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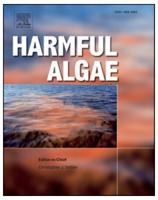

Marine phycotoxin levels in shellfish—14 years of data gathered along the Italian coast

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ABSTRACT

Along the Italian coasts, toxins of algal origin in wild and cultivated shellfish have been reported since the 1970s. In this study, we used data gathered by the Veterinary Public Health Institutes (IZS) and the Italian Environmental Health Protection Agencies (ARPA) from 2006 to 2019 to investigate toxicity events along the Italian coasts and relate them to the distribution of potentially toxic species. Among the detected toxins (OA and analogs, YTXs, PTXs, STXs, DAs, AZAs), OA and YTX were those most frequently reported. Levels exceeding regulatory limits in the case of OA (≤2,448 µg equivalent kg^{-1}) were associated with high abundances of *Dinophysis* spp., and in the case of YTXs (≤22 mg equivalent kg^{-1}) with blooms of *Gonyaulax spinifera, Lingulodinium polyedra,* and *Protoceratium reticulatum*. Seasonal blooms of *Pseudo-nitzschia* spp. occur all along the Italian coast, but DA has only occasionally been detected in shellfish at concentrations always below the regulatory limit (≤18 mg kg^{-1}). *Alexandrium* spp. were recorded in several areas, although STXs (≤13,782 µg equivalent kg^{-1}) rarely and only in few sites exceeded the regulatory limit in shellfish. *Azadinium* spp. have been sporadically recorded, and AZAs have been sometimes detected but always in low concentrations (≤7 µg equivalent kg^{-1}). Among the emerging toxins, PLTX-like toxins (≤971 µg kg^{-1} OVTX-a) have often been detected mainly in wild mussels and sea urchins from rocky shores due to the presence of *Ostreopsis* cf. *ovata*. Overall, Italian coastal waters harbour a high number of potentially toxic species, with a few HAB hotspots mainly related to DSP toxins. Nevertheless, rare cases of intoxications have occurred so far, reflecting the whole Mediterranean Sea conditions.

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1. Introduction

Harmful Algal Blooms (HABs) are natural events that can have negative consequences for public health, aquaculture, environment and/or recreational activities (Hallegraeff et al., 2004). HABs can be harmful in several ways, causing water discolorations, mucilage aggregates, bottom anoxia, and production of a broad diversity of toxins that can harm animals and humans (Glibert et al., 2018; Hallegraeff et al., 2004). In presence of toxin-producing microalgae, fish and shellfish consuming them may accumulate toxins, often without apparent damage, and transfer them to human consumers. The range of phycotoxins found during marine HABs includes azaspiracids (AZAs), brevetoxins (BTXs), ciguatoxins (CTXs), domoic acids (DAs), gymnodimines (GYMs), okadaic acids and derivatives (OAs), palytoxins (PLTXs), pectenotoxins (PTXs), saxitoxins and derivatives (STXs), spirolides (SPXs) and yessotoxins (YTXs). Each of these biotoxin groups includes several analogues, and the toxicity of each analogue is expressed as a fraction of the toxicity of the most toxic one, applying the Toxicity Equivalency Factors (TEFs) (FAO/WHO 2016). The European Regulation (EC) No 853/2004 sets the maximum toxin limits for each biotoxin group for the commercialization of bivalve mollusks for human consumption: 160 μg of AZA equivalent kg^{-1} for AZAs, 20 mg kg^{-1} for DA, 800 μg of STX equivalent kg^{-1} for STXs, 160 μg of OA equivalent kg^{-1} for OA, DTX and PTX together, and 1 mg YTX equivalent kg^{-1} for YTX-group (European Council, 2004). These legal limits have remained in force until the present day with the exception of YTXs, for which the limit was raised to 3.75 mg YTX equivalent kg^{-1} in 2013, with the Regulation (EC) No 786/2013 (European Commission, 2013), and of PTXs, which were deregulated in 2021 with the Regulation (EC) No 2021/1374 (European Commission, 2021a). Moreover, the recent findings of toxins typically found in extra European areas (for this reason considered as emerging marine biotoxins (EMBs)) led the European Commission (EC) to request the European Food Safety Authority (EFSA) to develop a series of scientific opinions concerning these marine biotoxins, for which regulatory limits have not been established yet. These EMBs include palytoxin-like compounds (PLTXs), cyclic imines (CIs), and tetrodotoxins (TTXs) (Antonelli et al., 2022; EFSA 2010, 2009b; Knutsen et al., 2017).

Marine biotoxin-related symptoms on human consumers depend on the nature of the phycotoxin and can include (i) nausea, vomiting, from short-term memory loss to coma and in severe cases death for DA, (ii) diarrhoea, nausea, vomiting and abdominal cramps for OAs and AZAs, (iii) vomiting/nausea, partial paralysis and respiratory distress for BTXs, (iv) nausea, vomiting, tingling of the mouth, slurred speech, paralysis (and can be fatal in extreme cases) for STXs (Neves et al., 2021; Nicolas et al., 2017). Marine biotoxins may cause huge economic losses particularly in coastal areas where commercial fisheries, aquaculture and tourism can be strongly affected. For example, fish and shellfish farms in areas where toxins and/or toxic microalgal species are detected are forced to close down until the HABs fade away, and have to run costly tests to verify the safety of seafood prior to putting it on the market. Accordingly, the national and local authorities responsible for safeguarding public health have instituted programs of toxin monitoring and risk management with the purpose of limiting adverse impacts of HABs. The Regulation (EC) No 2074/2005 established the official analytical methods for the detection of lipophilic marine biotoxins (OA, DTXs, PTXs, YTXs and AZAs) in bivalve mollusks, which were represented by the mouse bioassay (MBA) and the rat bioassay (RBA) (European Commission, 2005). However, these bioassays have ethical and methodological problems, such as high variability of results, insufficient detection capability and limited specificity. Alternative approaches were tested and the analytical method based on liquid chromatography coupled to mass spectrometry (LC-MS/MS) was validated and recognized as the official detection method by the Regulation (EC) No 15/2011 since December 2014 (European Commission, 2011; European Commission, 2021b).

In Italy, the earliest record of toxic microalgal blooms dates back to the 1970s (Pistocchi et al., 2012), but it was not until 1989 that the first toxic algal outbreak was scientifically substantiated by the evidence of the presence of *Dinophysis fortii, D. sacculus* and *D. acuminata* complex in the water column in concomitance with OA in mussels along the Emilia-Romagna coast (northern Adriatic Sea) (Boni et al., 1992; Fattorusso et al., 1992). From 1989 onwards, the records of toxic events and HABs have increased throughout the Italian coastline as well as for the rest of the Mediterranean Sea, mainly reflecting the increased awareness and intensified monitoring efforts associated with increased aquaculture production rather than a real increase of HAB frequency, intensity and/or distribution (Hallegraeff et al., 2021; Zingone et al., 2021)

In Italy, the authorities responsible for the monitoring of toxic phytoplankton in seawater (toxic or potentially toxic species from the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae, https://www.marinespecies.org/hab/, counted following the Utermöhl method (EUROPEAN STANDARD 2006)) and marine biotoxins in cultivated and wild shellfish are the Veterinary Public Health Institutes (Istituti Zooprofilattici Sperimentali: IZSs), and the Regional Environmental Protection Agencies (Agenzie Regionali Per l'Ambiente: ARPAs). In this paper, we collected and analysed data obtained by ARPAs and IZSs in Italian marine coastal areas from 2006 to 2019, with the aim of tracing the seasonal and interannual trends of the records of toxins at levels above the legal limit in seafood and relating them to the abundance of the main potentially toxic phytoplankton species found simultaneously in seawater.

2. Materials and methods

2.1. Sampling

Samples of mollusks (mainly *Mytilus galloprovincialis* and occasionally *Aequipecten opercularis, Bolinus brandaris, Crassostrea gigas, Murex* spp., *Nassarius* spp., *Octopus* sp., *Ruditapes decussatus, Ruditapes philippinarum, Solen capensis* and *Donax* sp.) and, rarely, sea urchins (see Table 1) were collected from 2006 to 2019 from aquaculture farms located along the coasts of Liguria, Tuscany, Lazio, Sardinia, Sicily, Apulia, Marche, Emilia-Romagna, Veneto and Friuli Venezia Giulia regions ([dataset] Accoroni et al., 2023b) with a fortnightly frequency for most regions. Unfortunately, data from Campania, Calabria, Basilicata, Abruzzo and Molise were not available for our analysis, therefore only literature data were used to integrate this survey along the Italian coast.

2.2. Toxin analyses

For toxin analysis, shellfish were opened, deprived of the shell, and washed with running water to remove any residues. Then, each sample was homogenized with a blender and stored at −20 °C until the analysis. Lipophilic toxins (OA and analogs, PTXs, YTXs, AZAs), STXs and DA were analyzed at the IZSs following the methods provided by the European legislation (Commission Regulation (EC) 2074/2005, European Commission, 2005). Briefly, for the lipophilic toxins, sample preparation, extraction and analysis were performed following the official method "EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS, vers. 5 January 2015″ (AESAN vers. 5 2015), as imposed by regulation Reg. (EU) 627/2019 (Reg. (EU) 627/2019). Basic chromatographic conditions with ammonium hydroxide (0.05 % v/v, pH 11), described in the official method were chosen. For the determination of domoic acid, the "EU-Harmonised Standard Operating Procedure for determination of domoic acid in shellfish and finfish by RP-HPLC using UV detection Version 1, June 2008″ was performed. For the determination of saxitoxins, Mouse Bioassay (MBA) (according to OMA AOAC 959.08) was performed until 2019 as it was the official method for PSTs analysis in bivalves (AOAC International, 1995). Table S1 summarizes the specific testing methodologies described above throughout the study period (2006–2019).

Table 1
Potentially toxic microalgal species identified in light microscopy with associated types of toxins (https://www.marinespecies.org/hab/) accumulating in marine invertebrate (vectors), along the Italian coasts from 2006 to 2019. AZA, azaspiracid; Cis, cyclic imines; DA, domoic acid; OA, okadaic acid; PLTXs, palytoxin-like compounds; STX, saxitoxin; YTX, yessotoxin.

Species	Toxins	Vectors
Bacillariophyceae		
Pseudo-nitzschia australis	DA	*Mytilus galloprovincialis*
Pseudo-nitzschia cf. *delicatissima*	DA	*Mytilus galloprovincialis*
Pseudo-nitzschia cf. *fraudulenta*	DA	*Mytilus galloprovincialis*
Pseudo-nitzschia cf. *pseudodelicatissima*	DA	*Mytilus galloprovincialis*
Pseudo-nitzschia galaxiae	DA	*Mytilus galloprovincialis*
Pseudo-nitzschia multiseries	DA	*Mytilus galloprovincialis*
Pseudo-nitzschia multistriata	DA	*Mytilus galloprovincialis*
Pseudo-nitzschia pungens	DA	*Mytilus galloprovincialis*
Pseudo-nitzschia subpacifica	DA	*Mytilus galloprovincialis*
Dinophyceae		
Alexandrium minutum	STX	*Mytilus galloprovincialis, Ruditapes decussatus, Donax* sp.
Alexandrium ostenfeldii	STX	*Mytilus galloprovincialis, Ruditapes decussatus, Donax* sp.
	CIs	*Mytilus galloprovincialis*
Alexandrium pacificum	STX	*Mytilus galloprovincialis, Ruditapes decussatus, Donax* sp.
Alexandrium tamarense	STX	*Mytilus galloprovincialis, Ruditapes decussatus, Donax* sp.
Alexandrium taylorii	STX	*Mytilus galloprovincialis, Ruditapes decussatus, Donax* sp.
	GDA	n.d.
Azadinium dexteroporum	AZA	*Mytilus galloprovincialis*
Dinophysis acuminata	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Dinophysis acuta	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Dinophysis caudata	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Dinophysis fortii	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Dinophysis ovum	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Dinophysis sacculus	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Dinophysis tripos	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Gonyaulax spinifera	YTX	*Mytilus galloprovincialis, Crassostrea gigas*
Gymnodinium catenatum	STX	*Mytilus galloprovincialis, Ruditapes decussatus, Donax* sp.
Lingulodinium polyedra	YTX	*Mytilus galloprovincialis, Crassostrea gigas*
Ostreopsis cf. *ovata*	PLTX-like	*Mytilus galloprovincialis, Octopus* sp., sea urchins
Phalacroma mitra	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Phalacroma rotundatum	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Prorocentrum lima	OA	*Mytilus galloprovincialis*
	CIs	*Mytilus galloprovincialis*
Prorocentrum rhathymum/ mexicanum	OA	*Aequipecten opercularis, Crassostrea gigas, Mytilus galloprovincialis, Ruditapes decussatus, Ruditapes philippinarum,* sea urchins
Protoceratium reticulatum	YTX	*Mytilus galloprovincialis, Crassostrea gigas*

Shellfish samples were considered positive when their phycotoxin content was above the thresholds indicated by the European Council (EC) Regulation No. 853/2004 and subsequent amendments. As regards the YTXs, positivity was determined according to the limits in force, which were raised up to 3.75 mg kg^{-1} in 2013, with the Regulation (EC) No 786/2013 (European Commission, 2013). Until February 2012, the IZSs utilized the mouse bioassay (MBA) for the determination of lipophilic marine biotoxins in bivalve mollusks. However, this method provided only semi/quantitative data (i.e., above or below the legal limits) and was replaced with the new official LC-MS/MS protocol in March 2012.

The general phycotoxins distribution map along the Italian coasts (Fig. 1) and the heatmaps showing the number of positive samples for a given phycotoxin were achieved using the entire dataset ([dataset] Accoroni et al., 2023b) (Fig. S1) and integrated with further information provided by the IZSs. In some regions (Apulia, Tuscany, Lazio, Friuli-Venezia Giulia, Veneto, Liguria and Sicily) the numbers of positive records were not available in some periods. Although regulatory limits have not been established yet, some sporadic data about EMBs including PLTXs, CIs, and TTXs were supplied by some IZS.

2.3. Phytoplankton

Seawater samples for phytoplankton analysis were collected in the areas of the aquaculture farms with bottles and/or 20 μm mesh-sized nets ([dataset] Accoroni et al., 2023a) following the Intergovernmental Oceanographic Commission guidelines (Reguera et al., 2016). Samples were preserved by adding acid or neutral Lugol's solution. Species identification was generally performed in light microscopy, hence it should be considered tentative in all cases requiring electron microscopy or molecular methods. Yet identification at times was supported by more detailed information obtained on specific samples at research institutions of the areas, but information about which samples were analyses by electron microscopy and/or molecular methods are not available. Toxic or potentially toxic species were counted following the Utermöhl method (EUROPEAN STANDARD 2006).

2.4. Statistical analyses

Statistical analyses were conducted using the Statistica (StatSoft Inc., Tulsa, OK, USA) software. The Shapiro-Wilks test was used to check data for normal distribution, while the Levene's test was used to assess homogeneity of variance. Non-parametric tests were used as tests did not reveal homogeneous variances and/or normal distributions. The R software version 4.1.1 (R Core Team, 2021) was used for the following analyses. Wilcoxon Test was applied, using the stats package, to assess differences in average abundances of toxic microalgae between instances when toxins (AZAs, DAs, OAs, PTXs, STXs and YTXs) exceeded the EU limit (i.e., positive records) and occasions when toxins were recorded below the EU limit, in each region. Principal Component Analysis (PCA) was performed on the number of positive records of toxins (okadaic acid analogue, yessotoxin and saxitoxin equivalent) and the phytoplankton abundances (*Alexandrium* spp., *Protoceratium reticulatum, Prorocentrum lima, Prorocentrum* spp., *Gonyaulax spinifera,*

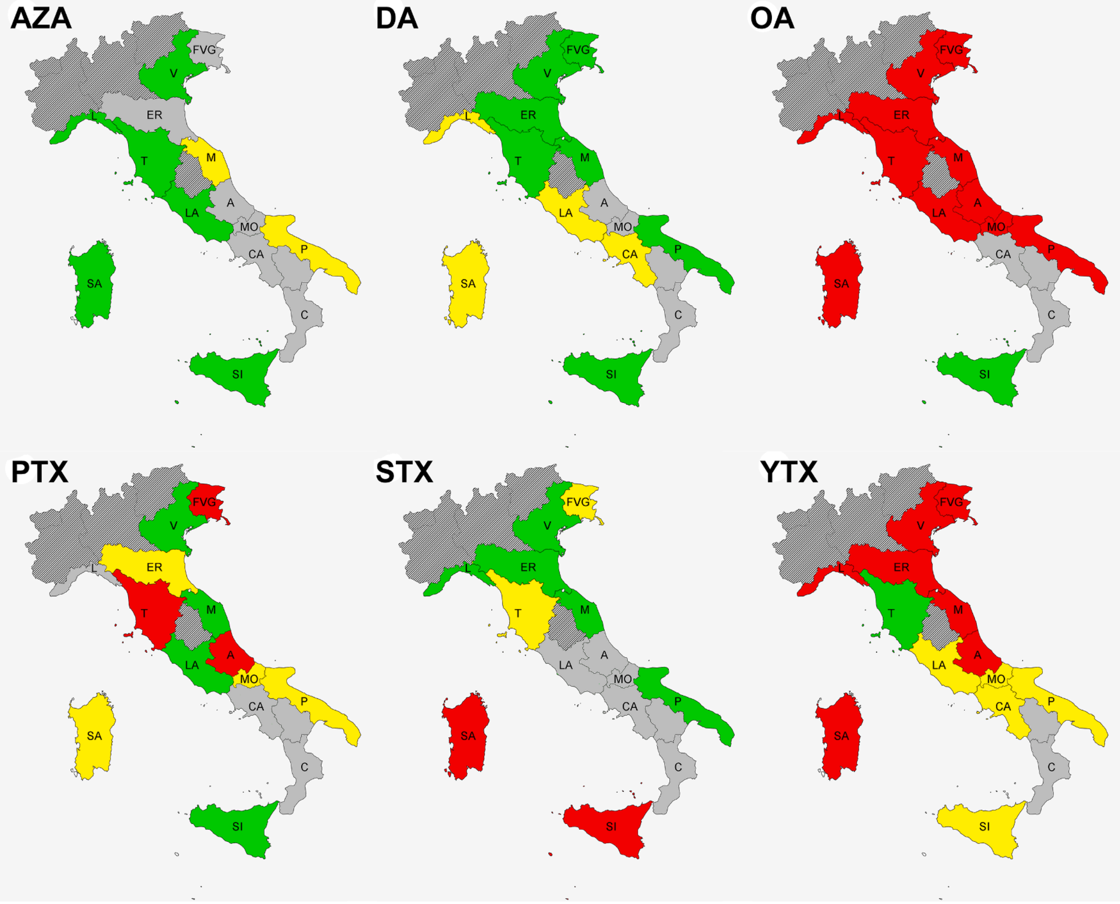

Fig. 1. Microalgal toxins (AZA, Azaspiracids; DA, Domoic Acid; OA, Okadaic Acid and analogues, PTX, Pectenotoxins, STX, Saxitoxins, YTX, Yessotoxins) in shellfish tissues along the Italian coasts (2006–2019). Red: regions in which toxin concentration was at least once above the EU limits; yellow: regions in which toxins were below the limits. Green: no toxins recorded. Grey: data not available. FVG = Friuli Venezia Giulia, V = Veneto, ER= Emilia-Romagna, M = Marche, A = Abruzzo, MO = Molise, P = Apulia, C = Campania, SI = Sicily, SA = Sardinia, CA = Campania, LA = Lazio, T = Tuscany, L =Liguria.

Lingulodinium polyedra and *Dinophysis/Phalacroma*), after rank transformation. The FactoMineR and factoextra packages were used for PCA calculation and representation. The Mann-Kendall trend test was used to check for upward or downward trends in the annual number of positive records of toxin for each region where data where present for more than three years (Kendall package, https://cran.r-project.org/web/packages/Kendall/index.html).

3. Results

3.1. Distribution of marine phycotoxins in seafood

The phycotoxin dataset consisted of 21,863 records obtained by the analysis of seafood samples collected from 2006 to 2019 along the Italian coasts, with a considerable difference in the amount of data among the Italian regions. Marche and Emilia-Romagna had the highest number of records (>1000 each), followed by Sardinia, Veneto, Friuli Venezia Giulia and Abruzzo (>100 each). Marche and Emilia-Romagna were the only regions with an almost homogeneous number of records per year (Table 2).

Table 2
Number of records of toxins in seafood samples analysed for phycotoxin content ([dataset] Accoroni et al., 2023b) per region and year.

	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	TOT
Marche	734	750	811	964	1237	375	903	1125	1198	1506	1501	2922	2278	555	16,859
Emilia-Romagna	402	438	403	306	281	314	246	209	202	336	242	57	33	33	3502
Sardinia	34	0	18	30	13	3	3	7	0	169	210	47	109	10	653
Veneto	1	1	1	1	111	0	0	0	0	87	0	132	22	0	356
Friuli Venezia Giulia	0	0	0	0	0	0	20	0	0	0	39	71	43	0	153
Abruzzo	0	0	0	0	0	0	0	0	0	54	40	0	0	0	94
Sicily	0	2	0	0	0	0	3	0	10	28	18	10	0	14	85
Apulia	0	0	0	0	0	0	0	0	0	0	55	0	0	0	55
Molise	0	0	0	0	0	0	0	0	0	17	16	15	0	0	48
Tuscany	0	4	26	0	0	0	0	0	0	0	0	0	0	0	30
Liguria	0	0	0	0	0	0	3	0	0	0	0	0	8	4	15
Lazio	0	0	0	0	0	0	0	0	0	5	2	5	0	0	12
Campania	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1

AZAs, DAs, OAs, PTX, STXs and YTXs were recorded in shellfish tissues in several regions along the Italian coast over the sampling period (Fig. 1). AZAs were recorded only along the Apulia and Marche coasts ($\leq$7 μg of AZA equivalent kg^{-1}, Ancona), while DA was recorded in Campania, Lazio, Liguria and Sardinia (3.4 mg DA kg^{-1}, Santa Gilla), with concentrations below the EU limits in all cases. STXs were recorded in Tuscany and Friuli Venezia Giulia (recorded in trace, Marano lagoon) with concentrations never exceeding the EU limit. High concentrations exceeding the EU limit were recorded only in Sardinia ($\leq$ 2941 μg of STX equivalent kg^{-1}, Gulf of Oristano) and Sicily ($\leq$ 13,782 μg of STX equivalent kg^{-1}, Syracuse Bay). OA and analogues were recorded with above-threshold values in all regions for which information is available ($\leq$ 2448 μg of OA equivalent kg^{-1}, Cervia), with the exception of Sicily. YTXs were recorded with values above the EU limit in Friuli Venezia Giulia ($\leq$ 4.7 mg YTX equivalent kg^{-1}, Trieste), Veneto ($\leq$ 2.8 mg YTX equivalent kg^{-1}, Porto Viro), Emilia-Romagna ($\leq$ 22 mg YTX equivalent kg^{-1}, Ravenna), Liguria, Abruzzo ($\leq$ 2.7 mg YTX equivalent kg^{-1}, Teramo), Sicily and Marche ($\leq$ 2.7 mg YTX equivalent kg^{-1}, Sirolo), and below the EU limit in Apulia, Lazio, Molise ($\leq$ 0. 4 mg YTX equivalent kg^{-1}, Campobasso) and Campania coasts. Only in Tuscany YTXs were never detected. PTXs were recorded with concentrations exceeding EU limit in Friuli Venezia Giulia, Tuscany ($\leq$ 1174 μg of PTX equivalent kg^{-1}, Orbetello Lagoon) and Abruzzo, while they occurred with low values in Emilia-Romagna, Molise ($\leq$ 75 μg of PTX equivalent kg^{-1}, Campobasso), Apulia and Sardinia ($\leq$ 122 μg of PTX equivalent kg^{-1}, Orosei) coasts.

In the PCA analysis, the variance explained by the first two components (PCA1 and PCA2) was 55.4 and 23.9 %, respectively (Fig. 2). This analysis did not show a clear correlation between toxic microalga abundances and phycotoxin concentrations accumulated in recorded seafood. Instead, it emphasizes a connection between PSP toxicity and seafood samples from Sardinia, as well as between both YTX and OA toxicity and samples from Marche and Emilia-Romagna, in the northern Adriatic Sea.

A significant negative trend was observed for the number of positive records in seafood samples in Marche for OA (Mann-Kendall test, τ = -0.52, p <0.01) and YTX (Mann-Kendall test, τ = -0.829, p <0.001), in Emilia-Romagna for YTX (Mann-Kendall test, τ = -0.275, p < 0.001) and in Sardinia for STX (Mann-Kendall test, τ = -0.691, p < 0.05). No significant trends were observed for the other toxins in the other regions (Mann-Kendall test, p > 0.05).

3.1.1. *Emilia-Romagna*

This region was mainly subjected to OAs and YTX positivity (Table 3). OA and analogues were often recorded throughout the study period at levels above the regulatory limit, showing the 2015 as the worst year with 66 samples up to 274 analyses exceeding the EU limits ($\leq$ 2448 μg of OA equivalent kg^{-1}, Cervia, Table S2). In the same way, YTX concentrations often exceeded the regulatory limit, up to 59 times in 2007. YTX positives were mainly recorded in late summer and fall, and at times also in winter (Table S2). For both toxins, there was also a marked variability in the number of positive records among years in spite of an almost-homogeneous number of samples analysed each year. A decay of YTX positives after 2013 (when the threshold for YTX was raised from 1 to 3.75 mg kg^{-1}) was observed.

During the study period, 18 potentially toxic dinoflagellate taxa were recorded. Among them, 3 potential producers of STXs (*Alexandrium* sp., *A. ostenfeldii* and *A. minutum*), 11 of OA (and analogs) (*Dinophysis acuminata, D. acuta, D. caudata, D. fortii, D. ovum, D. sacculus, D. tripos, Prorocentrum* sp., *P. lima, P. rhathymum* and *Phalacroma rotundatum*), three of YTXs (*Gonyaulax spinifera, Lingulodinium polyedra, Protoceratium reticulatum*), and one of AZA (*Azadinium* sp.) were identified (Table S3).

Dinoflagellates potentially producing STXs (*Alexandrium* spp.) reached the maximum abundances of 24,520 cells l^{-1} in April 2007 and 11,040 cells l^{-1} in February 2008, while generally they showed abundances around 10^2–10^3 cells l^{-1}.

Similarly, YTX producers reached the maximum abundances of 64,034 cells l^{-1} (*G. spinifera*) and 1371,005 cells l^{-1} (*L. polyedra*) in October 2014, i.e. in the same period when high numbers of YTX limit-exceeding sample were detected, while generally they showed abundances around 10^2–10^3 cells l^{-1}. *Protoceratium reticulatum* showed the highest abundances in September 2006 (3920 cells $^{-1}$) in the same

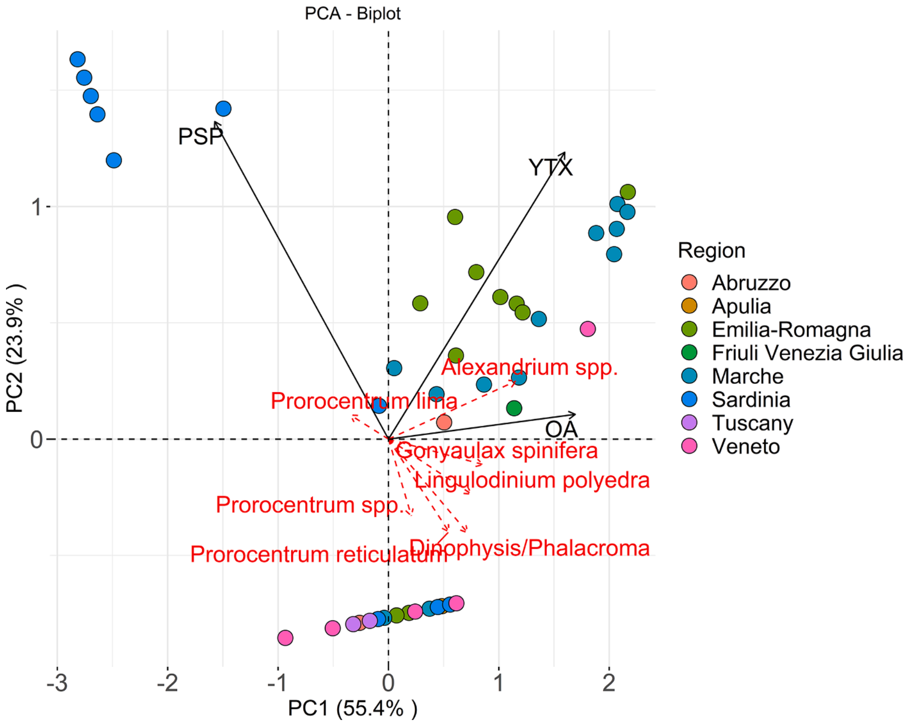

Fig. 2. Principal Component Analysis (PCA) based on correlation matrix of ranked number of positive records in seafood and abundance of toxic microalgae used as supplementary variables.

Table 3

Microalgal toxins (AZA, Azaspiracids; DA, Domoic Acid; OAs, Okadaic Acid and analogues, PTX, Pectenotoxins, STX, Saxitoxins, YTX, Yessotoxins) in shellfish tissues sampled along the coasts of Emilia-Romagna, Marche, Sardinia, Friuli-Venezia Giulia, Liguria, Veneto and Sicily. Red: toxin concentrations at least once above the EU limits. Yellow: toxin concentrations below the EU limits. Green: no toxins recorded. White: data not available. Samples were considered positive based on the EU limit in place at that time.

Emilia-Romagna	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
STX	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
DA	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
OA	🟥	⬜	🟨	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥	⬜	🟥
PTX	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
YTX	🟥	🟥	🟥	🟥	🟨	🟨	🟥	🟥	🟩	🟥	🟩	⬜	🟨	⬜
AZA	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜

Marche	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
STX	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
DA	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	⬜	⬜
OA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟥	🟥	🟩	🟥	🟩	🟨
PTX	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
YTX	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟩	🟩	🟨
AZA	⬜	⬜	⬜	⬜	⬜	⬜	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨

Sardinia	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
STX	🟥	⬜	🟥	🟥	🟥	🟥	🟥	⬜	⬜	🟨	⬜	⬜	🟥	⬜
DA	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟨	⬜	⬜	⬜	⬜
OA	⬜	⬜	⬜	⬜	🟥	⬜	⬜	⬜	⬜	🟥	🟥	🟥	🟥	🟥
PTX	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟨	⬜	⬜	⬜
YTX	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟥	⬜	⬜	⬜	⬜	⬜	⬜
AZA	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜

Friuli Venezia Giulia	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
STX	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	⬜	⬜	⬜	🟨	🟩	⬜
DA	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	⬜	⬜
OA	🟩	🟩	🟩	🟩	🟥	🟩	🟩	🟩	🟩	🟩	🟥	🟥	🟩	⬜
PTX	⬜	⬜	⬜	⬜	🟥	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟩	⬜
YTX	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟥	⬜	⬜
AZA	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜

Liguria	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
STX	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	⬜	⬜
DA	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟨	🟩	🟨	🟨	⬜	🟩	🟩
OA	🟩	🟩	🟩	🟩	🟩	🟩	🟥	🟩	🟨	🟨	🟥	⬜	🟩	🟩
PTX	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜
YTX	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	⬜	⬜	⬜
AZA	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	⬜	⬜

Veneto	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
STX	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟨	🟨	⬜
DA	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	⬜	⬜
OA	🟥	🟥	⬜	🟥	🟥	⬜	🟥	🟥	🟥	🟥	⬜	🟥	🟨	⬜
PTX	⬜	⬜	⬜	⬜	⬜	⬜	🟩	🟩	🟩	🟩	🟩	⬜	🟨	⬜
YTX	⬜	⬜	⬜	⬜	🟥	⬜	🟥	🟥	🟥	🟥	⬜	🟨	🟨	⬜
AZA	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	⬜	⬜

Sicily	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
STX	🟩	🟥	🟩	🟩	🟩	🟩	🟥	🟩	🟩	🟥	🟥	🟥	⬜	🟥
DA	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟩	🟩	🟩	🟩	🟩	⬜	⬜
OA	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟩	🟩	🟩	🟩	🟩	⬜	⬜
PTX	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟩	🟩	🟩	🟩	⬜	⬜
YTX	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟨	🟨	🟨	🟨	⬜	⬜
AZA	⬜	⬜	⬜	⬜	⬜	⬜	⬜	⬜	🟩	🟩	🟩	🟩	⬜	⬜

period when high numbers of YTX limit-exceeding samples were recorded.

Dinophysis/Phalacroma species generally occurred in low abundances, but from spring to fall they reached concentrations $>10^3$ cells l^{-1} (except 2006, 2017, 2019), with the highest value of 9640 cells l^{-1} (*D. acuminata* complex) in June 2012. Three further high, but short–living, density values of *Dinophysis/Phalacroma* were recorded in May 2009 (8720 cell l^{-1}), in May 2018 (4480 cell l^{-1}) and in November 2018 (1680 cell l^{-1}) but no relationships with limit-exceeding seafood samples were observed.

Although AZA and DA never exceeded the EU limits, their potential producers, *Azadinium* sp. and *Pseudo-nitzschia* spp., were recorded with abundances up to 3120 cells l^{-1} in May 2018 and 10,110,096 cells l^{-1} in August 2018, respectively.

Significant higher abundances of *G. spinifera* and *P. rotundatum* were recorded in those periods in which the EU limits for YTXs were exceeded, than in those periods when no YTX positivity was recorded (Wilcoxon Test, for all $p < 0.001$).

3.1.2. *Marche*

This region was subjected to OA (and analogs) and YTX positivity (Table 3). OA and analogs were recorded throughout the study periods over the regulatory limit several times until 2012, with 2008 and 2010 being the worst years, with regulatory limit exceeded in 92 and 90 samples, respectively. The maximum OA and YTX concentrations were 2448 μg of OA equivalent kg^{-1} and 2.7 mg YTX equivalent kg^{-1}, (San Benedetto del Tronto and Sirolo, respectively). In the same way, YTX concentrations exceeded the regulatory limit until 2015, with up to 44 positives in 2006 (Table S4). A clear seasonality of OA and analogs was not observed before 2012. Only after 2012, when positive records became less frequent, they were mainly distributed in autumn. Regarding YTXs, they were mainly recorded in summer and autumn, rarely up to winter (Table S4). The highest number of limit-exceeding samples for both toxin types were observed in autumn.

During the study period, 24 potentially toxic taxa were detected in the region. Among them 17 dinoflagellates, one producer of STX (*Alexandrium* sp.), 12 of OAs (and analogs) (*Dinophysis* sp., *D. acuminata, D. caudata, D. fortii, D. ovum, D. sacculus, D. tripos, Phalacroma* sp., *P. mitra, P. rotundatum, Prorocentrum* sp. and *P. lima*) and three of YTXs (*G. spinifera, L. polyedra, P. reticulatum*), together with 7 diatoms, all potential DA producers (*Pseudo-nitzschia* sp., *P.* cf. *delicatissima, P. fraudulenta/subfraudulenta, P. multistriata, P.* cf. *pseudodelicatissima* and *P. pungens*) were identified (Table S5).

Although STXs dinoflagellates reached relatively high abundances especially in July 2010 (54,547 cells l^{-1}), STXs never exceeded the EU limits in the studied period. In the same way, *Pseudo-nitzschia* spp. were often recorded with high abundances (up to 4082,510 cells l^{-1} for *P.* cf. *delicatissima* in April 2010), but DA never exceeded the EU limit in shellfish samples.

Among the OA (and analogs) dinoflagellates, *Dinophysis/Phalacroma* showed the highest abundance of 6800 cells l^{-1} in May 2013 (among which 5600 cells l^{-1} of *D. fortii*), followed by the YTX producer dinoflagellate *G. spinifera* with 2549 cells l^{-1} in November 2014. Significant higher abundances of *D. fortii, D. acuminata, D. tripos*, and *D. caudata.* were recorded, in those periods in which the EU limits for OAs were exceeded than in those periods when no OAs positivity was recorded (Wilcoxon Tests, $p < 0.01$). Significant higher abundances of *L. polyedra* and *D. caudata* were recorded, when in those periods in which the EU limits for YTXs were exceeded, than in those periods when no YTX positivity was recorded (Wilcoxon Test, for all $p < 0.001$).

3.1.3. *Sardinia*

This region was subjected to STX and OA positivity mainly in the first part of the study period (i.e., until 2012) and during the 2015–2019 period, respectively (Table 3), with maximum of 2941 μg of STX equivalent kg^{-1} and 1480 μg of OA equivalent kg^{-1} (Gulf of Oristano and Cagliari, respectively). STX levels above the regulatory limit were recorded almost every year until 2012. Subsequently, positive samples were only recorded in May 2018. OA (and analogs) concentrations exceeded the regulatory limit only in the last years (from 2015) with the highest number of positive samples (60 over 210 records) in 2016. Positive samples for YTXs were found in April 2013 ($\leq$ 1.5 mg of YTX equivalent kg^{-1}, Gulf of Olbia). Other toxins as DA and PTXs were recorded in 2015 and 2016, respectively, but below the EU regulatory limits. However, it is important to highlight that the number of available records were not homogeneous throughout the study period, most of them being concentrated between 2015 and 2018 (Table S6). STXs were mainly recorded in autumn/winter, and OA and analogs were recorded in winter/spring (Table S6).

From 2011 to 2019, 13 potentially toxic taxa occurred. Among them, three STX (*Alexandrium* sp., *A. minutum* and *A. pacificum*) and 9 OA (and analogs) dinoflagellates (*Dinophysis* sp., *D. acuminata, D. caudata, D. fortii, D. sacculus, D. tripos, Phalacroma rotundatum, Prorocentrum lima* and *P. rhathymum*) were identified in addition to one diatom species, *Pseudo-nitzschia* sp., a potential ASP producer (Table S7).

STX dinoflagellates reached the highest abundance in May 2018 with 11,080 cells l^{-1} in concomitance with STX samples exceeding the EU limits. From 2015, OA positivity in seafood coincided with high *Dinophysis/Phalacroma* abundances, up to 479,000 cells l^{-1} in 2016 (mainly due to *D. acuminata* and *D. sacculus*), and with the occurrence of *Prorocentrum lima* and *P. rhathymum*, which showed the highest abundances in 2012 and in 2018 (6000 and 1100 cells l^{-1}), respectively. Although *Pseudo-nitzschia* spp. were often abundant, DA never exceeded the EU limits in the study period. Overall, no significant differences in the abundances of toxic microalgae were recorded between those periods in which phycotoxins exceeded the EU limits in analyzed seafood and those periods when toxins were recorded below the EU limit (Wilcoxon Test)

3.1.4. *Friuli Venezia Giulia*

This region was subjected to OA, PTX and YTX positivity (Table 3). OA (and analogs) exceeded the EU limits in 2010, 2016 and 2017 ($\leq$ 1815 μg of OA equivalent kg^{-1}, Trieste) whereas PTX and YTX concentrations exceeded them in 2010 and 2017, respectively ($\leq$ 4.7 mg of YTX equivalent kg^{-1}, Trieste). STX was also recorded but with concentrations below the regulatory limit.

From 2006 to 2019, up to 16 potentially toxic dinoflagellate (15) and one diatom species were detected. Among the identified dinoflagellates, two species potentially produce STX (*Alexandrium* sp. and *A. taylorii*), 8 OA (and analogs) (*Dinophysis* sp., *D. acuminata, D. caudata, D. fortii, D. sacculus, D. tripos, Phalacroma rotundatum, Prorocentrum lima*), three YTX (*G. spinifera, L. polyedra, P. reticulatum*), one BTX (*Karenia papilionacea*) and one AZP (*Azadinium* sp.). *Pseudo-nitzschia* sp. was the only identified diatom potentially able to produce DA (Table S8).

STX dinoflagellates reached exceptional high abundances in 2017 (up to 26,160 cells l^{-1} in August) in concomitance with records of STX in seafood samples at levels below the EU limits (Table 3). Among the OA (and analogs) dinoflagellates, *Prorocentrum lima* reached the highest abundance in September 2011 (8800 cells l^{-1}), followed by *Dinophysis/Phalacroma* with maximum concentrations reached in 2015, 2016 and 2017, when OA-limit exceeding samples were recorded (except for 2015), with up to 2560 cells l^{-1} in September 2016. Among YTX dinoflagellates, *G. spinifera* and *L. polyedra* reached the highest abundance in 2016 (200 and 2720 cells l^{-1}, respectively) and *P. reticulatum* in 2017 (440 cells l^{-1}), at the same times of the records of YTX-limit exceeding samples. *Pseudo-nitzschia* spp. were often recorded, with the peak of 8784,619 cells l^{-1} in 2015, but DA never exceeded the EU limit in shellfish samples. Overall, no significant differences in abundances of toxic microalgae were recorded in periods in which phycotoxins exceeded the EU limits in analyzed seafood compared to those periods when toxins were recorded below the EU limit (Wilcoxon Test).

3.1.5. Liguria

This region was subjected to YTX and OA positivity (Table 3). OA concentrations were found above the EU limits in 2012 and 2016 and below it in 2014 and 2015. DA was recorded in 2013, 2015 and 2016 without exceeding the EU regulatory limits. YTX were detected from 2006 to 2016 but reached values above the regulatory limits only in 2006.

During the last two years of sampling, water samples were analyzed revealing 18 potentially toxic taxa. Among them, three STXs dinoflagellates (*Alexandrium* sp., *A. minutum* and *Gymnodinium catenatum*), 8 OA (and analogs) (*Dinophysis* sp., *D. acuminata, D. acuta, D. caudata, D. fortii, D. sacculus, D. tripos* and *Phalacroma rotundatum*), five YTX (*Gonyaulax* sp., *G. spinifera, Lingulodinium* sp., *L. polyedra* and *Protoceratium reticulatum*), and two DA diatoms (*Pseudo-nitzschia* sp., *Pseudo-nitzschia simulans*) were identified. In particular, among the OA (and analogs) dinoflagellates, *Dinophysis/Phalacroma* showed the highest abundance with 12,210 cells l^{-1} in 2016 when OA exceeded the EU limits. The maximum concentration of *Pseudo-nitzschia* spp. (10^6 cell l^{-1}) was recorded in 2016 when DA was also detected, with values below the regulatory limits. Overall, no significant differences in abundances of toxic microalgae were recorded in those periods in which phycotoxins exceeded the EU limits in analyzed seafood compared to those periods when toxins were recorded below the EU limit (Wilcoxon Test).

3.1.6. Apulia

This region was subjected to positivity for OA and analogs, whose concentrations exceeded the EU limits in 2009, 2011, 2012, 2015 and 2016. PTXs, YTXs and AZAs were sometimes recorded but never exceeding the regulatory limits.

Water samples analyzed since 2010 revealed 32 potentially toxic taxa. Among them, five STX-producing dinoflagellate species (*Alexandrium* sp., *A. catenella, A. minutum, A. tamarense* and *Gymnodinium catenatum),* 12 OA (and analogs) (*Dinophysis* sp., *D. acuminata, D. caudata, D. fortii, D. ovum, D. sacculus, D. tripos, Phalacroma* sp., *P. rotundatum, Prorocentrum* sp., *P. lima* and *P. rhathymum*), four YTX (*Gonyaulax* sp., *G. spinifera, Lingulodinium polyedra, Protoceratium reticulatum*), one BTX (*K. papilionacea*) and 10 DA diatoms (*Pseudo-nitzschia* sp., *P. australis, P.* cf. *delicatissima, P. fraudulenta/subfraudulenta, P. galaxiae, P. multiseries, P. multistriata, P.* cf. *pseudodelicatissima, P. pungens* and *P. seriata*-group) were identified (Table S9). In particular, among the OA (and analogs) dinoflagellates, *Dinophysis/Phalacroma* showed abundances higher than 10^3 cells l^{-1} almost every year coinciding with OA values above the EU limits (i.e., from 2011 to 2014 and in 2016, with the highest value of 3327 cells l^{-1} in 2011). Overall, no significant differences in abundances of toxic microalgae were recorded in those periods in which phycotoxins exceeded the EU limits in analyzed seafood compared to those periods when toxins were recorded below the EU limit (Wilcoxon Test).

3.1.7. Tuscany

This region was subjected to OA (and analogs) and PTX positivity. OA concentrations exceeded the EU limits in 2007, 2008 and 2009 ($\leq$ 393 μg of OA equivalent kg^{-1}, Orbetello Lagoon, Pigozzi et al., 2009). Moreover, STX were recorded in 2011 at concentrations below the EU limits.

During the study period, water samples were analyzed revealing 22 potentially toxic taxa. Among them, three STX dinoflagellates (*Alexandrium minutum, A. tamarense* and *Gymnodinium catenatum*), 8 OA (and analogs) (*Dinophysis acuminata, D. caudata, D. fortii, D. ovum, D. sacculus, D. tripos, Phalacroma rotundatum, Prorocentrum lima*), three YTX (*Gonyaulax spinifera, Lingulodinium polyedra, Protoceratium reticulatum*), two BTX (*Karenia brevis* and *K. papilionacea*) and 6 DA diatoms (*Pseudo-nitzschia fraudulenta, P. galaxiae, P. multistriata, P. seriata*-group, *P. subpacifica* and *P. turgidula*) were identified (Table S10). No particular correlations were observed between potentially toxic algae abundances and regulatory limit-exceeding samples. Only the highest abundances of *Gonyaulax spinifera* recorded in 2008 (11,400 cells l^{-1}) corresponded to YTX positivity in seafood in that year. Overall, no significant differences in abundances of toxic microalgae were recorded in those periods in which phycotoxins exceeded the EU limits in analyzed seafood compared to those periods when toxins were recorded below the EU limit (Wilcoxon Test).

3.1.8. Veneto

This region was subjected to OA (and analogs) and YTX positivity (Table 3). OA (and analogs) concentrations exceeded the EU limits in 2006, 2007, 2009, 2010, 2012–2015 and 2017 ($\leq$ 1864 μg of OA equivalent kg^{-1}, Porto Viro), while in 2018 concentrations were found below EU limits. Instead, YTX concentrations were found above the limits in 2010 and from 2012 to 2015 ($\leq$ 2.8 mg of YTX equivalent kg^{-1}, Porto Viro), while in 2017 and 2018 the amount was below the law limits. OA positivity was recorded mainly in late summer-fall. Moreover, STXs and PTXs were recorded at concentrations non-exceeding the EU limits in 2017 and 2018 ($\leq$ 420 μg of STX equivalent kg^{-1}, Venezia), respectively (Table 3).

During the period considered, 13 potentially toxic taxa were detected in the samples analyzed. Among them, one STX dinoflagellate (*Alexandrium* sp.), 7 OA (and analogs) (*Dinophysis* sp., *D. sacculus, D. fortii, D. tripos, D. caudata, D. acuminata, Prorocentrum lima*), three YTX ones (*G. spinifera, L. polyedra, P. reticulatum*), one AZP (*Azadinium* sp.) and one DA diatom (*Pseudo-nitzschia* sp.) were identified (Table S11).

Potential STX-producing dinoflagellates never exceeded 400 cells l^{-1}. Such low abundances were recorded in spring 2017 (when STX was recorded in seafood without exceeding the EU limit) and in summer 2018. Regarding OA (and analogs) dinoflagellates, maximum concentrations of *Dinophysis/Phalacroma* was recorded in summer 2008, with 180,000 cells l^{-1}. Significantly higher abundances of *Dinophysis* spp. (in particular *D. fortii, D. tripos* and *D. caudata*) and *Phalacroma* spp. were observed in those periods when OA exceeded the EU limit compared to those when no OA positivity was recorded (Wilcoxon Test, both $p <$ 0.001).

Among YTX dinoflagellates, *Gonyaulax spinifera* never exceeded 10^3 cells l^{-1}, except for February 2007 and July 2015 (in concomitance with YTX positivity in seafood), when the maximum abundances reached 4800 and 2582 cells l^{-1}, respectively. *Lingulodinium polyedra* and *Protoceratium reticulatum* showed the highest abundances in spring 2006 (500 cells l^{-1}) and 2017 (800 cells l^{-1}) respectively. YTX were recorded in 2017 with high but non exceeding regulatory levels.

3.1.9. Abruzzo

This region was subjected to OA (and analogs), YTX and PTX positivity, with concentrations exceeding the EU limits in 2015 and 2016 for OA and analogs ($\leq$ 758 μg of OA equivalent kg^{-1}, Chieti), and only in 2015 for both YTXs ($\leq$ 2.7 mg YTX equivalent kg^{-1}, Teramo) and PTXs ($\leq$ 94 μg PTX equivalent kg^{-1}, Chieti). Further samples contaminated by YTXs and PTXs were recorded in 2016 but not exceeding the EU limits (Schirone et al., 2018).

3.1.10. Molise

This region was subjected by OA (and analogs) positivity, with concentrations exceeding the EU limits in 2015 and 2016 ($\leq$ 409 μg of OA equivalent kg^{-1}, Campobasso). Further samples contaminated by OA (and analogs) below the EU limits were recorded in 2017. Moreover, YTX and PTX were recorded at concentrations below the EU limits in 2015, 2016 and 2017 for YTX and only in 2015 for PTX ($\leq$ 0. 4 mg YTX equivalent kg^{-1} and 75.1 μg PTX equivalent kg^{-1}, Campobasso, Schirone et al., 2018).

3.1.11. Campania

In this region, for which only literature data are available, only DA was reported in 2015 samples and never above the EU regulatory limit (Rossi et al., 2016). However, a couple of cases of DSP (Diarrhetic Shellfish Poisoning) toxins below the regulatory limit and the presence of several species producing different kinds of toxins were reported in years preceding the present study (Zingone et al., 2006).

3.1.12. Sicily

This region was subjected to STXs, whose concentrations exceeded the EU limits in 2007, 2012, 2015, 2016, 2017 and 2019 ($\leq$ 13,782 µg of STX equivalent kg^{-1}). Also, YTXs below the regulatory limits were recorded from 2014 to 2017 (Table 3).

During the decade considered 8 potentially toxic taxa were detected in the samples analyzed. Among them, four STX dinoflagellates (*Alexandrium* sp., *A. catenella, A. minutum, A. pacificum*), one OA (and analogs) (*Prorocentrum lima*) and one YTX (*Gonyaulax spinifera*), and two DA diatoms (*Pseudo-nitzschia* sp. and *P. multistriata*). *Alexandrium* spp. were recorded with maximum abundances above 10^5 cells l^{-1} in 2012, 2014, 2015 and 2017, years when STXs exceeded the regulatory limit in seafood, with the exception of 2014.

3.1.13. Lazio

In this region, OA and analogs was recorded in 2015, 2016 and 2017, with concentrations exceeding the EU limits in 2015 and 2017 ($\leq$ 224 µg of OA equivalent kg^{-1}, Latina).

3.2. Emerging marine biotoxins (EMBs) in Italy

In the last decade, several EMBs, i.e., palytoxin-like compounds (PLTXs), cyclic imines (CIs), and tetrodotoxins (TTXs) were reported along the Italian coasts (Fig. 3).

PLTXs have been detected in several regions with values often above the threshold of 30 µg/kg of shellfish meat suggested by EFSA (2009b). These high values were associated to intense proliferation of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata*, which has occurred along the Italian coasts in the last decades during summer and/or autumn (Accoroni et al., 2015; Accoroni et al., 2022; Mangialajo et al., 2011; Monti et al., 2007; Vassalli et al., 2018). In the Marche region, ovatoxin-a (OVTX-a) was recorded in mussels with concentrations up to 91 µg kg^{-1} of soft tissue in 2009 (Accoroni et al., 2011). In Tuscany, PLTXs were recorded in sea urchins, mussels and octopuses with maximum values in July 2008 in octopus (971 µg kg^{-1} OVTX-a). In Campania, PLTXs were often recorded at concentrations exceeding the EFSA threshold (30 µg/kg) in shellfish with concentration up to 600 µg kg^{-1} PLTXs (Sardo et al., 2020).

Among CIs, spirolides and gymnodimines were recorded in mussel samples collected in Marche in 2014 and 2015, with values up to 31 and 14 µg kg^{-1}, respectively (ARPAC 2008; Bacchiocchi et al., 2020). In May 2016, pinnatoxin G was recorded in mussels (6.8 µg kg^{-1}) sampled in Sardinia (Varriale et al., 2021).

TTXs were recorded in seafood samples collected in Marche in 2019 (8–76 µg kg^{-1} of soft tissue of mussel) (Bacchiocchi et al., 2021), in Sicily in April – July 2015, 2016, and 2017 (1.6 – 6.4 µg kg^{-1} of soft tissue), in Friuli Venezia Giulia in May- June 2017 and 2018 (trace-541 µg/kg of soft tissue of mussel) and in Veneto in May 2017 (TTX trace in wedge clams). TTXs were associated to *Prorocentrum cordatum*

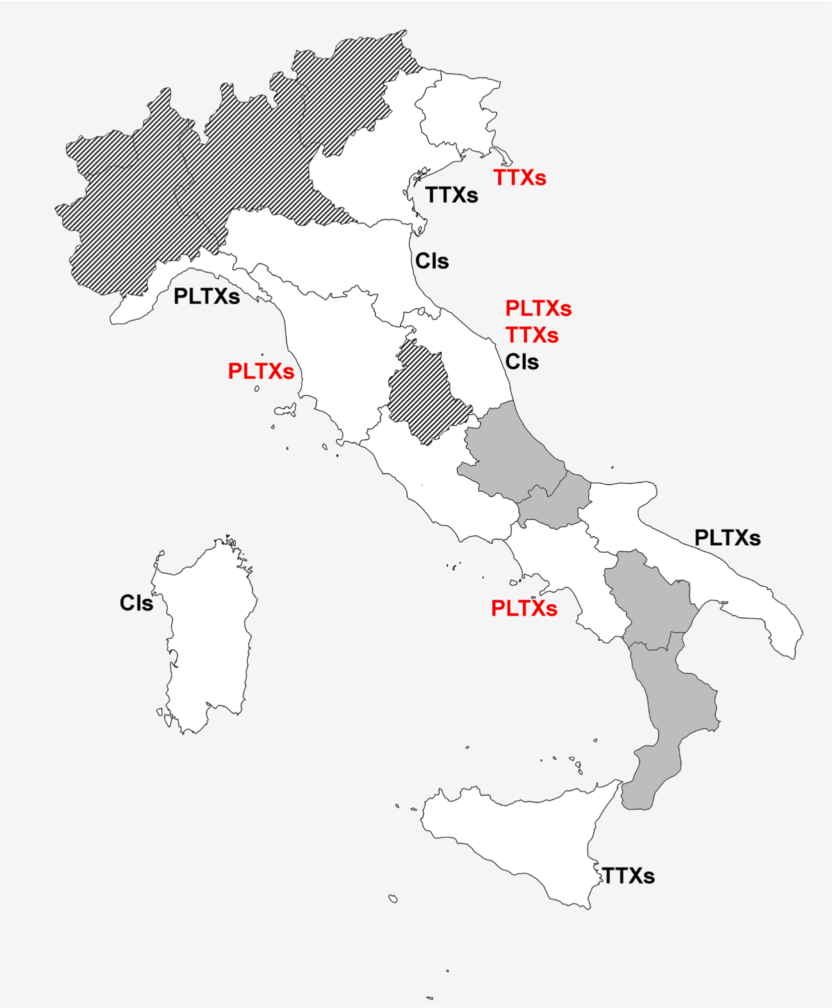

Fig. 3. PLTXs (Palytoxin-like compounds), CIs (Cyclic imines) and TTXs (tetrodotoxins) recorded in shellfish tissues seafood along the Italian coast. Red colour indicates toxin concentration above EFSA opinion. Grey: not available.

occurrence (Bane et al., 2014; Pratheepa et al., 2016; Rodríguez et al., 2017; Vlamis et al., 2015), and this dinoflagellate was recorded in the latter two regions in 2017 (with abundances up to 95,000 cell l^{-1} in June) and in May- August 2017 (with abundances up to 3445 cell l^{-1} recorded in June 2017), respectively.

4. Discussion

Shellfish collected along the coastal waters of Italy in the period considered (2006–2019) were subjected to the accumulation of most of the known HAB toxins. AZAs, DA, OA (and analogs), PTXs, STXs and YTXs were recorded in shellfish tissues in several regions along the Italian coasts over this considered period, as discussed below.

Although during the study period several potentially toxic microalgae were recorded (Table 1), a direct relationship between toxic algae abundances and toxin amount in contaminated seafood was often not observed. Finding a direct relationship between potentially toxic algae abundances and toxin amount in contaminated seafood is not obvious for several reasons. Firstly, not all strains of a certain potentially toxic species produce toxins in all environmental conditions, and both toxigenic and non-toxigenic strains can co-occur in a bloom (e.g. Eckford-Soper et al., 2013). Secondly, high toxin bioaccumulation in seafood may be detected not necessarily in concomitance of high abundances of a certain species, as (i) some toxigenic algae are able to contaminate seafood without exceeding 10^3 cells l^{-1} (e.g., *Dinophysis/Phalacroma*) (Reguera et al., 2014), ii) some others can contaminate seafood with relatively low abundances if persisting for a long time in the water column, (iii) different toxigenic algae may co-occur at low abundances in the same period, resulting in a plurispecific toxic bloom, and (iv) some toxin classes (e.g. lipophilic toxins) can remain in shellfish tissues long after a bloom has passed. Thirdly, algal growth and toxin production (and, therefore, algal abundances and toxin content) are not necessarily correlated, as they are influenced by different environmental parameters and by the physiology status/life cycle stage of the toxigenic alga (Accoroni et al., 2017; Lin et al., 2017). Finally, bioaccumulation is not immediate, but may need from weeks to months, and depends on several boundary conditions, such as the abundance and quality of the whole phytoplankton communities that affect the filtration rates (Andersen, 1998), the nature of toxins, the physiology of the shellfish (Reguera et al., 2014) and the residence time of the water mass containing the toxigenic microalgae, which affects the exposure of the non-motile filter-feeders to them. Moreover, it should be taken into account that in this study species identification was generally performed in light microscopy (only at times supported by more detailed information obtained on specific samples at research institutions, but which samples were analysed by electron microscopy and/or molecular methods are not known), and therefore species records should be considered with caution especially in cases requiring electron microscopy or molecular methods. In particular, *Gymnodinium catenatum* could be a misidentification of *G. impudicum* (Gómez, 2003), a non-toxic species, whereas *A. catenella* in the Mediterranean Sea should be *A. pacificum* (Zingone et al., 2021) or *A. tamarense*. In the same way, in the genus *Pseudo-nitzschia*, the record of *Pseudo-nitzschia simulans* –belonging to the *P. pseudodelicatissima* complex and detected until now only in Australia (Ajani et al., 2020) aside from its type description from Chinese coastal waters (Li et al., 2017)– and *P. multiseries* –reported only in Greek waters until now (Quijano-Scheggia et al., 2010)– could be misidentifications.

4.1. Lipophilic toxins

OA (and analogs) and YTXs in mollusks represented the most frequently reported cases of seafood contamination in Italy as well as in the rest of the Mediterranean Sea (HAEDAT). In this study the maximum concentrations of OA (2448 μg of OA equivalent kg^{-1}) were lower than those observed along the Atlantic margin of Europe ($\leq$ 24,862 μg of OA equivalent kg^{-1}, recorded in France in 2012, Bresnan et al., 2021), while YTX maximum concentrations (22 mg YTX equivalent kg^{-1}) were higher than those observed along the Atlantic margin of Europe ($\leq$ 3 mg YTX equivalent kg^{-1}, recorded in France in 2012, Bresnan et al., 2021). Those toxins have been recorded often above the legal limits especially in the northern Adriatic Sea, causing the closure of shellfish farms in addition to a few cases of DSP diagnoses in humans caused by consumption of seafood collected in the Adriatic (Pistocchi et al., 2012 and references therein) and Tyrrhenian Seas (Lugliè et al., 2011a).

There are several OA-producing dinoflagellates that have been observed along the Italian coasts with relatively low abundances in the water column, the most frequent of them belonging to the genus *Dinophysis*. The *Dinophysis acuminata*-complex, *D. caudata, D. tripos* and *D. fortii* are the most frequently reported species in those regions affected by OA seafood contamination above the EU limits. Their maximum abundances generally did not exceed 10^2 cells l^{-1}, with several exceptions, e.g. *D. acuminata* complex reaching 9640 cells l^{-1} in 2012 in Emilia-Romagna (value that breaks the previous Adriatic maximum record of ~ 2000 cells l^{-1} of *D. fortii* recorded in Slovenian waters (Francé et al., 2018), and 479,000 cell l^{-1} in 2016 in Sardinia. Indeed, *D. acuminata* complex is known to produce OA, DTX-1 and PTXs (Hattenrath-Lehmann et al., 2013; Mackenzie, 2019; Séchet et al., 2021) and, in this study, in some regions, abundances of *Dinophysis* spp. were higher in those seasons when OA exceeded the EU limit than in those when no OA positivity was recorded. Other OA-producing species recorded in Italy were those belonging to benthic *Prorocentrum* species, whose abundances were higher when OA exceeded the EU limit than when no OA positivity were recorded.

YTXs producer species are *Lingulodinium polyedra, Gonyalulax spinifera* and *Protoceratium reticulatum* (Paz et al., 2008). In this study, such dinoflagellates have been often recorded in regions where exceeding EU limits were detected in analyzed seafood, i.e., in Marche (*L. polyedra*) and Emilia-Romagna (*G. spinifera* and *P. reticulatum*).

4.2. Azaspiracids

In this study the maximum concentrations of AZA (7 μg of AZA equivalent kg^{-1}) were considerably lower than those observed along the Atlantic margin of Europe ($\leq$ 8970 μg of AZA equivalent kg^{-1}, recorded in Ireland in 2005, (Bresnan et al., 2021)). AZAs have been reported in shellfish from numerous sites along the Adriatic Sea coasts (Bacchiocchi et al., 2015), but they were never associated to a real bloom of dinoflagellates belonging to the genera *Azadinium* and *Amphidoma* known to produce those toxins (Krock et al., 2012; Percopo et al., 2013; Tillmann et al., 2009). Indeed, it should be taken into account a possible underestimation of the AZA dinoflagellates, considering that they are not easily identifiable under light microscope. Moreover, in a study conducted with *Mytilus galloprovincialis* exposed to *A. dexteroporum* (one of the two toxic *Azadinium* species recorded in Mediterranean Sea, i.e. *A. dexteroporum* and *A. poporum*, (Luo et al., 2017; Rossi et al., 2017) at bloom concentrations, maximum total AZAs' concentrations in mussels tissues resulted well below the maximum permitted level in the European regulation (Giuliani et al., 2019). Therefore, the record of no limit-exceeding samples in Italy could be due to the presence of relatively low-toxic *Azadinium* species which, in addition, never reached high abundances throughout the years.

4.3. Domoic acid

The presence of DA in the study period has only occasionally been found in shellfish from Adriatic, Ligurian, Tyrrhenian (including southern Sardinian) coasts, and always with values below the regulatory limits, as already reported in previous studies in the same areas (e.g. Arapov et al., 2016; Ciminiello et al., 2005; Rossi et al., 2016; Ujević et al., 2010). In other Mediterranean areas (i.e., in southern Spain and France), the detection of DA has instead caused the closure of aquaculture plants in a few cases (HAEDAT; Amzil et al., 2001).

In this study the maximum concentrations (3.4 mg DA kg^{-1}) were strongly lower than those observed along the Atlantic margin of Europe ($\leq$ 2269 mg DA kg^{-1}, recorded in France in 2007, Bresnan et al., 2021). DA contamination in mussels in the Italian coasts could be related to the presence of potentially toxic *Pseudo-nitzschia* species, such as *P. pungens, P. calliantha, P. galaxiae, P. delicatissima, P. fraudulenta, P. multistriata* and *P. pseudodelicatissima* (Giulietti et al., 2021a; Totti et al., 2019; Turk Dermastia et al., 2020; this study), which can commonly cause seasonal blooms in several Italian coastal areas, with abundances up to several million cells·l^{-1} (e.g., Cabrini et al., 2012; Caroppo et al., 2005; Congestri et al., 2008; Ruggiero et al., 2015; Totti et al., 2019). However, the low DA levels even in bloom conditions could be explained considering that several strains of potentially toxic *Pseudo-nitzschia* species were proven to be non-toxic in experimental conditions (Giulietti et al., 2021b; Penna et al., 2013).

4.4. Saxitoxins

In this study the maximum concentration of STX (13,780 μg of STX equivalent kg^{-1}) does not reach the values of the Atlantic European margin (60,000 μg of STX equivalent kg^{-1}in Portugal in 1995, Bresnan et al., 2021) or the Norwegian Sea (i.e. 58,000 μg of STX equivalent kg^{-1}, Karlson et al., 2021). During the study period, STXs have been recorded along the Adriatic and Tyrrhenian Seas with concentrations in most cases not exceeding the EU limit. However higher values were recorded (i) in Sardinia, with a 3 or 4 limit-exceeding samples in one year, resulting in only a few cases of shellfish farm closures in the northern coast (Lugliè et al., 2011b, ii) in Sicily, where the first case of PSP toxins' contamination in mussels (10,851 μg STX equivalents kg^{-1} of shellfish tissue) was recorded in 2016 (Dell'Aversano et al., 2019), and (iii) in Liguria in 2012.

PSP events in the Mediterranean Sea have been related to toxins produced by *Gymnodinium catenatum* in the westernmost area and by species of the genus *Alexandrium* (FAO/WHO 2022). PSP events in Italy have been associated to *A. minutum, A. tamarense* and *A. pacificum* in Sardinia and Sicily (Dell'Aversano et al., 2019; Lugliè et al., 2017; this study) where dinoflagellate abundances reached value up to 10^5 cells l^{-1}. *Alexandrium* species were commonly recorded in other regions (e.g. Marche and Emilia-Romagna) where however no STX was detected in seafood. This could be explained considering that (i) *Alexandrium* spp. were only recorded at low abundances in those regions (generally ~ 10^2–10^3 cells l^{-1}, and always below 10^5 cells l^{-1}), and (ii) the genus also includes both non-toxic species and species producing other toxins, e.g. spirolides in *A. ostenfeldii* from Emilia-Romagna, Ciminiello et al., 2006)). However, STX positivity was recorded in Emilia-Romagna in 1994, when a maximum abundance of 73,000 cells l^{-1} of *A. minutum* was related to a contamination of up to 1920 μg STX equivalents kg^{-1} of shellfish tissue in *Mytilus galloprovincialis* sampled in that area (Honsell et al., 1996).

4.5. Trends of phycotoxin records in seafood along Italian coasts

There is increasing concern that climate change consequences (i.e. warming water temperature, changes in salinity, higher carbon dioxide levels, changes in rainfall, sea level rise, coastal upwelling alteration) might cause HABs to occur more often and in more waterbodies, and to be more intense (e.g. Hallegraeff, 2010; Wells et al., 2015). However, despite considerable interannual variability, overall, no increasing trend is detected in this 14-years study, in agreement with what reported by Zingone et al. (2021) for the whole Mediterranean area. The absence of an appreciable trend was also noted along the Atlantic margin of Europe (Bresnan et al., 2021) and in coastal seas of Northern Europe (Karlson et al., 2021).

In this study, a significant and negative trend is observed only in specific cases, such as in Marche for OA and YTX, in Emilia-Romagna for YTX and in Sardinia for STX. However, some trends could be only apparent and reflect changes in methodological approaches.The decrease of positive OA and YTX after 2012 is linked to (i) the change in the legal limit from 1 to 3.75 mg YTX equivalent kg^{-1} for YTX-group (European Commission, 2013) and (ii) the new official LC-MS/MS protocol for the determination of marine lipophilic toxins in bivalve mollusks (European Commission, 2011) replacing the obsolete MBA, which could have led to a high number of false positive records (EFSA 2009a) over the previous years. A study comparing results obtained through LC-MS/MS and those from the same samples analyzed via MBA for OA, DTX1, DTX2, YTX, PTX2, and AZA1 revealed consistent findings in 69 % of the samples, while 26 % of MBA results exhibited 'false-positive' outcomes in relation to the analyzed toxins (no 'false negatives' were observed, Chapela et al., 2008).

4.6. Emerging marine biotoxins

In the last years, the EFSA stressed the need to implement, from both a scientific and a legislative point of view, the information on Emerging Marine Biotoxins (EMBs) (EFSA 2010, 2009b; Knutsen et al., 2017), including palytoxin-like compounds (PLTXs), cyclic imines (CIs), and tetrodotoxins (TTXs), for which a regulatory limit has not been provided by EU as yet.

One of the most important classes of emerging toxins of the last two decades in the Mediterranean Sea are palytoxin-like (PLTX-like) compounds. These compounds are produced by several species of the benthic dinoflagellate genus *Ostreopsis*, whose blooms have become regular phenomena in summer and/or autumn along all the Italian coasts in the last decades (Emilia-Romagna and Veneto are the only exceptions). PLTXs during *Ostreopsis* blooms have been recorded in seafood in Marche, Tuscany, Campania, Liguria and Apulia, often with concentration exceeding the threshold of 30 μg kg^{-1} indicated in the Scientific Opinion published by the European Food Safety Authority for PLTXs in mollusks (EFSA, 2009b).

Regarding CIs, spirolides, gymnodimines and pinnatoxins were recorded in mussels' samples collected in Marche, Emilia-Romagna and Sardinia. The widespread dinoflagellates *Alexandrium ostenfeldii* and *A. peruvianum* are the only spirolide (SPX)- and GYM- producer organisms known to date (Cembella et al., 1999; Cembella et al., 2000; Harju et al., 2016; Van Wagoner et al., 2011). The first record of SPX in Italy dates back to 2003, when a bloom of *A. ostenfeldii* occurred along the Emilia-Romagna coast (Ciminiello et al., 2006; Pigozzi et al., 2006).

Until recently, TTXs have been considered to be confined to the Indo-Pacific area, especially Japan, where pufferfish are caught and eaten as fugu, a traditional culinary delicacy, resulting in several tens of severe intoxications every year, with fatal outcomes in some cases (Noguchi et al., 2011). Since 2003, pufferfish has been reported in the Eastern Mediterranean Sea (Bentur et al., 2008; Katikou et al., 2009) and the first European case of TTX intoxication occurred in Spain in 2006 in a patient who had eaten gastropods (*Charonia lampas lampas*) from Portugal. Subsequently, several other cases of shellfish contamination by TTX were reported in the UK, Greece, the Netherlands, and Spain since 2015 (Gerssen et al., 2018; Rodriguez et al., 2008; Turner et al., 2015; Vlamis et al., 2015). It has been demonstrated that TTX and its analogues in marine species are produced by a wide range of host-associated bacteria, such as *Vibrio, Bacillus, Aeromonas, Shewanella, Alteromonas* and *Pseudomonas*, which naturally inhabit the animals' gut (Pratheepa et al., 2016). However, even the planktonic dinoflagellate *Prorocentrum cordatum* has been suspected to be involved in TTXs production, because a correlation between the occurrence of TTX in shellfish and of *P. cordatum* in seawater has been noted (Bane et al., 2014; Pratheepa et al., 2016; Vlamis et al., 2015) and TTX-like compounds have also been found in *P. cordatum* cultures (Rodríguez et al., 2017). In this study, TTXs were detected in seafood from Sicily, Veneto, Marche and Friuli-Venezia Giulia, in the latter two regions exceeded the EFSA-opinion limit of 44 μg equivalent kg^{-1}, but only in Friuli

Venezia-Giulia and Veneto *P. cordatum* was recorded in high abundances (above 10^3 cells l^{-1}).

4.7. Conclusions

Despite many countries have put in place stringent quality control procedures to ensure the safety of seafood, about 2000 cases a year of poisoning are still reported in the world, 15 % of which are lethal (Hallegraeff, 2010). Overall, even if the Italian coastal waters host a high number of potentially toxic species, the cases of intoxications are relatively rare, and the impact on aquaculture appears to be limited to a few hot spots in the northern Adriatic Sea, with DSP as the main concern. A variety of toxins have been detected in several instances in microalgae strains from these waters, while seafood toxicity, when detected, has commonly remained below the safety limits.

Finally, no clear trends (only sometimes negative trends were observed in a few regions for OA, YTX and STX) in occurrence nor areal expansions emerge for either potentially toxic microalgae or seafood contamination in Italian coasts, in line with what observed in the rest of Mediterranean Sea (Zingone et al., 2021).

CRediT authorship contribution statement

Stefano Accoroni: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing, Investigation. **Monica Cangini:** Data curation, Investigation, Writing – review & editing. **Roberto Angeletti:** Investigation. **Carmen Losasso:** Investigation. **Simone Bacchiocchi:** Investigation. **Antonella Costa:** Investigation. **Aurelia Di Taranto:** Investigation. **Laura Escalera:** Formal analysis, Writing – review & editing. **Giorgio Fedrizzi:** Investigation. **Angela Garzia:** Formal analysis. **Francesca Longo:** Investigation. **Andrea Macaluso:** Investigation. **Nunzia Melchiorre:** Investigation. **Anna Milandri:** Data curation, Investigation. **Stefania Milandri:** Data curation, Investigation. **Marina Montresor:** Conceptualization, Writing – review & editing. **Francesca Neri:** Formal analysis, Writing – review & editing. **Arianna Piersanti:** Investigation. **Silva Rubini:** Investigation. **Chiara Suraci:** Investigation. **Francesca Susini:** Investigation. **Maria Rosaria Vadrucci:** Investigation. **Alessandro Graziano Mudadu:** Investigation. **Barbara Vivaldi:** Investigation. **Barbara Soro:** Investigation. **Cecilia Totti:** Conceptualization, Supervision, Writing – review & editing. **Adriana Zingone:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data underlying this paper are available at the Mendeley Data (http://data.mendeley.com) repository

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Supplementary materials

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