New evidence of pectenotoxins in farmed bivalve molluscs from Sardinia (Italy)

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Abstract

Several planktonic dinoflagellates can produce lipophilic phycotoxins that represent a significant threat to public health as well as to shellfish and fish farming. Poisoning related to some of these toxins is categorised as diarrhetic shellfish poisoning. We analysed 975 shellfish samples from Tortolì in the central-eastern region of Sardinia (Italy) from January 2016 to March 2020, to investigate the prevalence of different lipophilic marine biotoxins in mollusc bivalves. The results highlighted the predominant presence of toxins belonging to the okadaic acid group in all samples with toxin concentrations exceeding legal limits, and revealed the new occurrence of pectenotoxins in oysters and clams with a winter seasonality in recent years. The origin of shellfish toxicity was associated with the same *Dinophysis* species, mainly *D. acuminata*. Based on both these results and other precedents, monitoring and recording systems are strongly recommended.

Introduction

Toxic events associated with the consumption of bivalve molluscs contaminated by natural toxins produced by microalgae occur worldwide each year (Allen *et al*., 2006). These toxins are easy to accumulate in shellfish fat and may lead to cases of human poisoning by trophic transfer. Most toxins are lipophilic, and are related to diarrhetic shellfish poisoning depending on the symptoms they produce. They are grouped in different classes, including okadaic acid (OA), pectenotoxins (PTXs), yessotoxins, and azaspiracid groups. Based on toxicological studies using mouse bioassays, Renyan *et al*. (2011) reported that PTXs are not diarrheagenic after oral administration and do not exhibit the same mechanism of action as the OA-group of toxins (Farabegoli *et al.,* 2018). PTXs show hepatotoxicity to mice following intraperitoneal injection and cytotoxicity in several mammalian cells with tumorigenic properties; however, they have low toxicity via oral administration (Ito *et al.,* 2008). PTXs have never been linked to any human intoxication (EFSA, 2009), nevertheless, the health standards for live bivalve molluscs concerning OA, *Dinophysis* toxins, and PTXs together are 160 micrograms of OA equivalents per kg (Reg EU 853). PTXs are exclusively produced by dinoflagellates of *Dinophysis* (Farabegoli *et al.,* 2018) such as *D. acuminata* Claparède & Lachmann, *D. norvegica* Claparède & Lachmann, *D. acuta* Ehrenberg, *D. fortii* Pavillard, and *D. caudata* Kent. They are found in different bivalve molluscs (including scallops, mussels, and cockles) from several geographic areas (Wilkins *et al*., 2006; Gonzales-Gil *et al.,* 2011).

To prevent the effects of marine toxins and to minimise the potential high risk to consumers of bivalve molluscs, a specific monitoring program has been implemented in Sardinia (Bazzoni *et al*., 2016, 2018; Mudadu *et al*., 2017), where shellfish production is a very important economic sector.

Nowadays there are different strategies for a quick and efficient determination of new toxins, with innovative multidisciplinary strategy, which have been developed in recent years. An example of an innovative approach is reported by Esposito *et al.,* 2019, where the data of a new multidisciplinary strategy (Fast Detection Strategy for Cyanobacterial blooms) based on remote sensing technique are reported.

Studies have confirmed that often, the presence and accumulation of toxic compounds are involved in events of diarrhetic shellfish poisoning (Bazzoni *et al.,* 2018). The aim of this study was to assess the occurrence of lipophilic toxins in harvested bivalve mollusc (mussels, clams, oysters, and cockles) samples from Tortolì (central-eastern Sardinia, Italy) over a period of 5 years (2016-2020). The composition of biotoxins was analysed, paying particular attention to the content of PTXs, which were reported for the first time in oysters and clams. The detection of the biotoxins was analysed in relation to the presence of toxic microalgae.

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Materials and methods

Sampling area and sample collection

The Tortolì Lagoon (39°56′43″N, 9°40′37″E) is located in the central east coast of Sardinia (Italy) (Figure 1). Its area is approximately 2.422.440,722 m^2 and the perimeter 15.147,69 m. The lagoon receives fresh water from two different fluvial systems. The average depth of water is 2.3 m.

The study was conducted from January 2016 to March 2020. A total of 454 water samples were analysed to assess the presence and abundance of species causing potentially toxic diarrhetic shellfish poisoning: 84 in 2016, 111 in 2017, 110 in 2018, 119 in 2019, and 30 in the first three months of 2020. For the evaluation of PTXs, 975 mollusc samples (244 in 2016, 201 in 2017, 186 in 2018, 274 in 2019, and 70 in 2020) were collected, of which 639 were mussels (65.5%); 214, oysters (21.9 %); and 122, clams (12.5 %). Water and mollusc samples were collected bimonthly from five sampling points called 1, 2, 3, 4, and 1V (Figure 1). Stations 1 to 3 were dedicated to the production of mussels (*Mytilus galloprovincialis* Lamarck), station 4 to the production of oysters (*Crassostrea gigas* Thunberg), and the station 1V to the production of clams (*Ruditapes decussatus* Linnaeus).

Phytoplankton analysis

One litre of water was collected from each sampling point using clean polyethylene bottles, of which half a litre was fixed in situ with Lugol's iodine solution for the quantitative analysis of phytoplankton, and half a litre was without fixative for the observation of live microalgae. Cell numbers of *Dinophysis* species were counted in the Lugol's iodine-fixed samples under a light microscope (Olympus IX-73, Tokyo, Japan) and settling chambers of 25 mL, using the method described by Utermöhl (1958) and in accordance with the EU reference method UNI EN ISO 15204:2006. Abundance was expressed as the number of cells per litre. The quantitative detection limit of Utermöhl's method for a subsample of 25 mL is 120 cells/L (level of significance at 0.05).

Lipophilic toxin analysis

The presence of OA, *Dinophysis* toxins, PTXs, yessotoxins, and azaspiracids was determined in shellfish samples utilising liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (AB SCIEX QTRAP 4500), consistent with the official protocol (AESAN, Vigo, version 5, 2015) governed by the EC Regulation 15/2011. The different phases of extraction and analysis by LC-MS/MS are reported specifically in the report by Bazzoni *et al.* (2018).

Results

A total of 30 samples exceeded the maximum legal limit of 160 µg eq OA/kg e.p. (Reg 853/04 and subsequent amendment), among which 6 were sampled in 2016, 3 in 2017, 2 in 2018, 9 in 2019, and 10 in 2020. Bivalve molluscs were mainly contaminated by the OA-group toxins, which was the component that most frequently reached the highest values. PTX toxins (in particular PTX2) were reported for the first time in 2019 (January 2019). Between 2016 to 2018, the presence of PTXs was never reported. PTXs toxins were found between January to March in both 2019 and 2020. In 2019, a positive sample was also recorded in December (Table 1). The OA toxins were present in all samples along with the presence of PTX2.

PTX2 was reported in 10 samples, 9 of which exceeded the legal limit (5 in 2019 and 4 in 2020) from a total of 975 analysed samples (0.92%). The highest concentration of PTX2 (173 µg) was quantified in oysters in February 2019. This high value was enough to ensure these samples being beyond the regulatory limit without considering other toxins. However, PTXs contributed to exceeding the legal limits in only three samples (Table 1). In fact, in most samples with the presence of PTXs, it was a minority component in comparison to the OA toxin group (Figure 2); on average, it represented 26% of the toxins detected in the sample, with a maximum of 41% and a minimum of 10%. The presence of PTXs was always recorded in oyster and clam samples; in contrast, it was never detected in mussels. Every time the OA and PTXs toxin groups were found in molluscs, *Dinophysis* species were reported in the seawater, even days or weeks before the identification of toxin accumulation. *Dinophysis* species mostly present were *D. acuminata*, and to a lesser extent, *D sacculus* Stein. The average water temperature recorded when PTXs were present was 12°C and the average pH value was 7.3. Data on water temperature and pH are pre-

Figure 1. Study area and breeding points of the different species of molluscs.

Table 1. Values of pectenotoxins, okadaic acid and environmental parameters in samples.

Sampling Date	Sampling Station	Shellfish product	PTX2 µg PTX/Kg e.p.	OA µg OA/Kg e.p.	OA+PTX2 µg OA/Kg e.p.	Water pH	Water temperature °C
11/02/2019	4	Oysters	173	251	424	6.85	12.1
14/02/2019	4	Oysters	41	117	158*	6.64	9.4
18/03/2019	4	Oysters	58	214	272	7.03	14.7
16/12/2019	4	Oysters	56	121	177	6.85	12.2
04/02/2019	IV	Clams	48	453	501	8.70	13.6
11/02/2019	IV	Clams	47	402	449	8.80	12.1
02/02/2020	4	Oysters	55	126	181	7.30	12.6
12/02/2020	4	Oysters	52	207	259	6.70	12.7
17/02/2020	4	Oysters	103	309	412	7.07	8.0
24/02/2020	4	Oysters	82	168	250	7.30	13.5

sented in Table 1.

Discussion and conclusions

Sardinia is an area with a coastline of over 1800 km and has an extensive shellfish farming industry. Lipophilic toxin presence exceeding the legal limits has been reported since 2002 (Lugliè *et al*., 2011), although without cases of human intoxication. The toxicity, mainly associated with *Dinophysis* presence, has always been dominated by the occurrence of the OA toxin group, whereas other lipophilic groups have rarely been recorded, and always with low values (*e.g*., in Feraxi during the 2016; Bazzoni *et al*., 2018). Our study highlighted some interesting aspects: a) the detection of PTXs (particularly PTX2) in the last 2-year period (2019 and 2020) for the first time in this lagoon; b) the result of PTX presence only in certain periods of the year; c) PTX accumulation only in certain types of shellfish farmed. Regarding the first finding, it is known that the *Dinophysis* spp. are the only source of PTXs in marine waters (Reguera *et al.,* 2012). In Tortolì, analysis of harmful algae showed the presence of dinoflagellates belonging to *Dinophysis* in the water column. When PTXs were detected, *D. acuminata* was the toxic species most present, with the rare concurrent presence of *D. sacculus*. As reported by Reguera *et al*. (2012), harmful algal events arising from contamination of shellfish with toxins occur in one of two ways. Either a population of harmful plankton is carried into a shellfish production site by local currents, or a resident toxic algal population exists in a bay and can persist year on year owing to a dormant overwintering stage in its life cycle. It is possible that both conditions have occurred in the Tortolì Lagoon.

Regarding the positive record for PTXs only in some periods, in Tortolì, lipophilic toxins and *Dinophysis* spp. are not often detected at the same time. The temporal relation between the presence of *Dinophysis* spp. and bivalve mollusc toxicity is not always clear. The production and accumulation of toxins in microalgae can be affected by intrinsic, genetically controlled factors and the responses of different strains of the same species to environmental factors (Reguera *et al.,* 2012). Other determining aspects may be the dystrophic conditions of the lagoon, able to cause abrupt variations in spatial and temporal distribution of algae (Pigozzi *et al.,* 2009). Therefore, the presence of *Dinophysis* spp. in the water column is not always linked to the appearance of lipophilic toxins in shellfish. Strains of the same species may produce either OA, PTXs, or both (Fux *et al.,* 2011) and the others may not produce toxins.

Regarding the environmental factors, values of pH and water temperature determined in association with PTXs and OA detection were not different from those recorded in the other years in which these toxins were not present. Probably, environmental parameters were not the only factors determining the presence of lipophilic toxins, as reported in another study (Godhe, 2002). Nonetheless, positive results, and exceeding the legal limit, have always been detected in the late winter period. As already noted, PTXs were detected only in oysters and clams and never in mussels. In previous studies conducted in different farming areas of Sardinia (data not published), the presence of PTXs was found in mussels and rarely in clams. The toxicity observed in bivalve molluscs is not the result of a simple linear process, but of a balance from a chain of species-specific processes (Reguera *et al.,* 2014). Filtration rate is a specific characteristic of each bivalve mollusc species; however, this factor alone cannot explain the differences in the accumulation of toxins. Another factor could be the ability of each species to esterify toxins possibly present in the algae ingested, which determines their transformation and accumulation in the tissues of different molluscs (Pitcher *et al.,* 2017; Comeau *et al*., 2008).

PTXs were detected only in certain sampling points. The detection of PTXs may also have been influenced by the fact that the different species are bred at different areas in the lagoon, characterised by distinctive features, such as the depth and proximity to the sea. The production of toxins in the same algal species has already been shown (Fernandez *et al*., 2006) to vary considerably even within specimens collected in the same locality.

In conclusion, by showing the presence of PTXs for the first time, this study provides new information on lipophilic toxins in Sardinia, and offers new ideas for further investigations aimed at providing useful information on these toxin-producing *Dinophysis* species (in particular *D. acuminata* and *D. sacculus*), such as the presence of toxic and non-toxic strains.

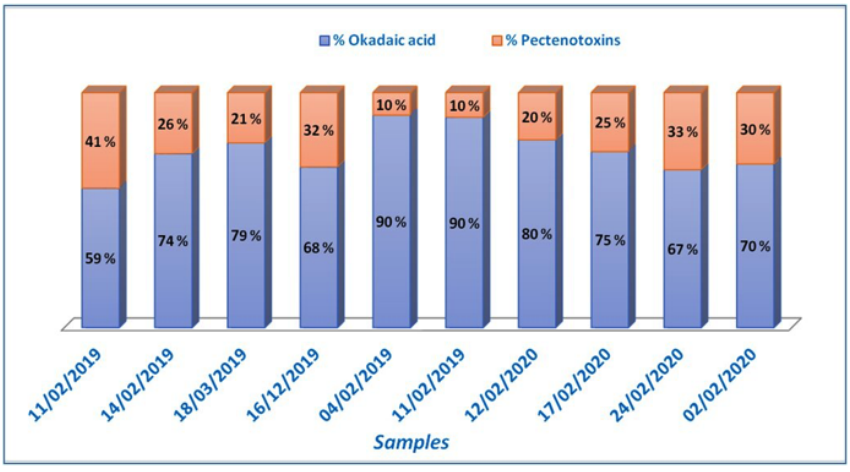

Figure 2. Proportion of okadaic acid and pectenotoxins content in positive samples.

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