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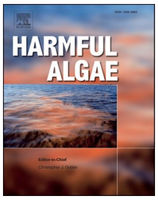

# A decade-long study on harmful dinoflagellate blooms and biotoxin contamination in mussels from the north-western Adriatic Sea (Mediterranean Sea)

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## ABSTRACT

The north-western Adriatic Sea is one of the most eutrophic areas of the Mediterranean Sea and an important aquaculture region, accounting for about 90% of Italian shellfish production. Since the 1970s, this area has experienced frequent harmful algal blooms, posing risks to consumers and coastal ecosystems. Despite the regular monitoring required by European legislation, long-term studies integrating taxonomic analysis of phytoplankton species, associated biotoxins, and environmental data remain limited. This study examines an 11-year dataset (2012–2022) from the Emilia-Romagna region coast, focusing on timing and occurrence trends of toxic dinoflagellates related to the shellfish toxicity. Phytoplankton species distribution and biotoxins content in farmed mussels were analysed, with particular attention to the presence of analogues. From 2012 to 2015, the most prevalent toxins were yessotoxins (YTXs, up to 8.44 mg $kg^{-1}$), primarily homo-YTX and 45OH homo-YTX, which were associated with autumn blooms of *Gonyaulax spinifera* and *Lingulaulax polyedra*. From 2015, okadaic acids and derivatives (OAs, up to 941 µg $kg^{-1}$) became the most prevalent phycotoxin group, associated to *Dinophysis fortii* and *Dinophysis acuminata* presence during autumn. This temporal shift was likely driven by increasing surface water temperature and salinity, which contributed to reduced water column mixing favouring oligotrophic species. Notably, low levels of YTXs persisted in mussels despite decreased abundances of producing species. Additionally, *Alexandrium* spp. cells were consistently detected, even in the absence of saxitoxins (STXs) in bivalves. These findings highlighted the need for integrated studies on strain-specific toxicity, phycotoxins accumulation in bivalves, and field monitoring.

## 1. Introduction

Marine coastal ecosystems are increasingly exposed to intense human activity, as well as to the growing occurrence of harmful algal blooms (HABs), which can negatively impact biodiversity and ecosystem functions. These events are characterised by the rapid proliferation and occasional dominance of a certain phytoplankton species, typically belonging to dinoflagellates or diatoms, which can cause water discolouration, mucilage formation, anoxia, and the production of toxins that can accumulate in seafood products, primarily in molluscs (e.g., mussels) (Anderson, 2009; Hallegraeff et al., 2004). Depending on the toxins involved, the consumption of contaminated seafood can lead to different courses of poisoning and thus illnesses, resulting in significant economic, social, and ecological impacts (Griffith and Gobler, 2020). Consequently, scientific interest in understanding and monitoring HABs dynamics has significantly increased (Gobler, 2020). Over the past decades, several phycotoxins have been identified together with their producers, whose presence and proliferation have been related to specific geographic areas (Hallegraeff et al., 2021). Within the European Union, a number of toxins are routinely monitored under EC Regulation 853/2004 (European Council, 2004), amended by EU Regulations 2019/627 and 2021/1709 (European Commission, 2019, 2021a), which

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establish the maximum permitted levels for each toxin group to ensure the safety of bivalve molluscs for human consumption. More than 140 toxin-producing phytoplankton species have been identified in the IOC-UNESCO taxonomic reference list (Lundholm et al., 2009), of which 84 have been recorded in the Mediterranean Sea, emphasising the importance of HAB surveillance and scientific investigation in this area (Zingone et al., 2021). Within this context, the Adriatic Sea, which is a shallow (average depth about 250 m, up to 20–30 m near the coast) semi-enclosed basin in the Mediterranean, stands out for its high susceptibility to HAB events. The vulnerability of this basin is attributable to its exposure to irregular and intense meteorological conditions, which promote the alternation between oligotrophic states and the influx of nutrients from continental runoff, potentially triggering anomalous and intense algal blooms at any time of the year (Garcés and Camp, 2012; Zingone et al., 2021). The north-western (NW) Adriatic Sea (north of 43° N, and east up to 13° E) is particularly eutrophic due to its proximity to the Po River delta and serves as a major aquaculture zone, contributing approximately to the 90% of Italy's shellfish production (Ciminiello and Fattorusso, 2006). However, since the 1970s, the frequent occurrence of HABs has posed a significant threat to these shellfish farms, resulting not only in substantial economic losses for the aquaculture industry, but also in heightened risks to consumer health. Consequently, water quality in this area is strictly monitored, particularly in relation to aquaculture activities. In the coastal region of Emilia-Romagna, located south of the Po River delta, the predominant algal toxins belong to the class of lipophilic toxins, primarily comprising diarrhoeic shellfish toxins (DSTs), e.g., okadaic acid and derivatives (OAs), dinophysistoxins (DTXs) and pectenotoxins (PTXs), as well as yessotoxins (YTXs) (Pistocchi et al., 2012; Pompei et al., 2018). In this region, the accumulation of DSTs in seafood above the permitted regulatory levels has led to the closure of bivalve mollusc production sites on several occasions over the years (Pistocchi et al., 2012). The first recorded seafood contamination event, involving OAs accumulation in mussels, occurred in 1989 and was associated with the presence of various *Dinophysis* species in the seawater (Boni, 1992). Since that event, OAs have been consistently detected in mussels along the coast of the Emilia-Romagna region, typically in association with low concentrations of *Dinophysis* spp. and *Phalacroma* spp. (Pistocchi et al., 2012). A distinct species succession within the *Dinophysis* genus has been observed over the years, with *D. sacculus* and *D. acuminata* reaching their highest abundances during the spring-summer period, *D. caudata* peaking in late summer-autumn, and *D. fortii* showing maximum concentrations in October and November (Ravera et al., 2024; Reguera et al., 2014). In June 1995, mussels were found to contain both OAs and YTXs (Ciminiello et al., 1997), marking the first recorded instance of this class of lipophilic toxins in the Adriatic Sea, where YTXs are now considered endemic (Rubini et al., 2021). Following this discovery, the presence of this toxin group has led to numerous harvesting closures for shellfish farms, despite the regulatory limit being raised from 1.00 mg kg$^{-1}$ to 3.75 mg kg$^{-1}$ in August 2013 (European Commission, 2013). YTXs are primarily produced by dinoflagellates within the order Gonyaulacales, including *Protoceratium reticulatum, Lingulaulax polyedra* (formerly known as *Lingulodinium polyedra*), *Gonyaulax spinifera, Gonyaulax taylorii,* and *Gonyaulax montresoriae* (Álvarez et al., 2016; Huang et al., 2025; Paz et al., 2008). Among these, *G. spinifera* has been identified as the most potent producer of YTXs, with levels reaching up to 200 pg cell$^{-1}$ (without distinction between analogues) (Rhodes et al., 2006), followed by *P. reticulatum* (4.5 - 65.0 pg cell$^{-1}$) (Guerrini et al., 2007). Over 90 YTXs analogues have been identified from these dinoflagellates, although their effects on humans remain unclear. *G. spinifera* has been reported to produce primarily homoyessotoxin (homo-YTX) (Riccardi et al., 2009), while *P. reticulatum* is commonly associated with yessotoxin (YTX), though some studies suggest that homo-YTX production may also occur depending on the ecological ecotype (Barbosa et al., 2024; Satake et al., 1999; Wang et al., 2019). This aspect was further supported through the analysis of various cultured strains of *P. reticulatum* isolated from the NW Adriatic Sea, where YTX was identified as the main produced analogue (Ciminiello et al., 2003; Guerrini et al., 2007). The predominant production of homo-YTX by *L. polyedra* has been reported for a nearly monospecific bloom in the Adriatic Sea (Tubaro et al., 1998) and for several isolated strains (i.e. Paz et al., 2004; Tillmann et al., 2021). Nonetheless, the cell quota for these strains is typically reported to be relatively low, indicating a generally low toxin production potential compared to other YTXs-producing species. In addition, the existence of both non-toxic and toxic strains has been documented worldwide (Barbosa et al., 2024; Tillmann et al., 2021), making it difficult to predict which toxin analogues might be produced during a HAB. Paralytic shellfish toxins (PSTs), specifically saxitoxins (STXs), are predominantly associated with dinoflagellates of the genus *Alexandrium* (e.g., *A. minutum, A. ostenfeldii*, etc.), and are the primary causative agents of toxic events in various region of the Mediterranean Sea (Anderson et al., 2012). Although potentially PSTs-producing species are present in the Emilia-Romagna region, this toxin group does not pose a significant threat in this area, where STXs have only been detected in mussels on two occasions, coinciding with *Alexandrium* spp. cell densities in seawater reaching a maximum of 10$^{3}$ cell L$^{-1}$. Toxin levels exceeded regulatory limits in only one instance, in May 1994, when mussels were found to contain 1926.6 μg STXs kg$^{-1}$ (Ciminiello et al., 1995; Honsell et al., 1995; Milandri et al., 2008). Similar observations can be made for other algal toxin groups, namely azaspiracids (AZAs) and domoic acid (DA); indeed, although their levels in shellfish have never exceeded EU limits, potential producers of AZAs and DA (*Azadinium* spp. and *Pseudo-nitzschia* spp., respectively) have been periodically detected along the Italian coasts, frequently reaching high cell abundances, above 10$^{7}$ cell L$^{-1}$ (Accoroni et al., 2024).

In the Emilia-Romagna region, the monitoring of toxic phytoplankton in seawater and marine biotoxins in farmed and wild shellfish is regulated by the regional Health Surveillance Plan on edible lamellibranch molluscs (Emilia-Romagna, 2020). The authorities responsible for this monitoring and for the water quality general assessment are the Italian Reference Centre for Marine Biotoxins (FCRM, Cesenatico, Italy), and the Regional Environmental Protection Agency (ARPA Emilia-Romagna, Italy), respectively.

Despite the numerous studies on the presence of potentially toxin-producing phytoplankton species in the Adriatic aquaculture farming areas, only a few long-term studies on toxic phytoplankton have been conducted in this area (Bacchiocchi et al., 2015; Casabianca et al., 2022; Cerino et al., 2019; Cozzi et al., 2020; Rubini et al., 2021; Totti et al., 2019), although a comprehensive analysis at low taxonomic levels, integrating both phytoplankton species and their associated toxins and analogues, is lacking.

Based on long-term data collected from the Emilia-Romagna region's coast over the past decade, this study aimed to correlate the presence of potentially toxic phytoplankton species with shellfish toxicity in this area. To this purpose, a systematic examination of phytoplankton species at a refined taxonomic level was conducted, incorporating the presence of toxin analogues whenever data were available. Furthermore, potential correlations between the proliferation of toxin-producing species and several seawater environmental parameters were investigated.

## 2. Materials and methods

### 2.1. Study area and samplings

The study area encompassed ten sampling stations from major mussel production and harvesting sites along the NW Adriatic Sea, off the coast of the Emilia-Romagna region (Fig. 1, Table S1). These sampling stations were divided into three main geographic areas, namely Ravenna (RA), Cesenatico (FC), and Rimini (RN), as identified by the regional plans for bivalve aquaculture (Emilia-Romagna, 2021). Specifically, stations S-1–4 were in the RA area (northernmost), S-5–6 in the

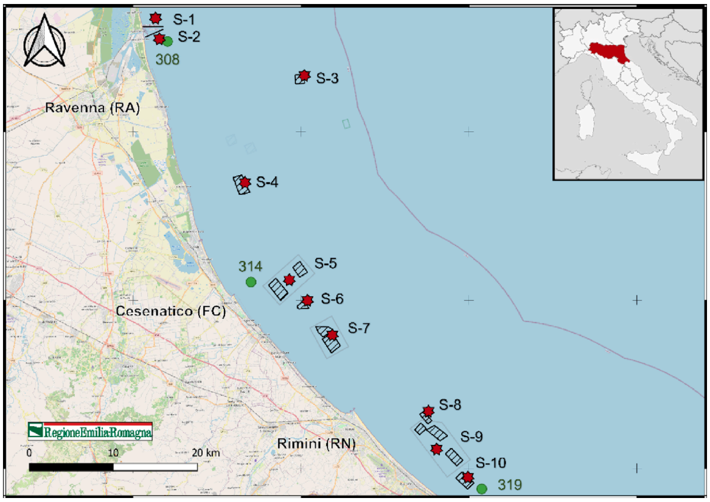


**Fig. 1.** Distribution map of the ten stations (i.e., S-1–10) corresponding to *M. galloprovincialis* production areas along the coast of Emilia-Romagna region (NW Adriatic Sea, lat. from 44.49783 to 43.98944 WGS84) here studied, and of the three ARPAE sampling stations (i.e., RA-308, FC-314, RN-319) considered for physicochemical parameters (green circles).

FC area (central), and S-7–10 were located in the RN area (southernmost). Seawater and mussel samples (*Mytilus galloprovincialis*) were collected once-twice a month from each station by the ASL Romagna Veterinary Service between 2012 and 2022, as part of the regional official monitoring programme.

### 2.2. Qualitative and quantitative phytoplankton analyses

From 2012 to 2019, seawater samples for phytoplankton analysis were collected using a bucket for surface water, and, since August 2019, a hose sampler (Reguera et al., 2014). After collection, samples were preserved by adding Lugol's solution, and cell abundances (cell L$^{-1}$) of toxic or potentially toxic species were determined following the Utermöhl method (European Standard, 2006).

### 2.3. Toxin analyses

In accordance with EC Regulation 2074/2005 and EU Regulation 2019/627, mussel samples were tested for the presence of lipophilic toxins, including OAs, DTXs, PTXs, AZAs, and YTXs, as well as for the hydrophilic STXs and DA (European Commission, 2019, 2005).

Since 2012, the quantification of lipophilic toxins has been conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS), following the "EU-Harmonised Standard Operating Procedure for the determination of lipophilic marine biotoxins in molluscs by LC-MS/MS, Version 5, 2015″. Until December 2018, the quantification of STXs was performed using the Mouse Bioassay (MBA) according to the OMA AOAC 959.08 method. Thereafter, from January 2019 onwards, STXs (e.g., STX, GTXs, C-toxins, etc.) were quantified using high-performance liquid chromatography with fluorescence detection (HPLC-FLD), following the "EURLMB SOP for the analysis of PSTs" according to the OMA AOAC 2005.06. The analysis of DA was carried out following the "EU-Harmonised Standard Operating Procedure for the determination of domoic acid in shellfish and finfish by RP-HPLC using UV detection, Version 1, 2008".

Samples were considered positive if toxin concentrations exceeded the regulatory limits set by EC Regulation 853/2004 and its subsequent amendments (European Council, 2004). Specifically, maximum allowed concentrations were 160 µg kg$^{-1}$ for the sum of OAs and DTXs, PTXs, and AZAs, 800 µg kg$^{-1}$ for STXs equivalent, and 20 mg kg$^{-1}$ for DA, whilst the limit for YTXs was revised from 1.00 mg kg$^{-1}$ to 3.75 mg kg$^{-1}$ in 2013 (European Commission, 2013). In the present study, PTXs were not considered relevant, as they were detected in only two samples from 2015 and, in both cases, concentrations were slightly above the limit of quantification of the official method (i.e., 45 and 49 µg kg$^{-1}$ PTXs in November 2015 in RA and FC, respectively). This irrelevance was further confirmed by the EU Regulation 2021/1374, which led to the deregulation of this class of toxins (European Commission, 2021b).

### 2.4. Environmental data

Environmental data were provided by the official monitoring programme conducted by the Regional Environmental Protection Agency of Emilia-Romagna (ARPAE), which operates a network of 41 monitoring stations distributed along the coast, to evaluate the overall ecological status of coastal waters (L.R. 39/78, L.R. 3/99, and L.R. 44/95). From this network, three most representative and relevant monitoring stations were selected, one for each of the three study areas (i.e., RA, FC, RN), based on their proximity to at least one mussel sampling site, namely RA-308, FC-314, and RN-319 (Fig. 1, Table S1).

Data collected from surface seawater (0.5 m depth) included temperature (°C), salinity (psu), dissolved oxygen (mg L$^{-1}$), chlorophyll-*a* (µg L$^{-1}$), and inorganic nutrient concentrations, i.e., nitrates, nitrites, ammonium, and phosphates (µg L$^{-1}$).

### 2.5. Data analyses

#### 2.5.1. Dataset construction

The abundance of potentially toxic phytoplankton in seawater (cell L$^{-1}$) and the concentration of phycotoxins in mussels (µg or mg kg$^{-1}$) were grouped according to the Emilia-Romagna Official Gazette (Emilia-Romagna, 2021) and assigned to the ten stations. The dataset was structured to focus on toxin groups that have led to the closure of shellfish production areas at least once over the years in the NW Adriatic Sea. Thus, the presence of STXs, OAs, and YTXs in mussels and the corresponding concentrations of potentially toxin-producing dinoflagellates, were comprised. To ensure consistency across the 11-year study period, only dinoflagellate species that were consistently monitored throughout the entire decade were included in the dataset:

- PSTs-producing species and morphologically similar or co-occurring species not confirmed as PSTs producers: *Alexandrium* spp., *A. minutum, A. ostenfeldii, A. insuetum, A. taylorii;*
- DSTs-producing species: *Dinophysis acuminata* complex (*D. acuminata, D. sacculus, D. ovum*), *D. caudata, D. fortii, Phalacroma rotundatum;*

- YTXs-producing species: *Protoceratium reticulatum, Gonyaulax spinifera, Lingulaulax polyedra.*

Prior to data visualization and statistical analyses, the data underwent normalisation by assigning random values between zero and the detection limit for samples with concentrations below the detection threshold itself. Furthermore, missing values due to lack of samplings were replaced by either the median or the mean of the corresponding group, for biotic and abiotic variables, respectively. This methodological approach ensured a consistent treatment of non-detects and missing data, thus minimising potential biases in the analyses.

### 2.5.2. *Data visualization and statistical analyses*

Heatmaps were constructed to represent biotoxin levels in mussels, considering the maximum value for each sampling zone (RA, FC, RN) and time (years and months). This approach aimed to highlight periods of farm closures due to high biotoxin concentrations. Additional heatmaps, bar plots, and clustering graphs were also generated using group-specific average values of biotoxin levels and potentially toxin-producing phytoplankton, to visualise seasonal and geographical variations. Time series of potentially toxic phytoplankton cell abundances (cell $L^{-1}$) and biotoxin concentrations (μg or mg $kg^{-1}$) were plotted for all the ten stations (S1–10) over the decade considered (2012–2022), also including the relative abundance of toxin analogues, when available.

Data were tested for normality using the Shapiro-Wilk test and homogeneity of variances with Leven's test. Subsequently, permutational multivariate analysis of variance (PERMANOVA) was performed to assess differences in the abundance of potentially toxin-producing phytoplankton in seawater, biotoxin concentrations in mussels, and surface water physicochemical parameters, considering years (2012–2022), months (January-December), and sites (RA, FC, RN) as fixed factors. PERMANOVA for the multivariate analysis of phytoplankton abundance was based on Bray-Curtis similarity using log-transformed data, while analyses of biotoxin concentrations and environmental variables, whether univariate or multivariate, were conducted using Euclidean distances with untransformed and standardised data, respectively. When a significant effect of any factor or interaction was detected, pairwise comparisons were performed with Bonferroni correction for multiple testing. To identify the phytoplankton species contributing most to the observed variability among groups of potential toxin producers, the similarity percentage analysis (SIMPER) was conducted. To explore correlations among physicochemical parameters, a principal component analysis (PCA) was conducted. To evaluate relationships between abiotic environmental data and biotic variables (i.e., phytoplankton species and biotoxins), a constrained ordination approach was employed. Based on preliminary detrended correspondence analysis (DCA), unimodal and linear methods were deemed appropriate to analyse phytoplankton species and biotoxins data, respectively. Thus, canonical correspondence analysis (CCA) was performed for log-transformed phytoplankton species data, whereas redundancy analysis (RDA) was applied to fourth root-transformed biotoxin data. The Mann-Kendall trend test was used to assess long-term trends in the annual variation of the studied variables. Multivariate correlation analyses using Kendall's tau coefficient were conducted to further explore the relationships between potentially toxin-producing phytoplankton species, biotoxin concentrations in mussels, and physicochemical seawater parameters.

All statistical analyses were conducted using R Studio v. 4.4.1 (R Core Team, 2022).

## 3. Results

The final dataset comprised 2640 records detailing biotoxin levels in *M. galloprovincialis* and the abundances of potentially toxin-producing dinoflagellates in mussel farms along the coast of the Emilia-Romagna region, covering the period from 2012 to 2022. During this period, STXs were not detected. However, 545 mussel samples were found to be contaminated with YTXs, of which 50 exceeded the regulatory limit, and 216 samples were contaminated with OAs, of which 68 surpassed the regulatory limit (Table 1, Table 2, Table 3).

### 3.1. *Yessotoxins and producing species*

From 2012 to 2015, the presence of YTXs in mussels at concentrations exceeding the regulatory limit led to the recurrent closure of mussel production sites in the study area (Fig. 2), despite the increase of the legal limit from 1.00 to 3.75 mg $kg^{-1}$ in 2013 (European Commission, 2013). From 2016 onward, YTXs concentrations in mussels have remained below the regulatory threshold, although relatively low levels have been detected annually (< 1.92 mg $kg^{-1}$). Indeed, the Mann-Kendall test confirmed a significant downward trend in the concentration of these compounds in mussels over the analysed decade ($\tau = -0.13$, $p \leq 2.22 \times 10^{-16}$).

During the years when harvesting closures occurred, the highest concentrations of YTXs were recorded predominantly in autumn (September - October), persisting into early spring (March - April) (Fig. 2). The PERMANOVA analysis confirmed significant interannual differences in toxin presence ($R^2 = 0.088$, $F = 42.09$, $p = 0.01$), and further highlighted significant seasonal variations ($R^2 = 0.060$, $F = 25.73$, $p = 0.01$). Post-hoc analyses corroborated that, during colder months (i.e., September, October, November, December), YTXs concentrations were significantly higher than the rest of the year, thus aligning with the periods of harvesting closures. Spatial differences in toxin distribution across the three monitored sites were also observed ($R^2 = 0.012$, $F = 29.65$, $p = 0.01$), with the northernmost area (RA) differing from the central area (FC), and the latter differing from the southernmost area (RN).

The YTXs-producing species *G. spinifera*, *P. reticulatum*, and *L. polyedra* were consistently detected in seawater samples throughout the investigated period, showing no substantial fluctuations in occurrence despite the declining trend of YTXs in mussels (Table 1). This apparent discrepancy may be attributed to a progressive reduction in the abundance of the three dinoflagellates within aquaculture sites, as confirmed by the Mann-Kendall test, which demonstrated a significant downward trend in their abundance ($-0.23 < \tau < -0.19$, $p \leq 2.22 \times 10^{-16}$).

A general temporal and spatial variability in the community structure of YTXs-producing species was observed during the decade-long monitoring, as also confirmed by PERMANOVA (Year: $R^2 = 0.079$, $F = 25.22$, $p = 0.01$; Month: $R^2 = 0.016$, $F = 4.79$, $p = 0.01$; Site: $R^2 = 0.003$, $F = 5.25$, $p = 0.01$), with the highest abundances occurring in September, October, November, and December in the RA area. According to SIMPER analysis, *G. spinifera* was the dominant species blooming during autumn and reaching peak abundances up to $10^6$ cell $L^{-1}$ mainly during the years with harvesting closures (i.e., 2012–2016). In subsequent years (i.e., 2017–2020), *P. reticulatum* became more prevalent, peaking in late spring and early summer (May and June). In 2022, the community structure shifted back to a *G. spinifera*-dominated assemblage (Table 1).

To further investigate these patterns, the relative composition of YTXs analogues (YTX, 45OH YTX, homo-YTX, and 45OH homo-YTX) detected in mussels from 2012 to 2015 was analysed. The spatio-temporal distribution of these analogues mirrored the trends observed for YTXs during the entire study period. Homo-YTX and 45OH homo-YTX were the most abundant analogues, reaching their highest concentrations in mussels during September, October, November, and December (Fig. 3a). During this period, *G. spinifera* and *L. polyedra* dominated the phytoplankton community, thus being the primary contributors to the production of these analogues. In contrast, *P. reticulatum* exhibited peak abundances in spring and summer. A slight temporal delay was observed between toxin-producing phytoplankton presence in seawater and toxin levels in mussels. Additionally, a spatial gradient in

**Table 1**
Summary of the mussel samples exceeding the regulatory limit for YTXs, the presence of potentially YTXs-producing species in seawater, and the maximum abundances of these species recorded from 2012 to 2022 along the Emilia-Romagna region coast, with the corresponding month and sampling site.

| Years | N° of positive mussel samples (YTXs) | N° of seawater samples with YTXs-producing species presence | | | YTXs-producing species | | | |
|---|---|---|---|---|---|---|---|---|
| | | *G. spinifera* | *P. reticulatum* | *L. polyedra* | Max abundance (cell $L^{-1}$) | Species | Month | Site |
| **2012** | 22 | 69 | 19 | 34 | $2.3 \times 10^5$ | *G. spinifera* | Oct | RA |
| **2013** | 18 | 89 | 34 | 22 | $1.7 \times 10^4$ | *G. spinifera* | Oct | FC |
| **2014** | 9 | 153 | 33 | 58 | $1.4 \times 10^6$ | *G. spinifera* | Oct | RA |
| **2015** | 1 | 25 | 15 | 5 | $6.2 \times 10^3$ | *G. spinifera* | Aug | RA |
| **2016** | 0 | 96 | 40 | 38 | $3.2 \times 10^3$ | *G. spinifera* | Jul | RA |
| **2017** | 0 | 110 | 30 | 65 | $3.2 \times 10^3$ | *P. reticulatum* | May | RN |
| **2018** | 0 | 84 | 24 | 39 | $8.0 \times 10^2$ | *L. polyedra* | May | FC |
| **2019** | 0 | 322 | 98 | 123 | $3.5 \times 10^3$ | *P. reticulatum* | Jun | RN |
| **2020** | 0 | 285 | 92 | 115 | $2.5 \times 10^3$ | *P. reticulatum* | Sept | RA |
| **2021** | 0 | 215 | 67 | 81 | $1.2 \times 10^3$ | *G. spinifera* | Jul | RA |
| **2022** | 0 | 323 | 124 | 105 | $5.6 \times 10^2$ | *G. spinifera* | Jul | RA |

**Table 2**
Summary of the mussel samples exceeding the regulatory limit for OAs, the presence of potentially DSTs-producing species in seawater, and the maximum abundances of these species recorded from 2012 to 2022 along the Emilia-Romagna region coast, with the corresponding month and sampling site.

| Years | N° of positive mussel samples (OAs) | N° of seawater samples with DSTs-producing species presence | | | | | | DSTs-producing species | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *D. acuminata* | *D. sacculus* | *D. ovum* | *D. caudata* | *D. fortii* | *P. rotundatum* | Max abundance (cell $L^{-1}$) | Species | Month | Site |
| **2012** | 0 | 11 | 20 | 0 | 11 | 3 | 20 | $7.7 \times 10^3$ | *D. sacculus* | Jun | RA |
| **2013** | 0 | 28 | 17 | 0 | 22 | 16 | 33 | $2.2 \times 10^3$ | *D. acuminata* | May | RN |
| **2014** | 4 | 25 | 69 | 16 | 25 | 13 | 30 | $2.0 \times 10^3$ | *D. sacculus* | May | RN |
| **2015** | 19 | 15 | 18 | 0 | 14 | 16 | 13 | $7.6 \times 10^2$ | *D. fortii* | Nov | RN |
| **2016** | 4 | 24 | 21 | 0 | 13 | 8 | 18 | $3.1 \times 10^3$ | *D. acuminata* | May | RA |
| **2017** | 8 | 4 | 20 | 1 | 13 | 11 | 36 | $4.8 \times 10^2$ | *D. sacculus* | May | RA |
| **2018** | 0 | 18 | 20 | 0 | 13 | 5 | 15 | $2.5 \times 10^3$ | *D. sacculus* | May | RA |
| **2019** | 1 | 119 | 80 | 24 | 80 | 84 | 41 | $1.1 \times 10^3$ | *D. sacculus* | May | RA |
| **2020** | 10 | 108 | 6 | 3 | 13 | 33 | 29 | $5.4 \times 10^2$ | *D. acuminata* | Mar | FC |
| **2021** | 20 | 102 | 9 | 0 | 13 | 39 | 48 | $3.4 \times 10^2$ | *D. fortii* | Aug | RA |
| **2022** | 2 | 100 | 12 | 0 | 6 | 13 | 33 | $1.2 \times 10^4$ | *D. acuminata* | May | RA |

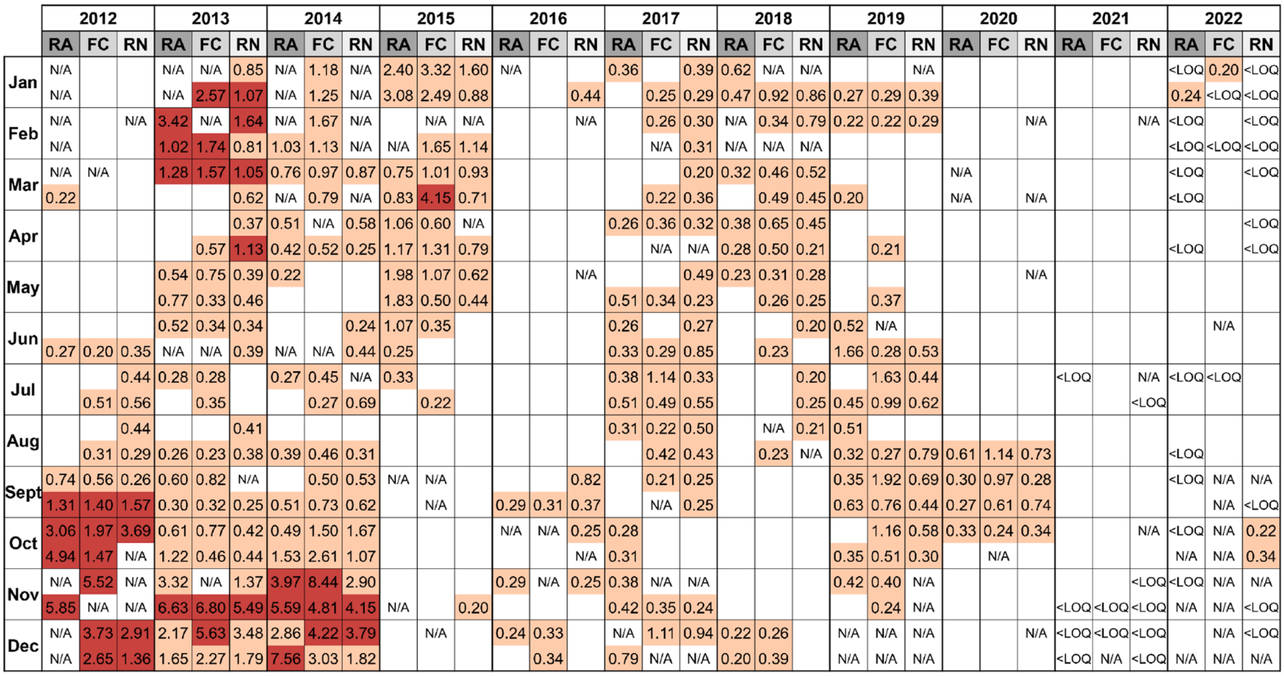
**Table 3**
Summary of the mussel samples exceeding the regulatory limit for STXs, the presence of potentially PSTs-producing species in seawater, and the maximum abundances of these species recorded from 2012 to 2022 along the Emilia-Romagna coast, with the corresponding month and sampling site.

| Years | N° of positive mussel samples (STXs) | N° of seawater samples with PSTs-producing species presence | | | | | PSTs-producing species | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *Alexandrium* spp. | *A. minutum* | *A. insuetum* | *A. ostenfeldii* | *A. taylorii* | Max abundance (cell $L^{-1}$) | Species | Month | Site |
| **2012** | 0 | 53 | 50 | 3 | 1 | 1 | $3.2 \times 10^3$ | *A. minutum* | May | FC |
| **2013** | 0 | 60 | 42 | 1 | 4 | 4 | $1.4 \times 10^3$ | *A. minutum* | May | FC |
| **2014** | 0 | 59 | 40 | 0 | 2 | 4 | $2.2 \times 10^3$ | *A. minutum* | May | FC |
| **2015** | 0 | 45 | 7 | 2 | 0 | 0 | $8.2 \times 10^3$ | *Alexandrium* spp. | Jun | RA |
| **2016** | 0 | 94 | 43 | 21 | 0 | 0 | $3.0 \times 10^3$ | *Alexandrium* spp. | Feb | FC |
| **2017** | 0 | 69 | 56 | 5 | 0 | 0 | $5.0 \times 10^3$ | *A. minutum* | Apr | RA |
| **2018** | 0 | 49 | 42 | 2 | 4 | 0 | $7.2 \times 10^2$ | *A. ostenfeldii* | Jan | RA |
| **2019** | 0 | 70 | 63 | 9 | 10 | 2 | $1.0 \times 10^4$ | *A. minutum* | Jun | RA |
| **2020** | 0 | 85 | 75 | 3 | 0 | 0 | $3.6 \times 10^3$ | *A. minutum* | Apr | RA |
| **2021** | 0 | 86 | 62 | 1 | 18 | 0 | $7.4 \times 10^2$ | *Alexandrium* spp. | Jun | RN |
| **2022** | 0 | 50 | 42 | 1 | 2 | 0 | $7.2 \times 10^2$ | *A. minutum* | May | FC |

toxin concentrations and producing species abundances was evident, with higher values recorded in the northern sites compared to the southernmost site, i.e. RA > RN (Fig. 3b,c) This evidence was confirmed by Jonckheere-Terpstra test that revealed a statistically significant monotonic decrease along the north-south axis for the two main YTXs analogues (homo-YTX, 45OH homo-YTX), and for *P. reticulatum* ($p < 0.05$).

Multivariate correlation analyses further confirmed significant positive associations between *G. spinifera* and YTX analogues ($\tau = 0.11, p = 1.2 \times 10^{-5}$ for YTX; $\tau = 0.10, p = 9.1 \times 10^{-5}$ for homo-YTX; $\tau = 0.09, p = 3.9 \times 10^{-4}$ for 45OH homo-YTX), as well as between *L. polyedra* and homo-YTX ($\tau = 0.09, p = 2.1 \times 10^{-4}$) and 45OH homo-YTX ($\tau = 0.06, p = 1.9 \times 10^{-2}$). In contrast, *P. reticulatum* exhibited negative correlations with all analogues ($p < 0.05$ for homo-YTX and 45OH homo-YTX).

In line with these findings, the highest YTXs concentration detected in mussels between 2012 and 2015 (Fig. S1–8), which was recorded in November 2014 at the FC site, corresponded to a prevalence of *G. spinifera* (2000 cell $L^{-1}$) and *L. polyedra* (120 cell $L^{-1}$). Moreover, two



| | 2012 | | | 2013 | | | 2014 | | | 2015 | | | 2016 | | | 2017 | | | 2018 | | | 2019 | | | 2020 | | | 2021 | | | 2022 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN |
| Jan | N/A | | | N/A | N/A | 0.85 | N/A | 1.18 | N/A | 2.40 | 3.32 | 1.60 | N/A | | | 0.36 | | 0.39 | 0.62 | N/A | N/A | | | N/A | | | | | | | <LOQ | 0.20 | <LOQ |
| | N/A | | | N/A | 2.57 | 1.07 | N/A | 1.25 | N/A | 3.08 | 2.49 | 0.88 | | | 0.44 | | 0.25 | 0.29 | 0.47 | 0.92 | 0.86 | 0.27 | 0.29 | 0.39 | | | | | | | 0.24 | <LOQ | <LOQ |
| Feb | N/A | | N/A | 3.42 | N/A | 1.64 | N/A | 1.67 | N/A | | N/A | N/A | | | N/A | | 0.26 | 0.30 | N/A | 0.34 | 0.79 | 0.22 | 0.22 | 0.29 | | | N/A | | | N/A | <LOQ | | <LOQ |
| | N/A | | | 1.02 | 1.74 | 0.81 | 1.03 | 1.13 | N/A | N/A | 1.65 | 1.14 | | | | | N/A | 0.31 | N/A | N/A | N/A | | | | | | | | | | <LOQ | <LOQ | <LOQ |
| Mar | N/A | N/A | | 1.28 | 1.57 | 1.05 | 0.76 | 0.97 | 0.87 | 0.75 | 1.01 | 0.93 | | | | | | 0.20 | 0.32 | 0.46 | 0.52 | | | | N/A | | | | | | <LOQ | | <LOQ |
| | 0.22 | | | | | 0.62 | N/A | 0.79 | N/A | 0.83 | 4.15 | 0.71 | | | | | 0.22 | 0.36 | | 0.49 | 0.45 | 0.20 | | | N/A | | N/A | | | | <LOQ | | |
| Apr | | | | | | 0.37 | 0.51 | N/A | 0.58 | 1.06 | 0.60 | N/A | | | | 0.26 | 0.36 | 0.32 | 0.38 | 0.65 | 0.45 | | | | | | | | | | | | <LOQ |
| | | | | | 0.57 | 1.13 | 0.42 | 0.52 | 0.25 | 1.17 | 1.31 | 0.79 | | | | | N/A | N/A | 0.28 | 0.50 | 0.21 | | 0.21 | | | | | | | | <LOQ | | <LOQ |
| May | | | | 0.54 | 0.75 | 0.39 | 0.22 | | | 1.98 | 1.07 | 0.62 | | | N/A | | | 0.49 | 0.23 | 0.31 | 0.28 | | | | | | N/A | | | | | | |
| | | | | 0.77 | 0.33 | 0.46 | | | | 1.83 | 0.50 | 0.44 | | | | 0.51 | 0.34 | 0.23 | | 0.26 | 0.25 | | 0.37 | | | | | | | | | | |
| Jun | | | | 0.52 | 0.34 | 0.34 | | | 0.24 | 1.07 | 0.35 | | | | | 0.26 | | 0.27 | | | 0.20 | 0.52 | N/A | | | | | | | | | N/A | |
| | 0.27 | 0.20 | 0.35 | N/A | N/A | 0.39 | N/A | N/A | 0.44 | 0.25 | | | | | | 0.33 | 0.29 | 0.85 | | 0.23 | | 1.66 | 0.28 | 0.53 | | | | | | | | | |
| Jul | | | 0.44 | 0.28 | 0.28 | | 0.27 | 0.45 | N/A | 0.33 | | | | | | 0.38 | 1.14 | 0.33 | | | 0.20 | | 1.63 | 0.44 | | | | <LOQ | | N/A | <LOQ | <LOQ | |
| | | 0.51 | 0.56 | | 0.35 | | | 0.27 | 0.69 | | 0.22 | | | | | 0.51 | 0.49 | 0.55 | | | 0.25 | 0.45 | 0.99 | 0.62 | | | | | | <LOQ | | | |
| Aug | | | 0.44 | | | 0.41 | | | | | | | | | | 0.31 | 0.22 | 0.50 | | N/A | 0.21 | 0.51 | | | | | | | | | | | |
| | | 0.31 | 0.29 | 0.26 | 0.23 | 0.38 | 0.39 | 0.46 | 0.31 | | | | | | | | 0.42 | 0.43 | | 0.23 | N/A | 0.32 | 0.27 | 0.79 | 0.61 | 1.14 | 0.73 | | | | <LOQ | | |
| Sept | 0.74 | 0.56 | 0.26 | 0.60 | 0.82 | N/A | | 0.50 | 0.53 | N/A | N/A | | | | 0.82 | | 0.21 | 0.25 | | | | 0.35 | 1.92 | 0.69 | 0.30 | 0.97 | 0.28 | | | | <LOQ | N/A | N/A |
| | 1.31 | 1.40 | 1.57 | 0.30 | 0.32 | 0.25 | 0.51 | 0.73 | 0.62 | | N/A | | 0.29 | 0.31 | 0.37 | | N/A | 0.25 | | | | 0.63 | 0.76 | 0.44 | 0.27 | 0.61 | 0.74 | | | | | N/A | <LOQ |
| Oct | 3.06 | 1.97 | 3.69 | 0.61 | 0.77 | 0.42 | 0.49 | 1.50 | 1.67 | | | | N/A | N/A | 0.25 | 0.28 | | | | | | | 1.16 | 0.58 | 0.33 | 0.24 | 0.34 | | | N/A | <LOQ | N/A | 0.22 |
| | 4.94 | 1.47 | N/A | 1.22 | 0.46 | 0.44 | 1.53 | 2.61 | 1.07 | | | | | | N/A | 0.31 | | | | | | 0.35 | 0.51 | 0.30 | | N/A | | | | | N/A | N/A | 0.34 |
| Nov | N/A | 5.52 | N/A | 3.32 | N/A | 1.37 | 3.97 | 8.44 | 2.90 | | | | 0.29 | N/A | 0.25 | 0.38 | N/A | N/A | | | | 0.42 | 0.40 | N/A | | | | | | <LOQ | <LOQ | N/A | N/A |
| | 5.85 | N/A | N/A | 6.63 | 6.80 | 5.49 | 5.59 | 4.81 | 4.15 | N/A | | 0.20 | | | | 0.42 | 0.35 | 0.24 | | | | | 0.24 | N/A | | | | <LOQ | <LOQ | <LOQ | N/A | N/A | <LOQ |
| Dec | N/A | 3.73 | 2.91 | 2.17 | 5.63 | 3.48 | 2.86 | 4.22 | 3.79 | | N/A | | 0.24 | 0.33 | | N/A | 1.11 | 0.94 | 0.22 | 0.26 | | N/A | N/A | N/A | | | N/A | <LOQ | <LOQ | <LOQ | | N/A | <LOQ |
| | N/A | 2.65 | 1.36 | 1.65 | 2.27 | 1.79 | 7.56 | 3.03 | 1.82 | | | | | 0.34 | | 0.79 | N/A | N/A | 0.20 | 0.39 | | N/A | N/A | N/A | | | | <LOQ | N/A | <LOQ | N/A | N/A | N/A |

**Fig. 2.** Concentration of YTXs in mussels (mg kg$^{-1}$) from the investigated production areas (RA, FC, RN) between 2012 and 2022. For each two-week period, the highest recorded value was selected. “N/A” indicates missing data, while empty cells denote YTXs concentrations below the limit of detection (<LOD). “<LOQ” indicates YTXs presence below the limit of quantification. Light red cells represent YTXs concentrations between the LOQ and the regulatory limit, which was 1.00 mg kg$^{-1}$ until August 2013 and increased to 3.75 mg kg$^{-1}$ thereafter. Red cells indicate YTXs levels equal to or exceeding the regulatory limit.

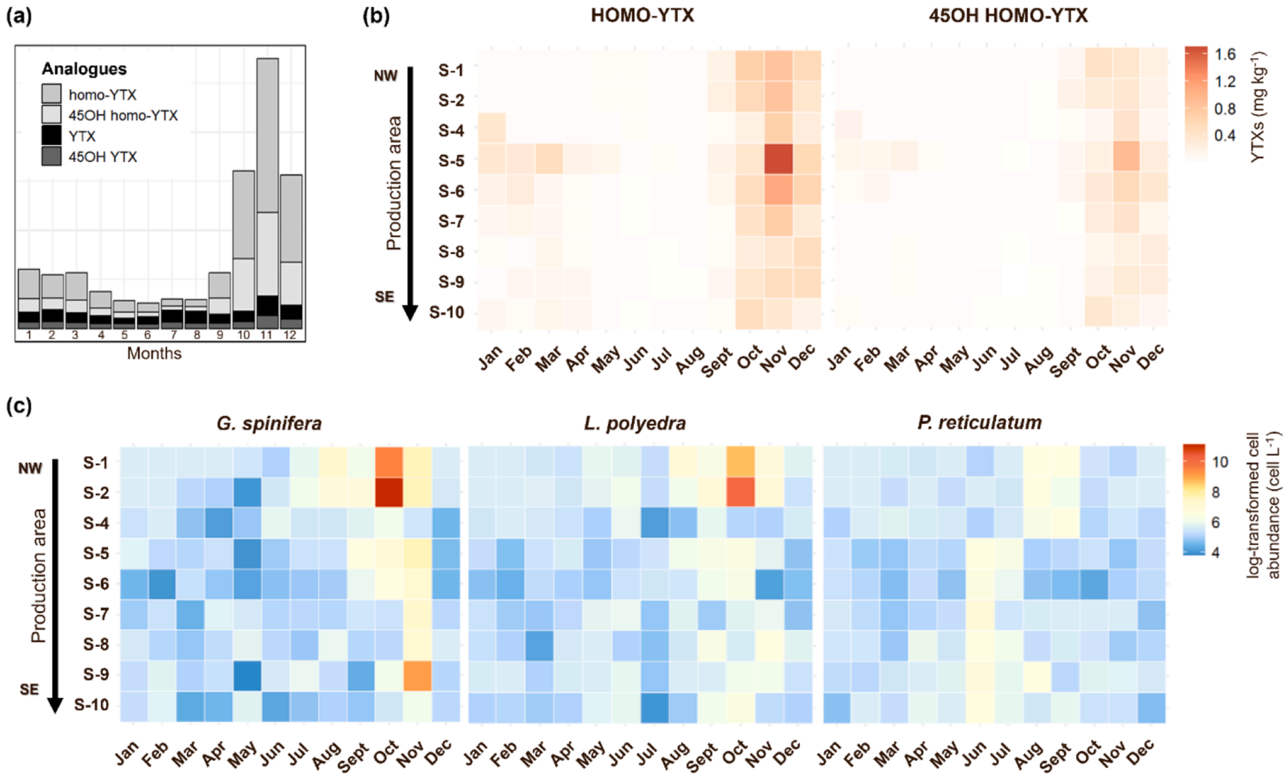


**Fig. 3.** (a) Bar plot showing the average YTXs content in mussels from the investigated production sites across the months, highlighting the relative composition of analogues between 2012 and 2015. (b) Heat map illustrating the average homo-YTX and 45OH homo-YTX concentration across production sites and months. (c) Heat map representing the average log-transformed abundance of potentially YTXs-producing phytoplankton species across production sites and months.

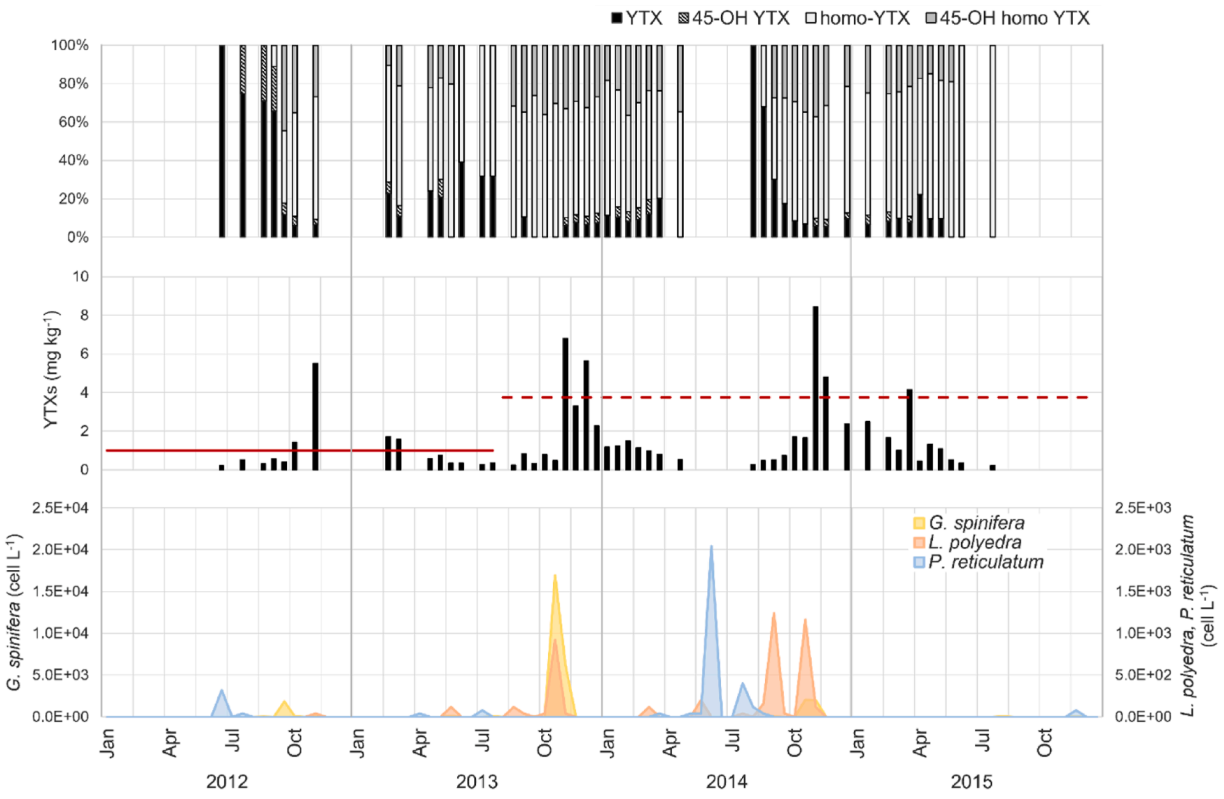
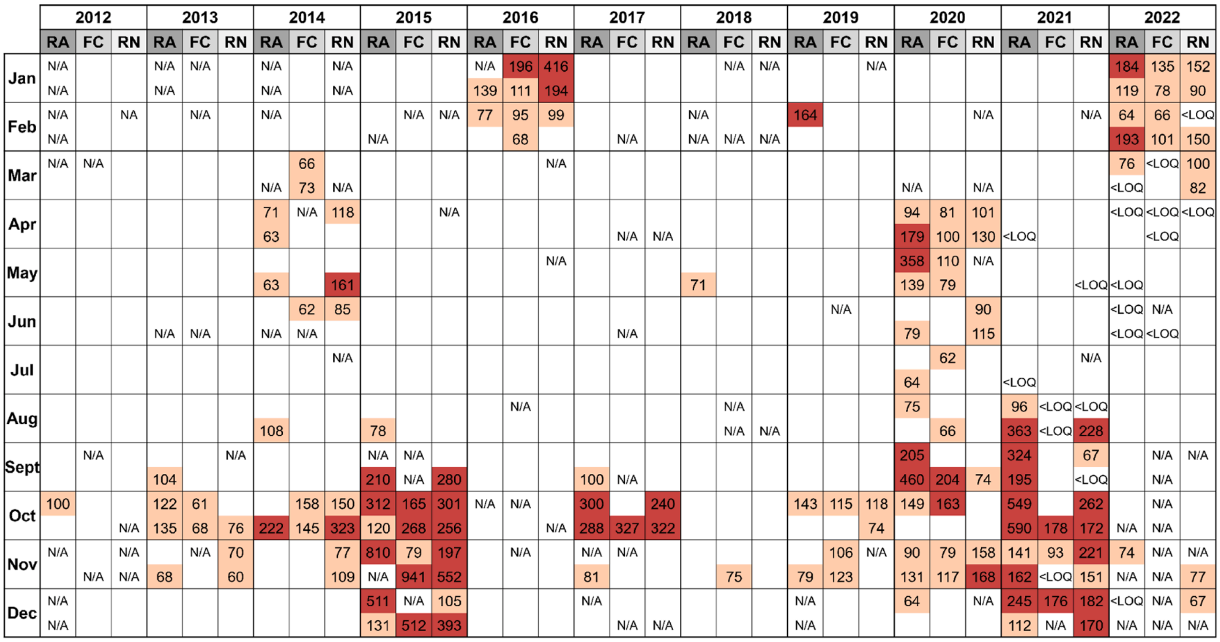


**Fig. 4.** Trends in YTXs concentration in mussels, relative composition of analogues, and cell counts of potentially YTXs-producing phytoplankton species in seawater from 2012 to 2015 at production site S-5 in the FC area. The solid red line in the second graph represents the regulatory limit for YTXs of 1.00 mg kg$^{-1}$ as defined by Reg. (EC) 853/2004, while the dashed red line represents the updated regulatory limit of 3.75 mg kg$^{-1}$ as defined by Reg. (EC) 786/2013.

weeks earlier, the same species had been recorded at densities of 2040 and 1160 cell L$^{-1}$, respectively, further highlighting the general delay observed between phytoplankton occurrence in seawater and toxins detected in bivalves. The mussel sample exceeding the regulatory limit contained a total YTXs concentration of 8.44 mg kg$^{-1}$, with homo-YTX (3.01 mg kg$^{-1}$) and 45OH homo-YTX (2.11 mg kg$^{-1}$) being the predominant analogues (Fig. 4).



| | 2012 | | | 2013 | | | 2014 | | | 2015 | | | 2016 | | | 2017 | | | 2018 | | | 2019 | | | 2020 | | | 2021 | | | 2022 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN | RA | FC | RN |
| Jan | N/A | | | N/A | N/A | | N/A | | N/A | | | | N/A | 196 | 416 | | | | | N/A | N/A | | | N/A | | | | | | | 184 | 135 | 152 |
| | N/A | | | N/A | | | N/A | | N/A | | | | 139 | 111 | 194 | | | | | | | | | | | | | | | | 119 | 78 | 90 |
| Feb | N/A | | NA | | N/A | | N/A | | | | N/A | N/A | 77 | 95 | 99 | | | | N/A | | | 164 | | | | | N/A | | | N/A | 64 | 66 | <LOQ |
| | N/A | | | | | | | | | N/A | | | | 68 | | | N/A | | N/A | N/A | N/A | | | | | | | | | | 193 | 101 | 150 |
| Mar | N/A | N/A | | | | | | 66 | | | | | | | N/A | | | | | | | | | | | | | | | | 76 | <LOQ | 100 |
| | | | | | | | N/A | 73 | N/A | | | | | | | | | | | | | | | | N/A | | N/A | | | | <LOQ | | 82 |
| Apr | | | | | | | 71 | N/A | 118 | | | N/A | | | | | | | | | | | | | 94 | 81 | 101 | | | | <LOQ | <LOQ | <LOQ |
| | | | | | | | 63 | | | | | | | | | | N/A | N/A | | | | | | | 179 | 100 | 130 | <LOQ | | | | <LOQ | |
| May | | | | | | | | | | | | | | | N/A | | | | | | | | | | 358 | 110 | N/A | | | | | | |
| | | | | | | | 63 | | 161 | | | | | | | | | | 71 | | | | | | 139 | 79 | | | | <LOQ | <LOQ | | |
| Jun | | | | | | | | 62 | 85 | | | | | | | | | | | | | | N/A | | | | 90 | | | | <LOQ | N/A | |
| | | | | N/A | N/A | | N/A | N/A | | | | | | | | | N/A | | | | | | | | 79 | | 115 | | | | <LOQ | <LOQ | |
| Jul | | | | | | | | | N/A | | | | | | | | | | | | | | | | | 62 | | | | N/A | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | | 64 | | | <LOQ | | | | | |
| Aug | | | | | | | | | | | | | | N/A | | | | | | N/A | | | | | 75 | | | 96 | <LOQ | <LOQ | | | |
| | | | | | | | 108 | | | 78 | | | | | | | | | | N/A | N/A | | | | | 66 | | 363 | <LOQ | 228 | | | |
| Sept | | N/A | | | | N/A | | | | N/A | N/A | | | | | | | | | | | | | | 205 | | | 324 | | 67 | | N/A | N/A |
| | | | | 104 | | | | | | 210 | N/A | 280 | | | | 100 | N/A | | | | | | | | 460 | 204 | 74 | 195 | | <LOQ | | N/A | |
| Oct | 100 | | | 122 | 61 | | | 158 | 150 | 312 | 165 | 301 | N/A | N/A | | 300 | | 240 | | | | 143 | 115 | 118 | 149 | 163 | | 549 | | 262 | | N/A | |
| | | | N/A | 135 | 68 | 76 | 222 | 145 | 323 | 120 | 268 | 256 | | | N/A | 288 | 327 | 322 | | | | | | 74 | | | | 590 | 178 | 172 | N/A | N/A | |
| Nov | N/A | | N/A | | N/A | 70 | | | 77 | 810 | 79 | 197 | | N/A | | N/A | N/A | | | | | | 106 | N/A | 90 | 79 | 158 | 141 | 93 | 221 | 74 | N/A | N/A |
| | | N/A | N/A | 68 | | 60 | | | 109 | N/A | 941 | 552 | | | | 81 | | | | 75 | | 79 | 123 | | 131 | 117 | 168 | 162 | <LOQ | 151 | N/A | N/A | 77 |
| Dec | N/A | | | | | | | | | 511 | N/A | 105 | | | | N/A | | | | | | N/A | | | 64 | | N/A | 245 | 176 | 182 | <LOQ | N/A | 67 |
| | N/A | | | | | | | | | 131 | 512 | 393 | | | | | N/A | N/A | | | | N/A | | | | | | 112 | N/A | 170 | N/A | N/A | N/A |

**Fig. 5.** Concentration of OAs in mussels (μg kg$^{-1}$) from the investigated production areas (RA, FC, RN) between 2012 and 2022. For each two-week period, the highest recorded value was selected. "N/A" indicates missing data, while empty cells indicate OAs concentrations below the limit of detection (<LOD). "<LOQ" denotes OAs presence below the limit of quantification. Light red cells represent OAs concentrations between the LOQ and the regulatory limit of 160 μg kg$^{-1}$, while red cells indicate OAs levels equal to or exceeding the regulatory limit.

### 3.2. Okadaic acid, derivatives and producing species

During the period from 2012 to 2022, several harvesting closures occurred along the investigated area due to the accumulation of OAs in mussels. A total of 68 samples exceeded the regulatory limit of 160 µg kg$^{-1}$, with instances recorded in all years except 2012, 2013, and 2018. The algal species potentially responsible for producing these toxins were detected every year, except for *D. ovum*, which was the least abundant among the analysed samples. Conversely, *D. acuminata* was the most frequently detected species (Table 2).

A seasonal pattern emerged, with OAs accumulation in mussels typically beginning in late August or early September and peaking in autumn and/or winter. This trend was consistent in 2014, 2015 (extending into 2016), 2017, 2019, 2020, and 2021 (extending into 2022). Notable exceptions were observed in 2014 and 2020, when closures occurred during spring (April and May) rather than in the expected autumn period (Fig. 5). The PERMANOVA analysis confirmed this temporal variability, with differences detected across months ($R^2 = 0.050, F = 16.84, p = 0.01$) and years ($R^2 = 0.042, F = 15.80, p = 0.01$). Post-hoc analyses revealed that October and November formed a distinct group characterised by elevated OAs levels in mussels, while September, December, and January exhibited intermediate OAs levels compared to other months (Fig. 6a). An apparent data cluster from 2019 to 2022 was also observed, reinforced by the results of the Mann-Kendall test, that indicated a slight but statistically significant increasing trend in OAs concentrations over the observed period ($\tau = 0.07, p = 2.1 \times 10^{-6}$).

The analysis of potentially OAs-producing phytoplankton species also demonstrated significant effects related to both seasonality and the sampling year (PERMANOVA, Month: $R^2 = 0.016, F = 4.35, p = 0.01$; Year: $R^2 = 0.065, F = 19.64, p = 0.01$). Post-hoc analyses once again revealed the clustering of data from 2019 to 2022 and highlighted compositional and abundance differences in the phytoplankton community of October and November compared to other months. Spring and summer months (May, June, and July) also exhibited significant differences, likely driven by the presence of species reaching high cell densities without being directly associated with toxin production. The SIMPER analysis indicated that the differences between the warmer months and the rest of the year were primarily driven by *D. sacculus* and *D. acuminata,* which exceeded $10^3$ cell L$^{-1}$ in late spring and summer (Table 2). Conversely, *D. caudata* appeared to be more closely associated with the late summer to autumn transition, particularly in October and November, when it co-occurred with *D. fortii* and *P. rotundatum* (Fig. 6b). These latter species, despite being very representative of the autumn period, never reached high cell densities, with a maximum of 1200 cell L$^{-1}$ reached by *D. fortii* in October 2014 in the RA area (Fig. S9). The Mann-Kendall test applied to phytoplankton species data revealed a slight but significant declining trend over time for most species ($-0.207 > \tau > -0.122, p \leq 2.2 \times 10^{-16}$), except for *D. acuminata*, which exhibited a slight but non-significant increasing trend ($\tau = 0.024, p = 0.09$).

To further investigate these relationships, a multivariate correlation

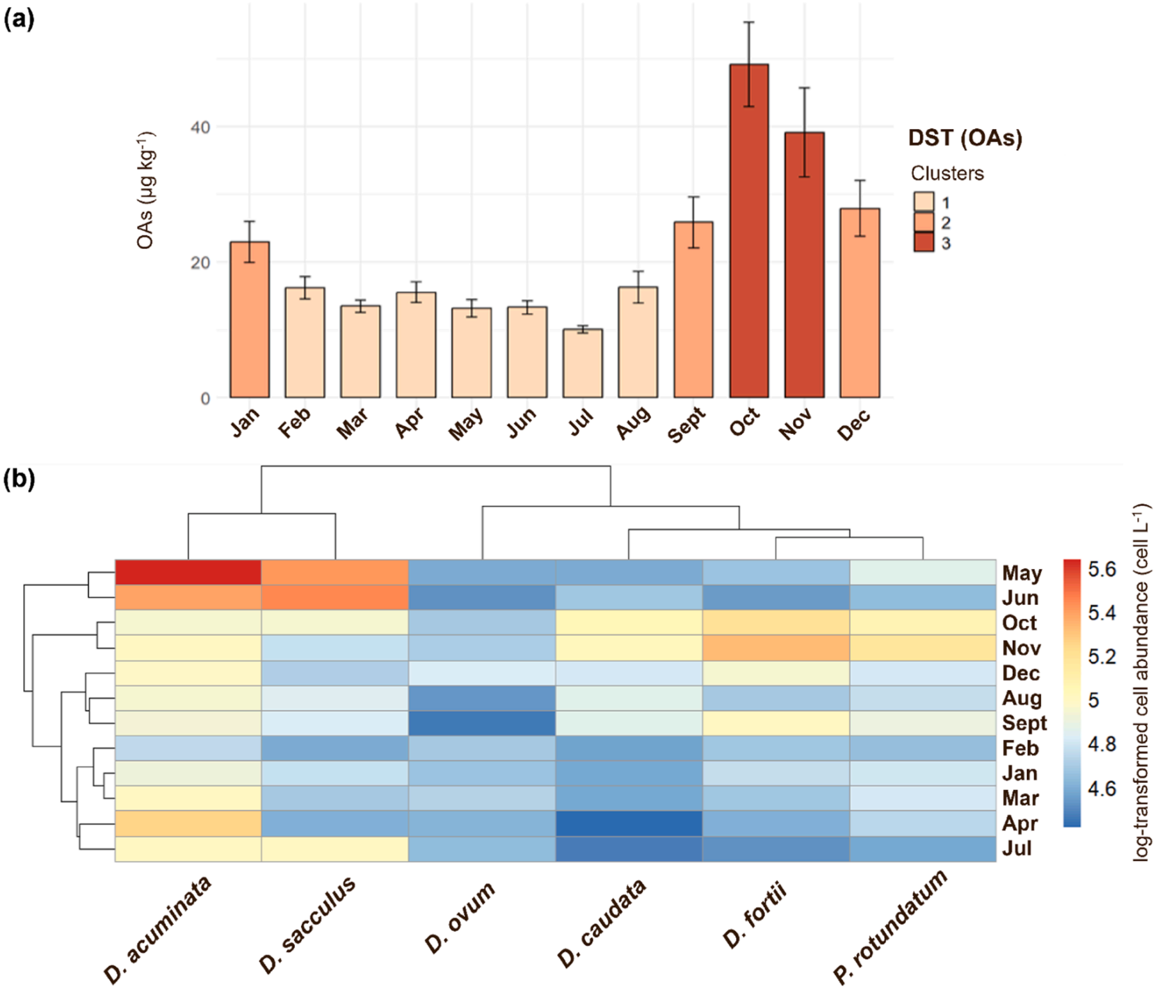


**Fig. 6.** (a) Bar plot showing the average OAs content in mussels from the investigated production sites across different months. Colours represent OAs concentrations, with lighter red indicating lower levels and darker red indicating higher levels. (b) Clustered heat map displaying the average log-transformed abundance of all potentially OAs-producing phytoplankton species over the months.

analysis was performed between OAs levels in mussels and phytoplankton abundances in seawater, which confirmed a weak but statistically significant positive correlation with *D. fortii* ($\tau = 0.04$, $p = 0.03$) and a non-significant positive correlation with *D. acuminata* ($\tau = 0.02$, $p = 0.20$). All other species showed negative associations with OAs levels, with *D. sacculus* being the only species exhibiting a statistically significant negative correlation ($\tau = -0.05$, $p = 1 \times 10^{-4}$). Given that seasonality was found to be a strong driver in structuring the phytoplankton community and its association with OAs accumulation in mussels, *D. fortii, D. caudata*, and *P. rotundatum* appeared to be the primary species linked to OAs. However, *D. acuminata* may also play a role, as suggested by its occasional presence outside its typical seasonal window, despite its cell abundances never exceeding $10^3$ cell $L^{-1}$ (Fig. 7, S-9–17).

The highest recorded OAs concentration in mussels during the study period was 941 μg $kg^{-1}$, detected in November 2015 in the FC area, coinciding with seawater concentrations of 40 cell $L^{-1}$ for *D. fortii* and *D. caudata* (Fig. 7). On the same sampling day, another mussel production site within the same area recorded *D. acuminata* and *D. fortii* at 160 cell $L^{-1}$ each (Fig. S13). During the same two-week period, the RA area exhibited OAs levels of 810 μg $kg^{-1}$ in mussels, concurrent with 480 cell $L^{-1}$ of *D. acuminata* and 40 cell $L^{-1}$ of *D. fortii* in seawater (Fig. S12).

Notably, even in exceptional cases such as 2014 and 2020, when harvesting closures occurred in spring rather than autumn, the same species were detected in seawater. For instance, in April 2020, OAs levels of 179 μg $kg^{-1}$ in mussels sampled from the RA area coincided with seawater concentrations of 20 cell $L^{-1}$ for *D. fortii. D. acuminata* was also detected at nearby sites, at abundances of 120–140 cell $L^{-1}$ (Fig. S11).

### 3.3. Saxitoxins producing species

Although STXs were not detected in mussel samples from the investigated aquaculture sites during the study period, the potentially PSTs-producing species *Alexandrium* spp., *A. minutum*, and *A. ostenfeldii* were detected annually across all sites, along with *A. insuetum* and *A. taylorii*. Among these, *A. minutum* exhibited the highest abundances, particularly during the spring and early summer months of April, May, and June. The maximum recorded cell density was observed in June 2019 in the RA area and reached the 10040 cell $L^{-1}$ (Table 3). However, this phytoplankton group did not form large-scale blooms over the analysed decade.

PERMANOVA analysis of the transformed abundances of all potentially PSTs-producing species confirmed this seasonal pattern, revealing significant differences across months ($R^2 = 0.060$, $F = 17.94$, $p = 0.01$), years ($R^2 = 0.060$, $F = 19.91$, $p = 0.01$), and sites ($R^2 = 0.002$, $F = 3.30$, $p = 0.01$). Post-hoc comparisons further supported a consistent seasonal trend, with significant higher abundances in April, May, and June. This seasonal effect accounted for a substantial portion of the variability in the distribution of these dinoflagellates and exhibited significant interannual variability (PERMANOVA, Month x Year, $R^2 = 0.086$, $F = 2.58$, $p = 0.01$), suggesting that fluctuations in seasonal occurrence contributed to community dynamics. Further post-hoc analyses revealed a temporal shift, with the study period clustering into two distinct intervals: 2012–2018 and 2019–2022. Although the Mann-Kendall trend test did not detect a significant increasing or decreasing trend in the overall abundance of PSTs-producing species over the decade, a slight increasing tendency was observed for the unidentified species grouped under *Alexandrium* spp. ($\tau = 0.04$, $p = 0.001$). Despite statistically significant spatial differences among sites, these variations accounted minimally to the overall community variability. The northern (RA) and the southern (RN) sites exhibited lower abundances of this phytoplankton group compared to the central (FC) site.

SIMPER analysis identified *Alexandrium* spp. and *A. minutum* as the primary contributors to the observed spatio-temporal differences, as they consistently exhibited the highest abundances and were the most prevalent taxa across sites and years. Conversely, *A. insuetum* was detected in only 48 out of 2640 samples, exceeding $10^3$ cell $L^{-1}$ only once (April 2016, RN, 1480 cell $L^{-1}$) (Fig. 8). Similarly, *A. ostenfeldii* was recorded in 41 out of 2640 samples, surpassing $10^3$ cell $L^{-1}$ in only three

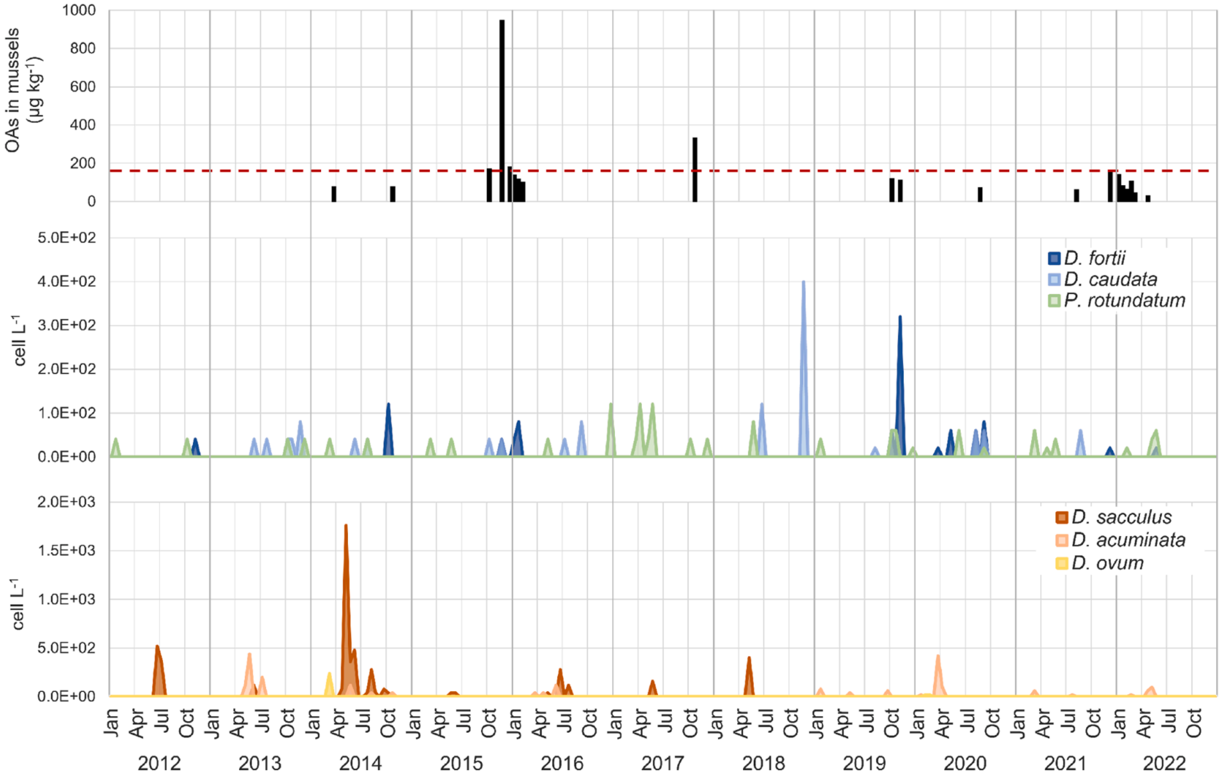


**Fig. 7.** Trends of OAs concentration in mussels and counts of potentially OAs-producing phytoplankton species in seawater from 2012 to 2022 at production site S-6 in FC area. The dashed red line in the first graph represents the regulatory limit for OAs as defined by Reg. (EC) 853/2004 (160 μg $kg^{-1}$).

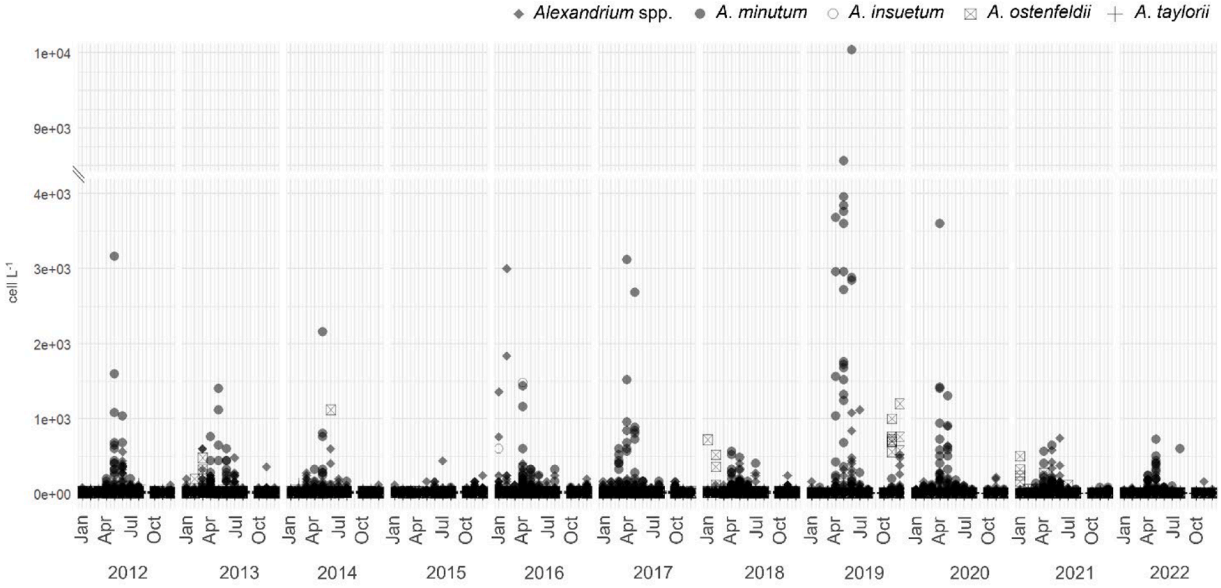


**Fig. 8.** Counts of potentially PSTs-producing phytoplankton species in seawater from 2012 to 2022 at all investigated production sites.

occasions: July 2014 in RN (1120 cell L$^{-1}$), November 2019 in RA (1000 cell L$^{-1}$), and December 2019 in FC (1200 cell L$^{-1}$) (Fig. 8). *A. taylorii* was detected in only 11 samples and never exceeded the $10^2$ cell L$^{-1}$, reaching a maximum abundance of 200 cell L$^{-1}$ in May 2019 in FC (Fig 8).

Given the low abundance of these three species and their limited contribution to the total community structure, SIMPER analysis revealed distinct seasonality patterns for *A. ostenfeldii, A. insuetum*, and *A. taylorii*, differing from the overall seasonal trend dominated by *Alexandrium* spp. and *A. minutum*. In particular, *A. ostenfeldii*, despite its occurrence in summer 2014, was predominantly associated with autumn and winter months over the study period.

### 3.4. Physicochemical parameters

The annual variations of the physicochemical parameters measured at the mussel production and harvesting sites in the Emilia-Romagna region coast are reported in Table S2. At the three selected ARPAE stations (i.e., 308, 314, 319), surface seawater temperature ranged from 2.95 to 29.47 °C, with the highest value recorded in August 2018 at RA and the lowest in February 2012 at RN. Minimum, maximum, and mean values exhibited a slight increase over the decade, despite the Mann-Kendall test indicating the absence of significant annual trends ($\tau = 4097$, $p = 0.58$). Conversely, a significant increasing trend was detected for salinity ($\tau = 70{,}577$, $p = 2.28 \times 10^{-21}$), which ranged from 8.23 to 38.57 throughout the study period. The highest salinity value was recorded in July 2022 at RN, whereas the lowest occurred in November 2014 at RA. This trend aligned with the overall temporal pattern of salinity, which exhibited marked fluctuations, with peaks in 2012, 2017, and 2022 and lower values in 2014 and 2016.

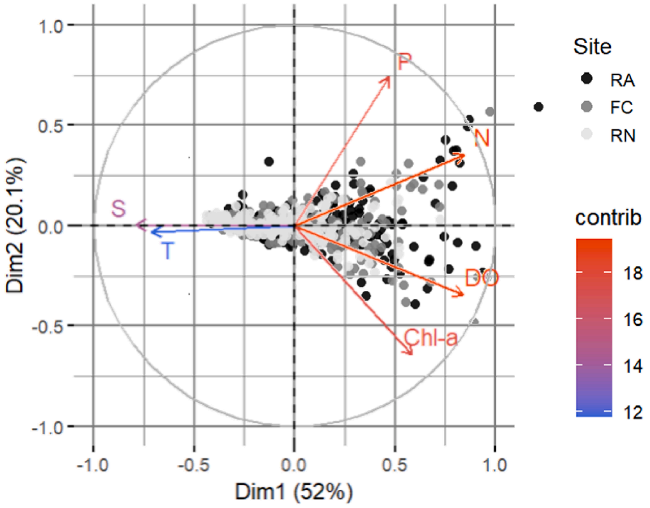


**Fig. 9.** Environmental characteristics of the coast of Emilia-Romagna region based on Principal Component Analysis (PCA). Factor map of individuals and variables for samples collected at different study sites, illustrating the projection of environmental variables onto two principal components. The colour and length of the vectors represent the variables' contribution to each component. Abbreviations. S = salinity, T = temperature, DO = dissolved oxygen, Chl-*a* = chlorophyll-*a,* N = nitrogen nutrients, P = phosphates.

For all other environmental variables, the Mann-Kendall analysis revealed decreasing trends over the decade. Dissolved oxygen concentrations ($\tau = -30{,}763$, $p = 3.52 \times 10^{-5}$) ranged from 3.56 mg $^{-1}$ (September 2016, RN) to 17.17 mg L$^{-1}$ (February 2021, FC), with no pronounced fluctuations. Similarly, chlorophyll-*a* concentrations ranged from 0.10 µg $^{-1}$ (September 2013, RN) to 54.40 µg $^{-1}$ (March 2015, FC). Phosphate concentrations ($\tau = -11{,}406$, $p = 0.12$) peaked at 27.8 µg $^{-1}$ in November 2014 at RA, but did not show significant annual variations. In contrast, nitrogenous nutrients ($\tau = -41{,}787$, $p = 1.91 \times 10^{-8}$), including the sum of nitrate, nitrite, and ammonium, exhibited pronounced fluctuations, with the lowest average values recorded in 2012, 2017, and 2022 and the highest in 2014 and 2018. The overall concentration of these compounds ranged from 0 to 1848 µg L$^{-1}$, with the maximum value recorded in March 2018 at FC.

The principal component analysis (PCA) of the complete environmental dataset (Fig. 9) demonstrated that temperature, salinity, dissolved oxygen, and nitrogenous nutrient concentrations collectively accounted for the most significant proportion of variability (52%), while phosphate and chlorophyll-*a* explained an additional 20%. The PCA ordination supported the findings of a post-hoc analysis, which identified the most pronounced spatial differences between the southernmost site (RN) and the two northern sites (FC: $R^2 = 0.016$, $F = 8.32$, $p = 0.001$; RA: $R^2 = 0.052$, $F = 28.37$, $p = 0.001$). The PCA results indicated that these differences were predominantly driven by lower phosphate and chlorophyll-*a* concentrations at RN. The post-hoc analysis also highlighted 2012, 2014, and 2022 as potentially key years in terms of environmental variability, as they frequently exhibited significant differences compared to other years. Furthermore, the years from 2018 to 2022 appeared to form a distinct cluster, displaying lower intragroup variability compared to the period preceding 2018.

### *3.5. Influence of the physicochemical parameters on the potentially toxin-producing dinoflagellates*

The PERMANOVA analysis of abundance data for all potentially toxin-producing phytoplankton species from the three selected sites based on the proximity with ARPAE stations (i.e., S-2, S-5, S-10) confirmed a strong seasonal effect on community composition (Month: $R^2 = 0.043$, $F = 3.65$, $p = 0.01$). Additionally, a significant interannual variability was revealed (Year: $R^2 = 0.084$, $F = 7.84$, $p = 0.01$), indicating that different assemblages of toxic, as well as non-toxic species, played a key role in influencing mussel toxicity throughout the study period. Among the analysed species, only *L. polyedra* exhibited a significant positive correlation with surface seawater chlorophyll-*a* concentrations ($\tau = 0.067$, $p = 0.007$), suggesting that toxin-producing species represented only a minor fraction of the phytoplankton community in the study area from 2012 to 2022, with non-toxic species being more prevalent.

SIMPER analysis identified *Alexandrium* spp., *A. minutum*, and *L. polyedra* as the primary contributors to pairwise dissimilarities across all years. Furthermore, the years 2012 and 2013 were predominantly characterised by blooms of *G. spinifera*, which, along with *D. sacculus*, also prominently characterised 2014. In contrast, the period from 2019 to 2022 was marked by a higher prevalence of *D. acuminata* compared to other species.

Canonical correspondence analysis (CCA) was conducted to explore the relationships between physicochemical parameters and the abundance of potentially toxic phytoplankton species across the study period. Although the constrained ordination explained a small proportion of the total variance (1.8%), the analysis revealed ecologically significant

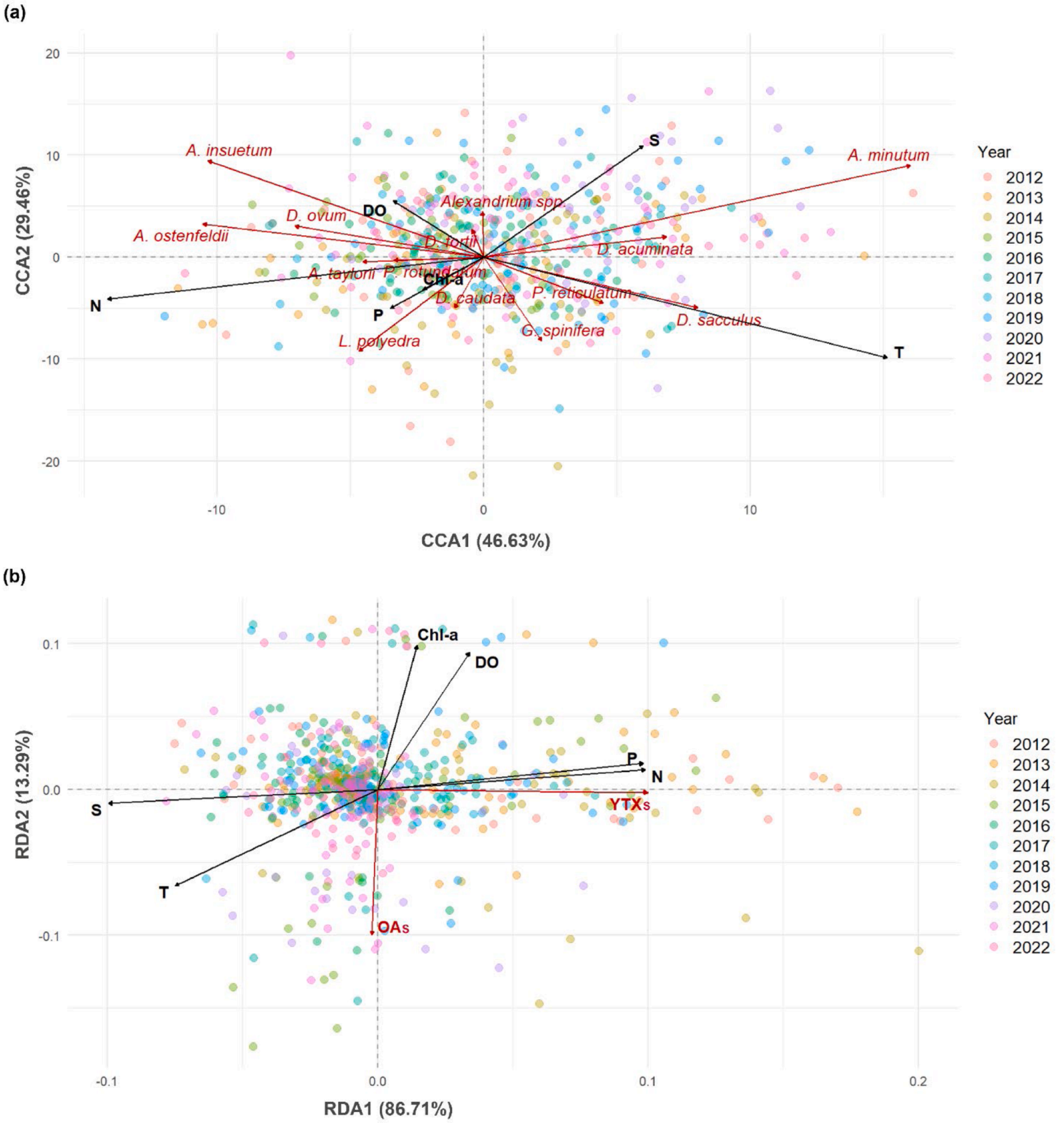


**Fig. 10.** (a) Canonical Correspondence Analysis (CCA) illustrating the relationships between potentially toxin-producing phytoplankton species and physicochemical parameters during the study period (2012–2022). (b) Redundancy Analysis (RDA) ordination diagram showing the relationships between biotoxin concentrations in mussels and physicochemical parameters over the same period.

patterns ($R^2 = 0.018$, $F = 2.40$, $p = 0.001$), particularly in relation to temporal trends and key environmental drivers. The biplot (Fig. 10a) showed that *A. minutum* and *D. acuminata* were associated with the most recent years, characterised by higher temperatures and salinity levels, suggesting that these parameters may have contributed to the increasing dominance of these species in recent years. Conversely, *G. spinifera* and *L. polyedra* were closely aligned with the years from 2012 to 2015, coinciding with harvesting closures due to the presence of YTXs in mussels from the study area. Notably, *L. polyedra* was associated with elevated phosphate concentrations and surface seawater chlorophyll-*a*, as well as low temperatures and salinity levels. Conversely, the other YTXs-producing species, *P. reticulatum*, together with *D. sacculus*, were associated with high temperatures, while *A. insuetum, A. ostenfeldii, A. taylorii*, and *D. ovum* exhibited relationships with high nitrogenous nutrients' concentrations and dissolved oxygen levels.

Redundancy analysis (RDA) was performed to investigate the relationship between environmental variables and the accumulation of phycotoxins in mussels. Environmental variables explained 2.8% of the total variance in toxin concentrations ($R^2 = 0.026$, $F = 3.43$, $p = 0.001$), with the first axis accounting for 86.7% of the constrained variance (Fig. 10b). This primary temporal gradient associated YTXs presence in mussels with increased nutrient concentrations (nitrogen and phosphorus) during the 2012–2015 period. In contrast, salinity was oriented in the opposite direction, aligning with more recent years (2019–2022), when elevated salinity levels were associated with the presence of *D. acuminata* and *A. minutum*. Along the second axis, OAs were linked to the years 2015, 2016, 2017, 2020, and 2021, all marked by DSTs-related harvesting closures, while chlorophyll-*a* and dissolved oxygen were oriented in the opposite direction.

## 4. Discussion

Since 1989, phycotoxins-contaminated shellfish have posed a serious issue in the Emilia-Romagna coastal region (NW Adriatic Sea, Italy) primarily due to lipophilic toxins, whose concentrations exceeding regulatory limits have led to prolonged closures of local shellfish farms (Pistocchi et al., 2012). Given the coexistence of multiple species in marine environments, laboratory studies employing monospecific algal cultures helped to identify the responsible species, the various toxin analogues produced, and, in some cases, the environmental factors influencing toxin production, particularly for issues emerging after 1996 (reviewed in Pistocchi et al., 2012). Nevertheless, culture-based studies do not always accurately reflect natural conditions (Lakeman et al., 2009). This study is the first to leverage extensive field data on both phytoplankton and toxins collected during a 11-year monitoring of shellfish farming sites in this area. This long-term dataset enabled the comparison of the ecological strategies of the main toxin-producing species, revealing an alternating dominance of these species over time.

Data collection began in 2012, following the implementation of Regulation (EU) 15/2011, which replaced the mouse bioassay with LC-MS/MS for lipophilic toxin analysis (European Commission, 2011). This methodological shift provided more detailed and accurate data, including the better characterisation of toxin analogues.

Over the 11-year period analysed, shellfish harvesting closures were primarily attributed to YTXs and to OAs, confirming the alternating pattern previously observed between 1989 and 2011 along the coast of Emilia-Romagna region, where these two toxin groups never caused simultaneous closures. During this previous 22-year period, distinct phases were evident: from 1989 to 1994, OAs were the primary cause of harvesting closures, followed by YTXs from 1995 to 2009. A transitional phase ensued from late 2015 onward, ultimately leading to the re-emergence of OAs as the predominant toxin group (Pistocchi et al., 2012; Pompei et al., 2018). During the first part of the subsequent and studied decade (2012–2022), closures were predominantly due to YTXs, which caused harvesting bans of mussel production areas until 2015. The highest concentrations of these compounds in mussels were detected during autumn and early winter, with a predominance of homo-YTX and its hydroxylated form, which were strongly associated with *G. spinifera* and *L. polyedra*. Recently, a new YTXs-producing species, *G. montresoriae*, was genetically linked to strains isolated from the study area (Huang et al., 2025), previously misidentified as *G. spinifera* (one of the two strains in Riccardi et al., 2009). This new species was not included among *G. spinifera* counts reported in this study, as taxonomic differences were well established before 2012, and its toxin profile is characterised primarily by YTXs, thus leading to unambiguous distinction. The predominance of homo-YTX and 45-OH homo-YTX in mussels collected from Emilia-Romagna (NW Adriatic coastal area) aligned with findings from north-eastern Adriatic regions, such as Croatia, where similar toxin analogues were prevalent in shellfish, albeit not directly linked to *G. spinifera* (Gladan et al., 2010). Instead, *L. polyedra* was highlighted as a key species in these dynamics due to its ability to form high-density blooms and to have a seasonal overlap with YTXs occurrence, as also observed in the northern area of the Po River delta (Rubini et al., 2021). In the present study, focused on the southern area of the Po River delta, *L. polyedra* was strongly associated with both chlorophyll-*a* concentrations in surface waters, confirming its bloom-forming potential (Pompei et al., 2018), and the presence of homo-YTXs in mussels. Although the role of *L. polyedra* in YTXs production has been debated (Barbosa et al., 2024; Boni et al., 2001), studies conducted on cultured strains worldwide have demonstrated its ability to produce YTXs, particularly homo-YTXs (Tillmann et al., 2021), which is also produced by different *P. reticulatum* strains, as reported in several studies (Barbosa et al., 2024; Satake et al., 1999; Wang et al., 2019). Blooms of these species and high YTXs levels in mussels were positively correlated with elevated nutrient concentrations in surface water and low temperature and salinity values, conditions typically found in autumn and winter and linked to water column mixing and upwelling environments (Chikwililwa et al., 2019). Furthermore, these factors are associated with freshwater inputs rich in nutrients, such as those derived from the Po River, explaining the observed spatial trend of *G. spinifera, L. polyedra*, and (also) *P. reticulatum*, which attested a higher abundance in the northern areas closer to the river delta (S-1 and S-2) than the southern ones (Fig. 3). Temporally, the general decline in YTXs-producing species and toxin levels in mussels over the study period may be linked to rising temperatures and salinity, potentially favouring oligotrophic-associated species such as *Dinophysis* spp. Despite this decline, YTXs were detected at sub-regulatory levels in almost all sampling years, raising concerns about chronic exposure and its potential effects on human health, which remain poorly understood (Rubini et al., 2021).

After 2015, closures in the Emilia-Romagna shellfish farms located south of the Po River were exclusively due to OAs, mainly detected in autumn and winter, with occasional spring occurrences. No detectable levels of DTXs or PTXs were found in the analysed samples, except in rare cases at negligible concentrations (personal observations). The presence of OAs in mussels was mainly associated with *D. fortii* and *D. acuminata*, being the first species identified as a DSTs producer (Yasumoto et al., 1980), and *D. acuminata* a cosmopolitan species linked to DSP events worldwide (Reguera et al., 2014). Both species have been documented as primary DSTs producers in the Adriatic Sea, particularly *D. fortii* (Gladan et al., 2011; Henigman et al., 2024; Ravera et al., 2024; Viličić et al., 2009), along with *D. tripos*, which lacked sufficient records to be included in this study. The role of *D. caudata* and *P. rotundatum* remained controversial, as they frequently co-occurred with *D. fortii* but did not exhibit a significant correlation with OAs levels in mussels. This aligns with previous observations indicating that *D. caudata* is a potential DSTs producer although in concomitance with other species as it is primarily associated with PTXs (Fernández et al., 2006). Conversely, *P. rotundatum* may act as a toxin vector by preying on ciliates that have fed on toxic *Dinophysis* spp. (González-Gil et al., 2011).

Among the *Dinophysis* species detected, *D. sacculus* was the only one negatively correlated with OAs presence in mussels, contrasting with findings from other Mediterranean studies where it was strongly associated with DSTs production, particularly when co-occurring with *D. acuminata* (Belin et al., 2021). Indeed, *D. sacculus* is more typical of spring and high-temperature conditions, while *D. acuminata* has a broader blooming period extending from spring to autumn.

All these DSTs-producing species thrive under strong water column stratification, low precipitation, and nutrient-poor conditions (Giacobbe et al., 1995; Vlamis and Katikou, 2014), since they are mixotrophic species that rely on a three-link food chain (cryptophyte-ciliate-dinoflagellate). This would explain the rapid uptake of OAs by mussels during the studied period, which closely mirrored *Dinophysis* presence, in contrast to the delayed YTXs accumulation pattern observed. Notably, the increasing bloom frequency of *D. acuminata* over the years coincided with rising OAs levels in mussels, a trend linked to climate-driven increases in surface temperature and salinity, which may further heighten DSP risk in shellfish farms.

Other species linked to increasing temperature and salinity in surface waters are *A. minutum* and, to a lesser extent, *Alexandrium* spp., encompassing species that could not be identified beyond the genus level through microscopy. The genus *Alexandrium* poses considerable challenges to morphological identification, due to the presence of both toxic and non-toxic cryptic species (Zingone et al., 2021). *A. minutum* itself may be mistaken for other morphologically similar species, such as *Alexandrium tamutum*, a non-toxic species identified in the northern Adriatic (Montresor et al., 2004). *A. minutum* was mostly associated with spring and early summer, consistent with observations from other Adriatic areas (Henigman et al., 2024; Valbi et al., 2019) and the Jonian Sea (Pistocchi et al., 2022), but in contrast to records from other Mediterranean regions, such as the Gulf of Oristano (Sardinia, Italy), where it typically blooms during winter periods, often coinciding with high STXs levels in mussels (Bazzoni et al., 2020). In the Adriatic Sea, this species has rarely led to STXs accumulation in mussels, with the exception of a single case in May 1994 (Honsell et al., 1995). Despite causing multiple blooms, reaching densities of $10^3$–$10^4$ cells $L^{-1}$, no toxicity events occurred during the studied period. This discrepancy has been reported globally (Hallegraeff et al., 2021) and may be attributed to the intrinsic toxicity of *A. minutum* strains in the northern Adriatic, warranting further culture-based studies, as well as interactions with other toxic and non-toxic phytoplankton species influencing bivalve filtration and toxins accumulation. In this study, *A. ostenfeldii*, responsible for spirolides accumulation in mussels farmed in Emilia-Romagna in 2003 (Accoroni et al., 2024; Ciminiello et al., 2006), remained below concern levels, with occurrences linked to low salinity in winter periods (Martens et al., 2016). Although *A. insuetum* and *A. taylorii*, are not confirmed producers of PSTs, they are included among the *Alexandrium* species to be monitored, as the former may be misidentified as a toxic *Alexandrium* species, while the latter may be associated with an alternative toxic profile predominantly producing goniodomin A (GDA), an emerging toxin (Tillmann et al., 2020).

In general, this study confirmed the Emilia-Romagna region as a hotspot for toxic events, considering the significant impact that recurrent toxic events have had in the study area, such as the closure of shellfish farms. Among the Italian coastal areas, the Adriatic Sea is particularly prone to such occurrences, as the Emilia-Romagna region, in conjunction with the nearby Marche region, is characterised by toxic events caused by the presence of toxic phytoplankton (mainly DST- and YTX-producing species), with significant impacts on aquaculture and the coastal environment (Accoroni et al., 2024).

## 5. Conclusion

This 11-year study was the first to investigate the relationships between the presence and abundance of toxic dinoflagellate species and the occurrence of their associated phycotoxins in farmed mussels in the NW Adriatic Sea off the Emilia-Romagna region. By relying exclusively on a long-term dataset of field observations, the retrieved data were also correlated with the physicochemical parameters measured in the surface waters to highlight toxin-producing species ecological strategies. While several toxin-producing species had previously been isolated in this area and chemically analysed, these relationships had never been explored solely through extensive field data analysis.

During the study period from 2012 to 2022, the most prevalent toxins detected in mussels were YTXs, mainly homo-YTX and its hydroxylated form, as well as OAs. The presence of these compounds above regulatory limits exhibited an alternating pattern, with 2015 marking a transitional year.

The phytoplankton species identified as the primary producers were *G. spinifera* and *L. polyedra* for YTXs (specifically for homo-YTXs), and *D. acuminata* and *D. fortii* for OAs, both characterised by predominantly autumnal blooms. The shift in the presence of these species was driven by an increase in surface water temperature and salinity, leading to a reduction in water column mixing, and thus favouring oligotrophic and mixotrophic species such as those belonging to the genus *Dinophysis*.

The increase in *D. acuminata* and OAs in mussels, along with the persistent presence of low concentrations of YTXs despite the decline of their producers, highlights the necessity for continuous monitoring, especially in the context of climate change. Furthermore, the occurrence of *A. minutum* blooms without the accumulation of STXs in mussels reinforces the need for further research on strain toxicity and phytoplankton interactions to understand the dynamics of toxins production and accumulation in mussels.

## CRediT authorship contribution statement

**Giorgia Zoffoli:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Laura Pezzolesi:** Writing – review & editing, Supervision, Conceptualization. **Mara Simonazzi:** Writing – review & editing. **Franca Guerrini:** Writing – review & editing. **Silvana Vanucci:** Writing – review & editing. **Anna Calfapietra:** Writing – review & editing, Investigation. **Sonia Dall'Ara:** Writing – review & editing, Investigation. **Irene Servadei:** Writing – review & editing, Investigation. **Rossella Pistocchi:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## 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.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2025.102870.

## Data availability

Data will be made available on request.

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