al., 2002 [58], the environmental parameters may not have been the only factors able to determine the presence of LTs during the 6-year period; the presence of similar environmental conditions was not always correlated with the accumulation of OA or PTX2 in shellfish. The toxicity observed in bivalve mollusks is not the result of a simple linear process, but rather a balance from a chain of species-specific processes [20].

PTXs were detected only in oysters and clams, bred in specific areas of the lagoon, characterized by distinctive features, such as the depth of and proximity to the sea. The production of toxins in the same algal species has already been shown [59] to vary considerably even within specimens collected in the same locality. As reported by some authors [60], each species may have the characteristic to esterify toxins possibly present in the algae ingested, in order to determine their transformation and accumulation in the tissues of different mollusks. For example, pectenotoxin metabolization via enzymatic oxidation has been reported in Japanese scallops, and hydrolysis to seco acid has been reported in mussels and other shellfish [61].

Our results show that species of the *Dinophysis* genus (in particular, *D. acuminata* and *D. sacculus*) were found every year, in all months, with greater frequency from January to March and from October to December. This is not in accordance with a previous study [62], carried out in the Adriatic Sea, where *D. acuminata* was mostly detected in spring and *D. sacculus* in late spring and summer. In accordance with previous authors [63], we noticed that *P. lima* and *P. mexicanum* were mostly present in the summer months.

The appearance of dinoflagellates may follow a series of environmental changes, e.g., enhanced availability of light and inputs of organic and inorganic matter [64], sometimes allowing the establishment of bloom prediction indices [65].

Although, in Sardinia, there have been numerous cases of non-compliance for PSP toxins [28], even recently [29], no non-compliance for PSP toxins was found in this study. The lack of PSP toxins could have been linked to the scarcity of algal cells capable of producing PSTs.

No non-compliance for ASP toxins was found. The absence of DA may be connected with the presence of the species *Pseudo-nitzschia* which is unable to produce toxins [66]. The presence of ASP toxins in bivalve mollusks bred in Sardinia was found in 2011 [67], and the levels found did not, in any case, exceed the legal limit (20 mg eq DA/kg p.e); however, in one sample, the concentration was close to the legal limit (18 mg/kg domic acid + epidomic acid). The *Pseudo-nitzschia* genus, responsible for the production of this toxin, is found constantly and continuously in Sardinian marine waters, representing a threat for regional shellfish productions.

The consequence of the accumulation of algal biotoxins in shellfish products is of two types: sanitary problems in humans, such as syndromes (PSP, DSP and ASP), and economic problems for shellfish farmers due to the ban on harvesting and the resulting loss of confidence in bivalve mollusks consumers.

HAB-related mortality events consisting of *Dinophysis* spp., *Prorocentrum* spp. producing OA and DA-producing *Pseudo-nitzschia* diatoms have been observed in combination with mortality events of a large number of marine mammals (*Tursiops truncates*) in Texas waters [68].

As regards STX, mortality events of marine mammals have been reported in Humpback whales (*Megaptera novaeangliae*) and in Bowhead whales (*Megaptera mysticetus*) in Alaska waters [69].

Further studies on the effects of marine biotoxins in aquatic mammals are needed, marine mammals being important barometers of coastal marine health [68].

5. Conclusions

Despite the fact that the percentage of non-compliant samples for the OA toxin group was low (3.5%), this fact has led to the closure of shellfish farms for some months a year, with consequent economic damage for producers.

A statistical correlation between the accumulation of the OA toxin group in mollusks, seasonality and environmental parameters, such as water temperature and the presence of HAS, was demonstrated, mainly due to the presence of *Dinophysis* species, which resulted in a seasonal trend, with the normal accumulation of toxins in winter months. Large datasets of a different nature are fundamental in order to better organize sampling strategies.

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