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Detection of *Dinophysis* species and associated okadaic acid in farmed shellfish: a two-year study from the western Mediterranean area

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Abstract

Introduction: Diarrhoetic shellfish poisoning (DSP), an alimentary intoxication known to lead to intestinal symptoms, and caused by toxins produced by some dinoflagellates (including several *Dinophysis*), represents a serious threat to public health. The aim of this paper was to provide information about the occurrence and abundance of potentially toxic harmful algal species causing DSP, and the associated concentration of okadaic acid (OA) toxins. The departing assumption was that in the study area there was an increase in the presence both of *Dinophysis* species and OA and its derivates that could result in a risk to the health of seafood consumers. **Material and Methods:** During 2015–2016, water and shellfish samples were collected in the Mediterranean area (Sardinia, Italy). *Dinophysis* cells were counted according to Utermöhl's method from water samples, while mass spectrometry was used to identify lipophilic toxins in molluscs. **Results:** A total of 46 non-compliant samples of *Mytilus galloprovincialis* were observed. Their non-compliance concerned their OA levels above the legal limit. Among toxic dinoflagellates, *D. acuminata* and *D. sacculus* were the species found mostly during DSP events. **Conclusion:** No cases of human intoxication have been reported, but continuous surveillance of toxic phytoplankton is necessary to predict and prevent its harmful effects on human health.

Keywords: shellfish, lipophilic biotoxins, *Dinophysis acuminata*, *Dinophysis sacculus*, Sardinia.

Introduction

Diarrhoetic shellfish poisoning (DSP) is an alimentary intoxication that can cause intestinal symptoms such as diarrhoea, nausea, vomiting, and abdominal pain in seafood consumers (24, 23). DSP has a high incidence that is increasing worldwide (7), representing a constant threat to public health. This poisoning is caused by a suite of toxins produced by some cosmopolitan microalgae belonging to the *Dinophyceae* class, including several *Dinophysis* and some benthic *Prorocentrum* species. *Dinophysis* includes a number of species distributed worldwide, 10 of which are able to synthesise diarrhoeic shellfish toxins (DST) (23), heat-stable and lipophilic polyether phycotoxins that include okadaic acid (OA) and dinophysistoxin derivatives (DTXs). The lipophilic toxins also include azaspiracids (AZAs), yessotoxins (YTXs), and pectenotoxins (PTXs). Seven of the DST analogues can cause DSP syndrome (22, 23). *Dinophysis* is considered the main source of DSP toxins in various species of shellfish, mainly filter-feeding bivalve molluscs such as mussels, scallops, clams, and oysters. In a number of countries, *Dinophysis* is one of the potentially high-risk genera for biotoxicity events. The impact of its presence can range from very mild to extremely toxic.

The present study was performed in shellfish farming areas of Sardinia (Italy, the Western Mediterranean Sea) and based on the results of a monitoring programme in 2015–2016. Its aim is to provide information about the occurrence and abundance of *Dinophysis* species and associated concentrations of DSP toxins that could be present in the shellfish. In consideration of the relevant risk related to food security that may derive from the presence of potentially toxic harmful algal species (HAS) and potential consequent human poisoning, a monitoring programme has been operational in Sardinia since 1988 in order to protect the health of

seafood consumer and the economic viability of aquaculture operators. Previous monitoring studies performed in aquaculture waters (25, 3) have revealed the presence of several potentially toxic HAS in Sardinia, some of which are widely distributed. The detection of different *Dinophysis* species is recurrent, but only a few of them (including *Dinophysis acuminata* Claparède & Lachmann and *Dinophysis sacculus* Stein) occur on a regular basis (3). Despite the fact, DSP toxicity episodes in shellfish have been rare. Lugliè *et al.* (18) reported only three cases through 2011, while shellfish farm closures for paralytic shellfish poisoning (PSP) events were more common (17).

Material and Methods

Study area. Sardinia is an island located in the middle of the Western Mediterranean Sea (Fig. 1). It has relevant economic and social interests in bivalve production and aquaculture, the mussel farm industry covering a surface area of about 1,300 ha. Mollusc and fish farms are present along the coast, both in marine and transitional waters. The coasts are subjected to intense naval traffic as they lie on the main route taken by ships crossing the Mediterranean Sea. Several Sardinian lagoons have a high trophic status (21) and show large seasonal variations in nutrient concentration due to polluted inputs from the respective watersheds, resulting from the inadequate purification of urban and agricultural wastewater. Moreover, lagoons and wetlands have been extensively reclaimed for a variety of human uses that have led to profound changes in the hydrological conditions.

Monitoring. Based on the Sardinian Regional Monitoring Programme (26), water samples were collected from a total of 19 coastal sites during 2015 and 2016 in order to assess the presence and abundance of potentially toxic HAS, as described in the Intergovernmental Oceanographic Commission (IOC) Taxonomic Reference List of Toxic Plankton Algae (http://www.marinespecies.org/hab/) (14).

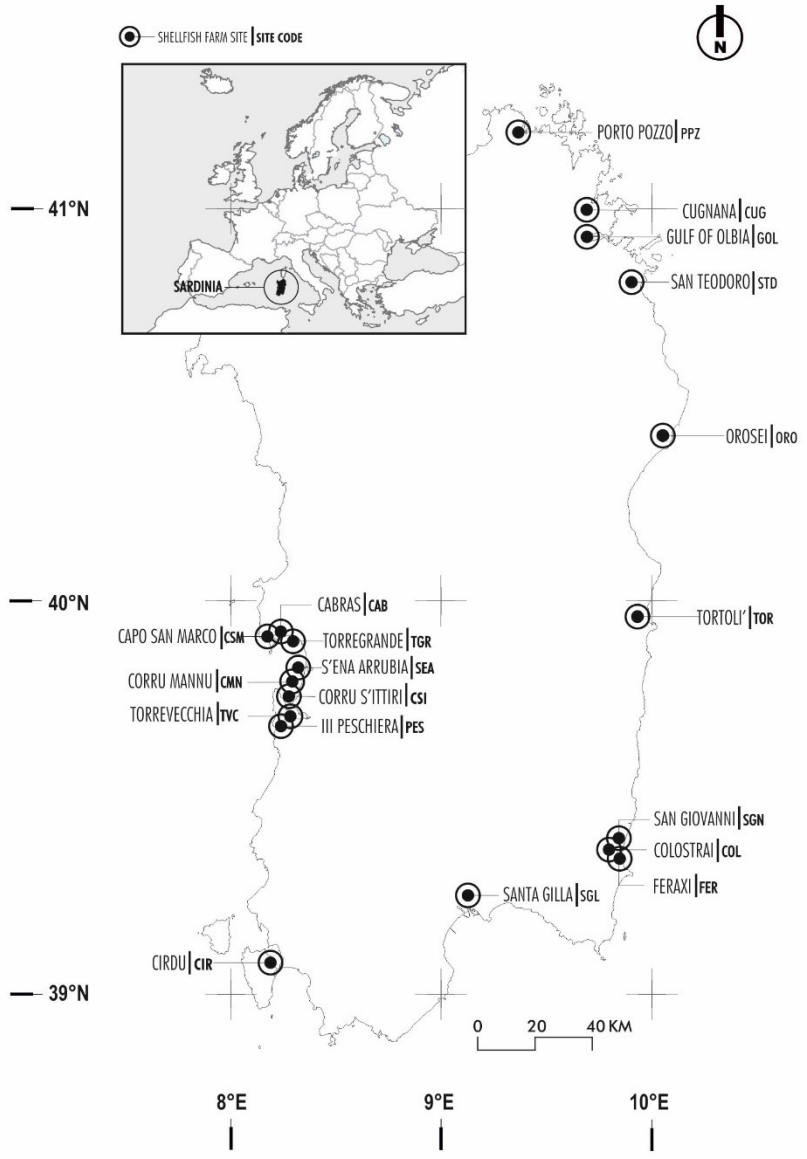

Fig. 1. Geographic locations of Sardinia and shellfish farm sampling sites (black circles)

For each site, one to five stations were sampled. Samples were collected fortnightly. Because of the sub-surface position of the mussels and the low mean depth (from 0.6 to 2 m) of the majority of the farming sites (3), in transitional water samples were taken manually at a depth of about 0.5 m from the surface into clean polyethylene bottles (PE). In marine areas, where the depth is greater, three water samples (from the surface, half column, and bottom) were collected by Niskin bottle and mixed together to obtain an integrated sample. All samples were fixed with Lugol's iodine. Either on the day of water sample collection or after no more than two days, mussel samples were obtained for the evaluation of diarrhoetic, amnaesic, and paralytic toxins. At each site, 3 kg of molluscs of commercial size (8) were collected.

Although the data collected included the quantitative analyses of all HAS found in the water samples and concomitant biotoxin groups, the present paper considered only those results regarding *Dinophysis* species (particularly *D. acuminata* and *D. sacculus*) and the combined DSP toxins, which often exceeded the permitted limit in the current regulations (9). *Dinophysis* was the most important genus in terms of relative abundance and frequency and due to repeated occurrences of DSP events during the study period.

***Dinophysis* species analyses.** The *Dinophysis* species were counted in fixed water samples (1 L). The cell count was performed according to Utermöhl's method (28) at ×200 magnification using settling chambers (from 5 to 25 cm^3 depending on phytoplankton cell abundance) under an inverted microscope (Olympus IX 73). When necessary, live samples were also observed for identification.

DSP toxin analyses. The shellfish samples harvested during 2015 and 2016 at the Sardinian monitoring areas were analysed to identify DSP toxins (OA, DTXs, PTXs, YTXs, and AZAs) by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in accordance with the official protocol (1) governed by EC Regulation 15/2011 (10). Analyses were performed at the Veterinary Public Health Institutes (IZS) of Piemonte, Liguria and Valle D'Aosta, Genova during 2015, and at IZS of Sardinia, Sassari, in 2016. Briefly, an aliquot (2 g) of muscle tissue was homogenised with 9 mL of methanol. After shaking, the extract was centrifuged and the supernatant was transferred to a flask. The extraction was repeated a second time. The extracts were combined and brought to a final volume of 20 mL with methanol. The determination of OA, AZAs, and YTXs was performed after filtering an aliquot of the methanolic extract through a syringe filter (0.45 µm and 0.2 µm, respectively in 2015 and 2016) and injecting it into an LC-MS/MS instrument (Thermo Scientific TSQ Vantage in 2015 and AB SCIEX QTRAP 4500 in 2016). A second aliquot was subjected to basic hydrolysis for the determination of the total OA-DTX content. Next, 125 µL of 2.5 M NaOH was added to 1 mL of extract and the mixture was heated at 76ºC for 40 min. Then, after cooling to room temperature, the mixture was neutralised with 125 µL of 2.5 M HCl and shaken for 0.5 min. This extract was filtered and injected into the LC-MS/MS instrument. The analysis was performed by high-performance liquid chromatography (HPLC), using a Thermo Scientific Accela 1250 pump in 2015 and a Perkin Elmer Flexar F15 in 2016. Chromatographic separation was obtained by a reversed phase HPLC column (Kinetex XB-C18 100 × 2.1 mm ID, 2.6 µm from Phenomenex in 2015, and X BRIDGE BEH C18 50 mm × 2.1 mm × 2.5 µm in the WATERS range in 2016). Different eluents were used. IZS of Genova used 6.7 mM of ammonia aqueous solution as eluent A and 6.7 mM of ammonia solution in acetonitrile: water 90:10 v/v as eluent B, at a flow rate of 450 µL/min; IZS of Sassari used 0.05 v/v% ammonia in water at pH 11 as eluent A, and 0.05 v/v% in 90% acetonitrile as eluent B at a flow rate of 400 µL/min. The analysis was carried out with a gradient elution and the injection volume was 5 µL. The mass spectral analysis was performed on a triple quadrupole mass spectrometer equipped with a heated ion spray interface (electrospray ionization - ESI). Detection and quantification of molecules were performed by selected reaction monitoring (SRM) in 2015 and by multiple reaction monitoring (MRM) in 2016. The detection limit of this analytical method for the OA toxin group was 60 µg OA eq/kg e.p.

Results

During the two-year study, a total of 2,755 water samples (1,715 in 2015 and 1,040 in 2016) and 2,561 shellfish samples (1,365 in 2015 and 1,196 in 2016) were examined. The OA toxin group was identified in 81 analysed samples (2.9%), 27 (1.6%) having been detected in 2015 and 54 (5.2%) in 2016. A total of 46 non-compliant samples with OA levels above the legal limit (160 µg OA eq/kg e.p.) were observed, as 23 each in 2015 and 2016 (Table 1). Positive samples were observed in the Feraxi (FER), Orosei (ORO), and Tortolì (TOR) sites during both 2015 and 2016. Positive samples were also found in 2015 in Colostrai (COL), Santa Gilla (SGL), and San Giovanni (SGN) and in 2016 in Cirdu (CIR) and San Teodoro (STD). During 2016, the OA toxin group was also found in Corru S'Ittiri (CSI), Golfo di Olbia (GOL), Peschiera (PES), and Torregrande (TRG), without toxin content exceeding the limits defined by law (Fig. 2).

The toxicity was dominated by the occurrence of the OA toxin group, while other components were rarely recorded. Specifically, in only a few cases (in TOR in January 2016 and in ORO in June 2016) PTX2 did co-occur with the OA toxin group, but never at high values; the maximum value was 122 µg PTX2 eq/kg e.p. in ORO, in coincidence with a toxic episode.

The results of HAS analyses showed that *D. acuminata*, followed by *D. sacculus*, were the species

most frequently identified during the positive events, although at variable relative abundance (Table 1). Rarely, *D. rotundata* Claparède & Lachmann and *Prorocentrum mexicanum* Osorio-Tafall were observed, always at negligible level of presence. *D. acuminata* and *D. sacculus* reached the highest values (almost 143×10^3 cell/L and 38×10^3 cell/L, respectively) in TOR during 2015, corresponding to an OA toxin group value (1,092 µg OA eq/kg e.p.) seven times higher than the allowed legal limit (Table 1). The highest level of toxin (1,480 µg OA eq/kg e.p.) was also found in 2015, in SGL, at which *Dinophysis* species were absent.

Most frequently, the increase in OA toxin group contamination was concomitant with a decrease in *Dinophysis* spp. abundance (Fig. 3a), or even with their absence (Fig. 3b). Only once the highest abundance of *Dinophysis* in seawater did coincide with the highest levels of toxins in mussels. This occurred in ORO, which was, together with TOR, the site where the OA toxin group was observed more frequently and where *Dinophysis* reached the maximum cellular abundance.

Toxicity has been mainly observed in the *Mytilus galloprovincialis* (Lamarck) mussel, while it has been found in rare cases in the other commercial bivalve species (Table 1).

Table 1. Abundance of *Dinophysis acuminata* and *D. sacculus* and quantification of okadaic acid toxin group among positive cases of diarrhoetic shellfish poisoning toxicity in 2015 and 2016

Sampling date	Site Code-station	Abundance (cell/L)		Okadaic acid toxin group (mg AO eq/Kg e.p.)	Shellfish products
		D. acuminata	*D. sacculus*		
23/05/2016	CIR-1	0	0	675.8	*Mytilus galloprovincialis*
19/02/2015	COL-1	0	0	341.3	*Mytilus galloprovincialis*
09/03/2015	COL-1	0	0	470.4	*Mytilus galloprovincialis*
03/02/2015	FER-1	0	0	1,269	*Mytilus galloprovincialis*
10/02/2015	FER-1	0	0	263.1	*Mytilus galloprovincialis*
19/02/2015	FER-1	0	0	516.2	*Mytilus galloprovincialis*
23/02/2015	FER-1	0	0	738.9	*Mytilus galloprovincialis*
26/02/2015	FER-1	0	0	317	*Mytilus galloprovincialis*
09/03/2015	FER-1	0	0	424.5	*Mytilus galloprovincialis*
19/03/2015	FER-1	0	0	383.1	*Mytilus galloprovincialis*
23/03/2015	FER-1	0	0	257.2	*Mytilus galloprovincialis*
07/01/2016	FER-1	1,040	120	355.8	*Mytilus galloprovincialis*
12/01/2016	FER-1	1,720	40	1,111	*Mytilus galloprovincialis*
19/01/2016	FER-1	0	0	790	*Mytilus galloprovincialis*
02/02/2016	FER-1	40	0	547	*Mytilus galloprovincialis*
09/02/2016	FER-1	40	0	778	*Mytilus galloprovincialis*
16/02/2016	FER-1	0	0	315	*Mytilus galloprovincialis*
19/02/2016	FER-1	n.s.	n.s.	273	*Mytilus galloprovincialis*
23/02/2016	FER-1	40	0	385	*Mytilus galloprovincialis*
03/03/2016	FER-1	0	0	317	*Mytilus galloprovincialis*
12/01/2016	FER-2	0	0	660	*Tapes decussatus*
27/04/2015	ORO-1	1,560	0	184	*Mytilus galloprovincialis*
27/04/2015	ORO-4	1,680	0	205	*Mytilus galloprovincialis*
15/06/2016	ORO-4	40,200	9,400	607	*Mytilus galloprovincialis*
29/06/2016	ORO-6	18,700	2,200	1,066	*Mytilus galloprovincialis*
04/07/2016	ORO-6	2,200	400	371	*Mytilus galloprovincialis*
18/07/2016	ORO-6	n.s.	n.s.	299	*Mytilus galloprovincialis*
25/07/2016	ORO-6	n.s.	n.s.	188	*Mytilus galloprovincialis*
03/02/2015	SGL-2	0	0	1480	*Mytilus galloprovincialis*
19/02/2015	SGL-2	0	0	704.8	*Mytilus galloprovincialis*
19/02/2015	SGL-3	0	0	498.8	*Mytilus galloprovincialis*
23/02/2015	SGN-4	0	0	618.9	*Mytilus galloprovincialis*
12/01/2016	STD-4	120	0	168	*Crassostrea gigas*
11/02/2015	TOR-2	1,800	0	192.5	*Mytilus galloprovincialis*
11/02/2015	TOR-3	142,662	37,895	1092	*Mytilus galloprovincialis*
26/02/2015	TOR-3	14,400	400	188.2	*Mytilus galloprovincialis*
11/03/2015	TOR-3	2,400	0	301.3	*Mytilus galloprovincialis*
18/03/2015	TOR-3	n.s.	n.s.	326.5	*Mytilus galloprovincialis*
23/03/2015	TOR-3	n.s.	n.s.	181.7	*Mytilus galloprovincialis*
15/04/2015	TOR-3	9,400	0	210.2	*Mytilus galloprovincialis*
07/03/2016	TOR-1	n.s.	n.s.	208	*Mytilus galloprovincialis*
14/03/2016	TOR-1	200	0	204	*Mytilus galloprovincialis*
07/03/2016	TOR-2	n.s.	n.s.	277	*Mytilus galloprovincialis*
14/03/2016	TOR-2	600	0	299	*Mytilus galloprovincialis*
07/03/2016	TOR-3	n.s.	n.s.	190	*Mytilus galloprovincialis*
14/03/2016	TOR-3	400	0	200	*Mytilus galloprovincialis*

n.s.: not sampled

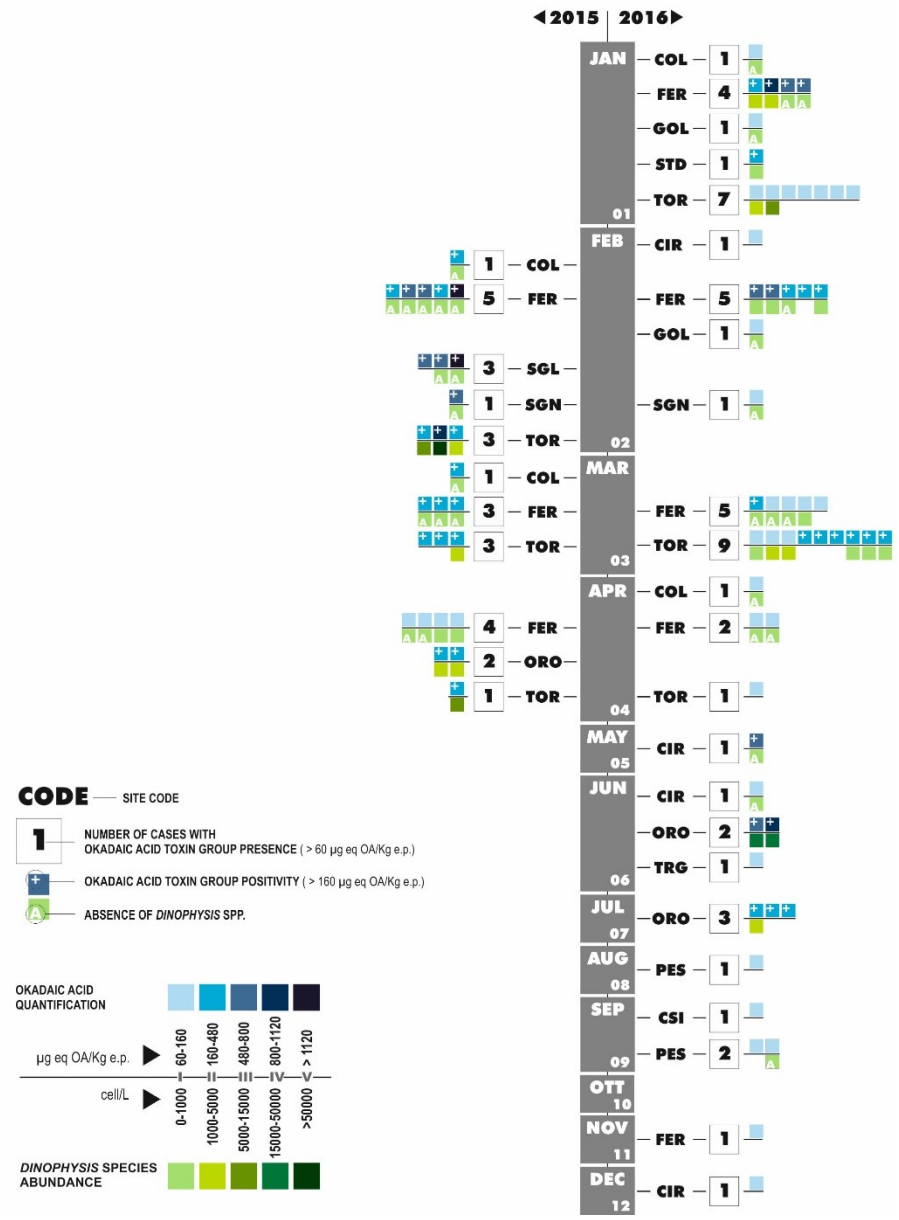

Fig. 2. Monthly numbers of cases (within the bigger squares) with okadaic acid toxin group in shellfish farms (identified with own site codes) during 2015 (left) and 2016 (right). Classes of okadaic acid toxin group quantification (smaller squares above the line) and *Dinophysis* spp. abundance (smaller squares below the line) are shown. Plus sign indicates values exceeding the legal limit for okadaic acid toxin group concentration. Letter A indicates the absence of *Dinophysis* spp.

Discussion

This two year-study performed in a monitoring area of the Mediterranean Sea documented the presence of lipophilic toxins causing DSP syndrome in molluscs. Although the percentage of contaminated samples was very low in some sites, positive shellfish were detected along the entire investigated coast.

Dinophysis species were commonly found throughout various sites during both study years. In particular, *D. acuminata* was the most geographically widespread dinoflagellate and it showed the highest cell abundance. Results from this study showed a spread of *D. acuminata* similar to those recorded in previous studies (3), but with higher abundance values.

Every time when the OA toxin group was found in molluscs, *Dinophysis* species were reported in the seawater, even days or weeks before the identification of the toxin accumulation. Therefore, toxin and the potential HAS were not often present at the same time. A direct relationship almost never occurred between *Dinophysis* spp. cell abundance in seawater and lipophilic toxin concentration in shellfish. A similar discrepancy between the abundance of *Dinophysis* in the water column and shellfish toxicity was previously recorded during events of DSP occurring in SGL and FER in 2002 and 2003, when the species implicated (*D. sacculus*, *D. caudata,* and *D. fortii*) reached a maximum concentration of 3×10^3 cell/L (3).

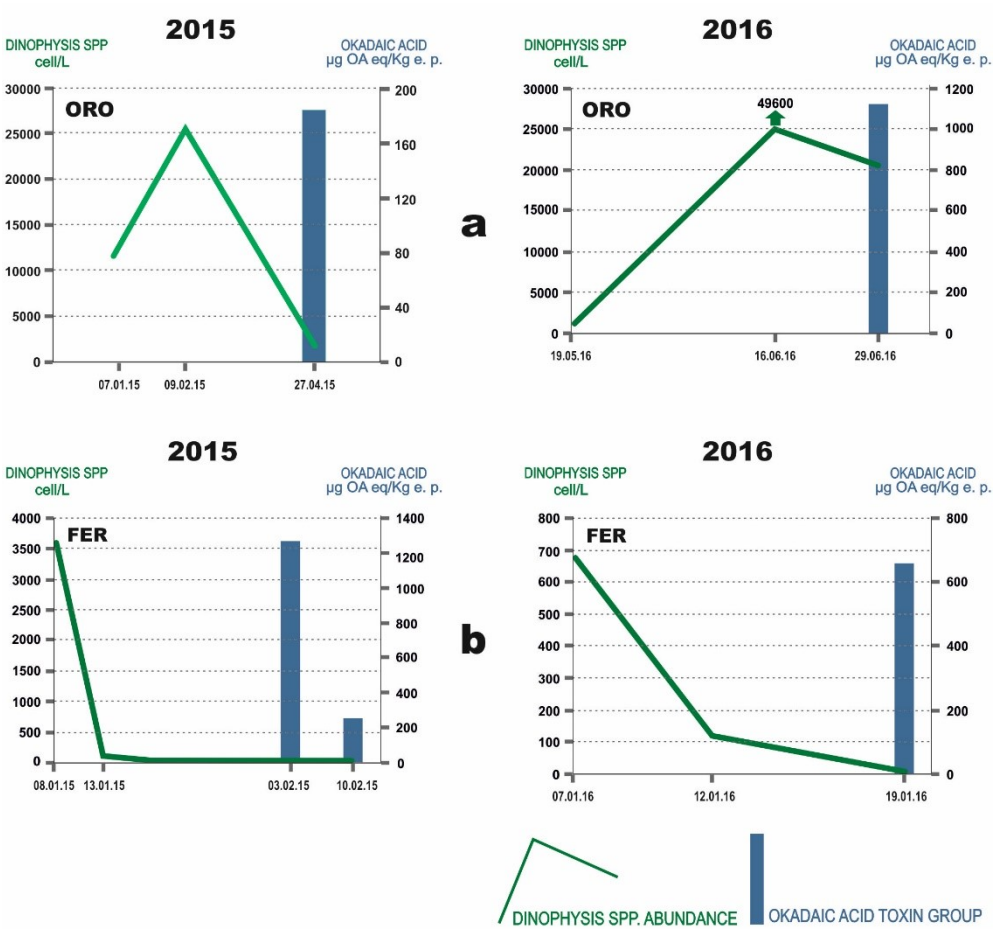

Fig. 3. Examples of the relationship between *Dinophysis* spp. cell abundance (green line) in seawater and the presence of okadaic acid toxin group (blue bar) in shellfish during 2015 (left side of figure) and 2016 (right side). The X-axis lists the dates of seawater and shellfish sampling; the left-hand Y-axis shows the values of *Dinophysis* species abundance (cell/L) and the right-hand Y-axis shows the okadaic acid toxin group concentration (µg OA eq/kg e.p.). a – increase in okadaic acid toxin group concentration concomitant with a decrease in *Dinophysis* spp. cell abundance in Orosei (ORO); b – presence of okadaic acid toxin group concomitant with *Dinophysis* spp. absence in Feraxi (FER)

Neither physico-chemical and environmental parameters nor the structure of the phytoplanktonic community was analysed in this study; however, several factors, including low salinity, stratification of waters, abundance of non-toxic accompanying species (possible alternative food sources for mussels), and DSP toxin content per *Dinophysis* cell may be responsible for the lack of temporal association between DSP-positive samples and the abundance of HAS (6, 12). This issue has been described in several reports (6, 29) in different geographical areas (the Netherlands and Norway respectively), but it is in contrast with results of other, studies (30, 16) which have reported such a relationship.

The OA toxin group was present only between February and April in 2015, but it was recurrent throughout 2016, except for in October (Fig. 2). The numbers of samples with OA concentrations over the legal limit were the same in 2015 and 2016 (23), despite the fact that the toxin was detected over a much longer period in 2016 than in 2015 (February-April in 2015 but January-December in 2016). Only in FER, TOR, and SGN there was a two-year correspondence in toxin detection from February to April (Fig. 2). The first four months seem to be the most critical period of the year (winter-early spring) for OA contamination due to its continued presence. However, during 2016, toxin positivity also occurred in the late spring-summer period, and in some cases it was rather severe (*e.g.* mussels from ORO showed OA levels above 1,000 µg eq/kg e.p. at the end of June, coinciding with *Dinophysis* spp. abundance > 20×10^3 cell/L). Toxins are frequently detected during the winter months in other Mediterranean areas, including Greece (7) and Turkey, where there is a clear seasonal trend (27).

Concurrent with the detection of levels exceeding the mussel toxicity limit, a ban on harvesting and an increased sampling plan (every two days) are implemented in the involved farms. Until the restoration of conformity, additional sampling is performed and the conduct of business by the shellfish industry is prohibited by law. Most toxic events are short-lived (a maximum of a few weeks), with a few exceptions. For

example, the OA toxin group was present for a long period (from January to April 2016) in FER, with values above the defined threshold for two months (Table 1). The closure of the mollusc farms and the resulting economic losses due to the blocked shellfish harvest have affected a number of areas, including Norway (20), Australia (2), Spain (19), France (13), and Greece (16).

Mussels have caused DSP outbreaks, sometimes quite serious, in several European countries (France, Spain, and Denmark), as well as in Canada, Chile, Argentina, and New Zealand (15 and references therein). This problem has also been reported in Italy. The first cases of gastroenteritis associated with DSP toxin-contaminated shellfish were reported in 1989 in the Northern Adriatic Sea (4). The most serious human intoxications occurred in 2010 when about 300 people in various Italian regions developed food poisoning (5). High toxicity levels related to the OA toxin group were also reported in the same shellfish product at the same time in Sardinia (data not published), but the control measures and the regional alert system ensured the protection of consumers. Despite the persistent presence of the OA toxin group, no reports of DSP intoxications were registered during this two-year monitoring programme.

In conclusion, regular surveys of farmed shellfish have simultaneously monitored for HAS and screened for marine biotoxins. OA was the principal group in the toxin profile of the shellfish samples and was found almost exclusively in *M. galloprovincialis*. Several DSP toxic episodes occurred over the two-year study period related mainly to *Dinophysis* species, which resulted in the closure of the farms. On these occasions, the total toxin content often increased when the *Dinophysis* abundance declined. This inverse relationship demonstrates that the risk of shellfish toxicity can persist even when cell abundance is reduced; therefore, it is important to take this finding into account in monitoring strategies. The results of this monitoring programme suggest that the risk associated with shellfish intoxication in Sardinia has increased in recent years due to the recurrent presence of *Dinophysis* species, which is implicated in mollusc contamination and the increased occurrence of OA and its derivatives. The monitoring programme does not ascribe shellfish intoxication risk to the widespread distribution of the HAS responsible for PSP and ASP, namely *Alexandrium* spp. and *Pseudo-nitzschia* spp. DSP has been occurring more frequently than in the past when PSP was considered the main toxin-related problem. Therefore, although no cases of human intoxication have been reported, continuous surveillance for potentially toxic phytoplankton and toxin bioaccumulation is necessary in order to predict their harmful effects, prevent human poisoning, and manage the negative consequences for aquaculture operators.

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