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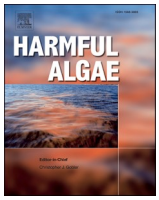

Okadaic acid as a major problem for the seafood safety (*Mytilus galloprovincialis*) and the dynamics of toxic phytoplankton in the Slovenian coastal sea (Gulf of Trieste, Adriatic Sea)

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ABSTRACT

This article presents the first results on shellfish toxicity in the Slovenian sea (Gulf of Trieste, Adriatic Sea) since the analytical methods for the detection of biotoxins (PSP, ASP, DSP and other lipophilic toxins) in bivalve molluscs were included in the national monitoring program in 2013. In addition to toxins, the composition and abundance of toxic phytoplankton and general environmental characteristics of the seawater (surface temperature and salinity) were also monitored.

During the 2014–2019 study period, only lipophilic toxins were detected (78 positive tests out of 446 runs), of which okadaic acid (OA) predominated in 97 % of cases, while dinophysistoxin-2 and yessotoxins only gave a positive result in one sampling event each. The number of samples that did not comply with the EC Regulation for the OA group was 17 or 3.8 % of all tests performed, all of which took place from September to November, while a few positive OA tests were also recorded in December, April, and May. This toxicity pattern was consistent with the occurrence pattern of the five most common DSP-producing dinoflagellates, which was supported by the development of warm and thermohaline stratified waters: *Dinophysis caudata, D. fortii, D. sacculus, D. tripos* and *Phalacroma rotundatum*. The strong correlation ($r = 0.611$, $p < 0.001$) between *D. fortii*, reaching abundances of up to 950 cells L^{-1}, and OA suggests that *D. fortii* is the main cause of OA production in Slovenian waters.

Strong interannual variations in OA and phytoplankton dynamics, exacerbated by the effects of anthropogenic impacts in this coastal ecosystem, reduce the predictability of toxicity events and require continuous and efficient monitoring. Our results also show that the introduction of the LC-MS/MS method for lipophilic toxins has improved the management of aquaculture activities, which was not as accurate based on mouse bioassays.

1. Introduction

Harmful algal blooms (HABs) are a natural phenomenon resulting from complex chemical, physical and biological interactions (Berdalet et al., 2016). They occur in almost all aquatic environments and cause negative impacts on ecosystems, coastal resources and human health. Of the several thousand microalgal species, about 200 are involved in the production of biotoxins (Lundholm et al., 2009 onwards). They accumulate in shellfish (and other seafood) and cause various symptoms in humans through the consumption of poisoned food. Toxic outbreaks usually occur after the increase of toxic phytoplankton in seawater, reaching densities from thousands (e.g. *Alexandrium* and *Karenia* spp.) to several million cells per liter (e.g. *Pseudo-nitzschia* spp.). On the other hand, even a few hundred cells per liter of dinoflagellates such as *Dinophysis* species can cause toxicity in shellfish.

Various aspects of HABs have been studied for decades, and the latest findings are summarized in the most recent Global HAB Status Report (Hallegraeff et al., 2021). Of greater relevance to our region is the overview of HABs in the Mediterranean, where about three quarters of toxic events are diarrhetic shellfish poisoning (DSP) (Zingone et al.,

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2021). In humans, DSP syndrome is caused by the lipophilic okadaic acid (OA) and its derivatives, the dinophysistoxins (DTXs), which are produced by several species of the genera *Dinophysis, Phalacroma* and *Prorocentrum*. In addition, there are other lipophilic toxins with dubious effects on humans, which in the past has often led to misinterpretations of the results of mouse bioassays and consequently to unnecessary bans on the sale of shellfish. For example, pectenotoxins (PTXs) produced by *Dinophysis* spp. (Draisci et al., 1996; Yasumoto et al., 1985) were deregulated in the European Union (EU) in 2021 with Regulation (EC) No 2021/1374 (European Commission, 2021) due to unproven adverse effects on humans (Boundy et al., 2020; EFSA, 2009a). The dinoflagellates *Lingulodinium polyedra, Protoceratium reticulatum* and *Gonyaulax spinifera*, which are also widespread in the Mediterranean and Adriatic (Zingone et al., 2021), produce yessotoxins (YTXs) and, like PTXs, have no harmful effects on humans (EFSA, 2009b; Tubaro et al., 2010). The same applies to spirolides (SPXs) and gymnodimines (GYMs), for which there is no information on cases of poisoning in humans. The last group of lipophilic toxins are the azaspiracids (AZAs). The species responsible are dinoflagellates of the genera *Azadinium* and *Amphidoma*, which are often neglected or misidentified due to their small size but have been found in several Mediterranean regions (Zingone et al., 2021). The first report of AZAs in Mediterranean mussels actually comes from the Adriatic Sea (Bacchiocchi et al., 2015). The rest of toxic events in the Mediterranean are attributed to paralytic shellfish poisoning (PSP) caused by dinoflagellates of the genus *Alexandrium* and *Gymnodinium catenatum*, while only a very small proportion of events are due to amnesic shellfish poisoning (ASP) caused by about 15 toxic species of the genus *Pseudo-nitzschia* (Zingone et al., 2021).

The Adriatic Sea, home to 34 potentially toxic species (Mozetič et al., 2019), reflects the same preponderance of DSP events. Despite the regular occurrence and sporadic outbreaks of toxic species from different taxonomic groups, DSP toxins and other lipophilic toxins such as YTXs and PTXs are most frequently detected above the legal limits in Adriatic shellfish (e.g. Accoroni et al., 2023; Ninčević Gladan et al., 2011; Rubini et al., 2021), while ASP and PSP toxins pose only a low risk for the time being (Arapov et al., 2016; Ciminiello et al., 1995, 2005; Ujević et al., 2010, 2012).

In Slovenia, the monitoring of toxic and potentially toxic phytoplankton species (hereinafter referred to as toxic species) in seawater and the quantification of biotoxins in shellfish from aquaculture facilities and occasionally in wild shellfish in the Slovenian sea (Gulf of Trieste, northern Adriatic) is supervised by the Administration for Food Safety, Veterinary Sector, and Plant Protection (AFSVSPP). This authority sets the national sampling plans on an annual basis. Official monitoring of toxins and phytoplankton in shellfish farms that cultivate mussels (*Mytilus galloprovincialis*) was introduced in the early 1990s. Using the mouse bioassay (Yasumoto et al., 1978), which was considered the reference method in the EU at the time (Directive 91/492/EEC) (Council of the European Communities, 1991), excessive levels of an unspecified mixture of lipophilic toxins were regularly detected in Slovenian mussels (Francé et al., 2013).

This study, covering the period 2014–2019, provides the first results on chemically specified lipophilic toxins in mussels from the Slovenian sea, which have not yet been published, as the LC-MS/MS analytical method (Commission Regulation (EC) No 2074/2005) (European Commission, 2005) was only officially included in the national monitoring program in 2013. We hypothesize that the toxic phytoplankton species present in seawater and the patterns of their occurrence are reflected in the occurrence and concentration of toxins in mussels depending on some environmental factors. Possible outbreaks of other toxins that have not yet been detected in Slovenian mussels, but could occur in the future, are also discussed.

2. Materials and method

2.1. Phytoplankton monitoring

The official systematic control of compliance for bivalve molluscs is laid down in the annual monitoring program for the control of toxic phytoplankton and toxicity in bivalve molluscs. Monitoring of phytoplankton is carried out by the National Institute of Biology, Marine Biology Station Piran (NIB-MBP), and the results on the presence and abundance of toxic phytoplankton are reported to the competent authorities (AFSVSPP). The same applies to the monitoring of toxins in bivalve molluscs, which is carried out by the National Veterinary Institute (NVI).

Shellfish farms are in three enclosed bays in the southeastern part of the Gulf of Trieste, the shallowest and northernmost part of the Adriatic Sea (Fig. 1). Seawater samples for phytoplankton are usually collected monthly from December to March, twice a month in April, May, June, July, August, and November, and three times a month in September and October in shellfish farms although some variation in sampling schedule is to be expected. For the purposes of this study, the period considered was January 2014 through December 2019.

Water samples for toxic phytoplankton identification and enumeration were collected using PVC tubing, which allows the entire water column to be sampled in a few liters (Sutherland et al., 1992) and especially dinoflagellates not to be overlooked, as they often concentrate in thin layers. Water samples fixed with formalin (2 % neutralized final concentration in 1 L) were analyzed on an inverted microscope within a few days using the standard quantitative Utermöhl method (Utermöhl, 1958). For larger species (e.g., *Dinophysis* spp., *Lingulodinium polyedra*, large *Alexandrium* species), the entire chamber bottom was counted at 200 × magnification. Smaller and more abundant species (e.g., *Pseudo-nitzschia* spp., small *Alexandrium* species) were counted in 200 fields of the chamber bottom at 400 × magnification.

In addition to phytoplankton data, environmental variables such as seawater temperature and salinity were also considered as ecologically relevant data for phytoplankton dynamics. Surface temperature and salinity measured at the oceanographic buoy 'Vida' (Seabird SBE temperature and conductivity probes at 2.5 m depth) were obtained from the database NIB-MBP. Daily measurements were averaged weekly.

2.2. Official monitoring of lipophilic, PSP and ASP toxins in bivalve molluscs

The national monitoring program provides a different sampling plan for each group of toxins. For the detection of lipophilic toxins, sampling was once a month in January, February, March, and December and three times a month from April to November. For PSP toxins, samples were collected once a month in June-November and twice a month in April-May; and for ASP, mussels were collected once a month in April, May, and December, and twice a month from June to November. However, some variations in the sampling schedule were possible. Between January 2014 and December 2019, 446 samples were analyzed for lipophilic toxins, 216 for ASP, and 165 for PSP toxins. Each sample consisted of 0.5 kg of live mussels transported to the laboratory in a cold chain.

Mussels were sampled by the competent authority in all Slovenian shellfish farms. Samples were taken to the NVI, where the samples were tested for OA group (OA, DTX-1 and 2), PTX-1 and 2, YTX, 45-hydroxyyessotoxin (45-OH-YTX), homo-yessotoxin (homo-YTX), 45-hydroxyhomo-yessotoxin (45-OH-homo-YTX), AZA-1, 2 and 3, spirolide C (SPX-C), GYM, saxitoxin (STX), neosaxitoxin (NEO), decarbamoyl saxitoxin (dcSTX), gonyautoxins (GTX1–5), decarbamoyl gonyautoxin 2 and 3 (dcGTX2,3), N-sulfocarbamoyl-gonyautoxin 1 and 2 (C1,2) and domoic acid (DA).

Lipophilic toxins were determined by liquid chromatography (Waters Alliance 2695, Waters, MA, USA) with triple quadrupole mass

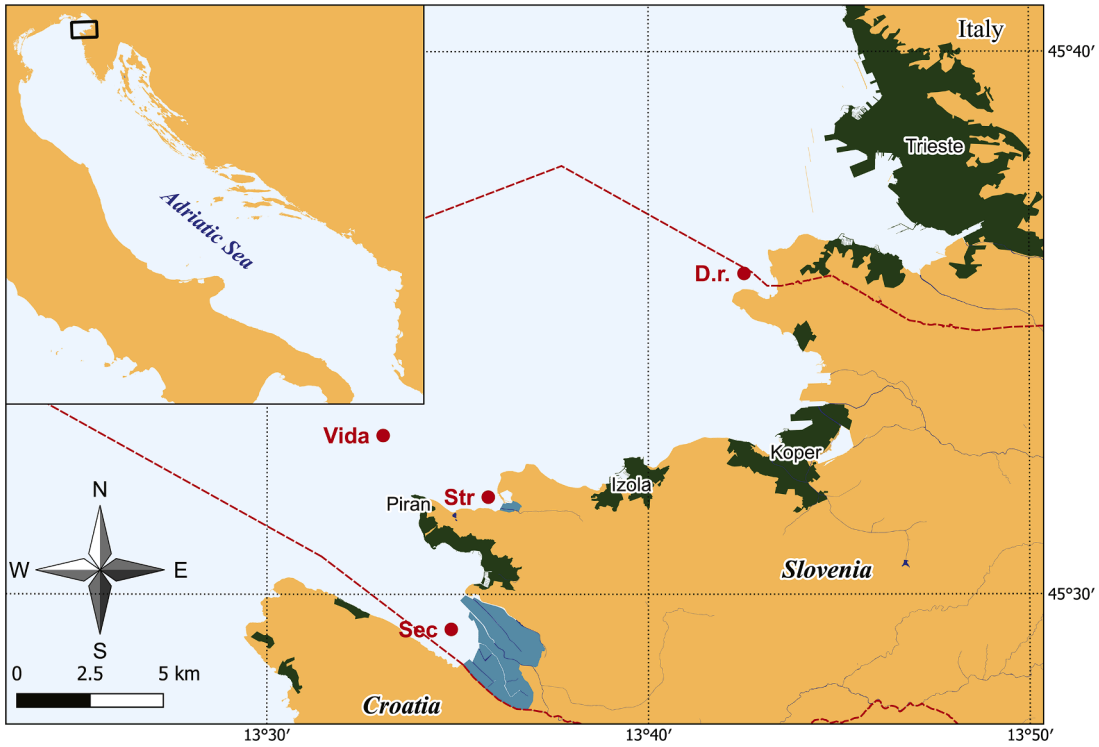

Fig. 1. Locations of the three mussel farms in three enclosed bays along the Slovenian coast (Gulf of Trieste, Adriatic Sea): Seča (Sec), Strunjan (Str) and Debeli rtič (D.r.). The location of the oceanographic buoy 'Vida' is also marked.

spectrometry (Quattro Micro API, Micromass, Waters, MA, USA) – LC-MS/MS, which is the reference method in the EU (European Commission, 2005).

Methanol, 4 mL, was added in a centrifuge tube containing 0.95–1.05 g of homogenized sample, vortexed and left in an ultrasonic bath for 5 min. The samples were centrifuged for 10 min at 3000 rpm. Supernatants were transferred in 10 mL measurement flasks. The extraction procedure was repeated once again. Combined supernatants were diluted with methanol to the mark of flasks. Aliquot, 5 mL, was transferred in the new centrifuge tube. The solvent was evaporated to dryness. The residues were dissolved in 1.0 mL of methanol and vortexed. Centrifuged solutions, 0.5 mL, were hydrolyzed with 62.5 µL 2.5 M sodium hydroxide at 76 °C for 40 min in a tightly sealed vial. 2.5 M hydrochloric acid, 62.5 µL, was used to stop the hydrolysis. Hydrolysis is required for the detection of DSP toxins, while this procedure does not interfere with the detection of other lipophilic toxins. Neutralized solutions were filtered through 13 mm 0.45 µm PTFE filters in the new vial. Matrix fortified calibration curve was prepared parallel with samples using fortified sample of blank matrices. DTX-1 and DTX-2 were monitored and quantified on the OA calibration curve.

The mobile phase consisted of A: 0.04 % ammonium hydroxide and B: 0.04 % ammonium hydroxide in 90 % acetonitrile at a flow rate of 0.3 mL min^{-1}. Mobile phase started at 80 % A with a linear gradient to 30 % A at 6 min, followed by a linear gradient to 80 % A from 8.0 to 8.5 min with a total run time of 14 min. Separation was achieved with the 2.1 × 50.0 mm, 5 µm column (XBridge C18, Waters Corp., MA, USA) maintained at 40 °C. Two ion transitions were used for quantification and qualification of each analyte. OA and YTX were measured in negative mode, all other analytes in positive mode.

The limit of detection (LOD) for the OA group, AZA, SPX, GYM was 10 µg kg^{-1} and for YTX 70 µg kg^{-1}. The analytical range of the toxins, except for YTXs, was between 32 (limit of quantification - LOQ) and 320 µg kg^{-1}. The analytical range for YTXs was between 200 (LOQ) and 1500 µg kg^{-1}. The relative expanded measurement uncertainty (coverage factor 2) at a concentration of 160 µg kg^{-1} was 10 % for OA group, 19 % for YTX, 16 % for AZA-1, 26 % for SPX and 17 % for GYM at a concentration of 1000 µg kg^{-1}. The concentrations of the identified toxins were converted into the toxic equivalent of the key toxin of the respective toxin group (OA group, AZAs, YTXs) using the toxic equivalence factors proposed by the EFSA (2008). The individual toxin concentrations in the OA group were corrected for the toxicity factor (for DTX-2 this is 0.6) (EFSA, 2008) and the OA equivalents were calculated. Finally, the sum of the toxins from the group was calculated and compared with the limit value. The accuracy for lipophilic toxins was as follows: for the OA group 100 %, for YTX 95 %, for AZA 101 % and for SPX and GYM 100 %, the precision was 5, 9, 8, 7 and 6 %, respectively.

For the determination of PSP toxins in mussels, an HPLC method with pre-column derivatization with peroxide or periodate was used, which complies with the EN 14526:2017 standard. The LOD for GTX1,4 and NEO was 100 µg kg^{-1}, and for GTX2,3, dcGTX2,3, GTX5 and STX it was 35 µg kg^{-1}. The LOQ for GTX1,4 was 300 µg kg^{-1}, while for NEO, GTX2,3, dcGTX2,3, GTX5, STX and dcSTX it was 100 µg kg^{-1}. The accuracy was 120, 71, 90, 100, 95, 95 and 100 %, respectively and the precision was 10, 8, 10, 17, 11, 7 and 12 %, respectively.

For the ASP toxins, the high performance liquid chromatography method coupled with a diode array detector (HPLC-DAD) was used (Quilliam et al., 1995). The LOD for DA was 1 mg kg^{-1}, the LOQ was 3 mg kg^{-1}, the accuracy was 93 % and the precision was 6 %.

The certified reference materials for OA, PTX-2, YTX, AZA-1, SPX-1, GYM, STX, dcSTX, NEO, GTX1,4, GTX2,3, GTX5, dcGTX2,3 and DA were purchased from the National Research Council Canada.

2.3. Statistical analysis

To test if the occurrence (positive results) and severity (concentrations above the detection limit) of toxicity events differ between shellfish farms, years, and months a non-parametric post-hoc analysis based on Kruskal–Wallis rank sum test (Kruskal and Wallis, 1952) was performed. As OA data are left-censored, concentrations of OA below the LOQ were replaced by half the LOQ for statistical analysis. Correlations

between monthly mean OA concentration, abundance of the most abundant DSP-producing phytoplankton species and all DSP-producing species, and seawater temperature and salinity were assessed using Spearman's rank correlation coefficients, which make no assumptions about distribution (Iman and Conover, 1982), and Holm's adjusted p-values.

The patterns of temporal (sampling dates) and spatial (sampling locations, i.e. three shellfish farms) distribution of the main DSP-producing species were analyzed using non-metric multidimensional scaling (NMDS; Legendre and Legendre, 2012). Ordination was based on the Bray-Curtis distance matrix. To obtain a visual representation of the OA concentrations associated with the phytoplankton data, their values were projected as the size of the circles (with a ln scale) representing each sample. Abiotic parameters (seawater temperature and salinity) were then fitted to the ordination using the *envfit* function to show their relationship with the phytoplankton and toxicity data.

Statistical analysis was performed using statistical software R version 4.2.2 (R Core Team, 2022). The vegan package was used for the NMDS and *envfit* (Oksanen et al., 2022), while for non-parametric post-hoc test based on Kruskal-Wallis the postHoc package was used (Labouriau, 2020). For all tests the significance level was set at 0.05.

3. Results

3.1. Toxic phytoplankton

During 2014–2019, a total of 32 toxic phytoplankton taxa were found in seawater at three shellfish farms, most of which were identified to the species level (Table 1). Toxic dinoflagellates and diatoms were grouped according to their toxins, and species abundances were aggregated across three sampling sites to show typical annual dynamics (Fig. 2).

Table 1
List of toxic and potentially toxic phytoplankton at three shellfish farms in the Slovenian sea in the period 2014–2019.

Species/taxon	Strunjan	Seča	Debeli rtič
ASP producers			
Pseudo-nitzschia delicatissima group	X	X	X
Pseudo-nitzschia seriata group	X	X	X
Pseudo-nitzschia cf. *galaxiae*	X	X	X
PSP producers			
Alexandrium insuetum	X	X	X
Alexandrium minutum	X	X	X
Alexandrium cf. *tamarense*	X	X	X
Alexandrium spp.	X	X	X
DSP producers			
Dinophysis acuminata		X	X
Dinophysis acutoides			X
Dinophysis caudata	X	X	X
Dinophysis fortii	X	X	X
Dinophysis hastata	X	X	X
Dinophysis nasutum	X	X	X
Dinophysis norvegica	X	X	
Dinophysis ovum	X	X	X
Dinophysis cf. *parva*	X		X
Dinophysis parvulum	X	X	X
Dinophysis sacculus	X	X	X
Dinophysis similis	X	X	X
Dinophysis schuettii			X
Dinophysis tripos	X	X	X
Dinophysis spp.	X	X	X
Phalacroma doryphorum			X
Phalacroma mitra	X	X	X
Phalacroma rapa	X		X
Phalacroma rotundatum	X	X	X
Phalacroma spp.	X	X	X
Prorocentrum lima		X	X
YTX producers			
Lingulodinium polyedra	X	X	X
Protoceratium reticulatum	X	X	X
NSP producers			
Karenia cf. *papilionacea*	X	X	X
Karenia spp.	X		

ASP producing diatoms of the genus *Pseudo-nitzschia* were the most abundant group and reached the highest values in the autumn months (outlier 7.4×10^5 cells L^{-1} in October). Their highest medians and maximum abundances were about 100- and 500-fold higher, respectively, than those of the most abundant dinoflagellates (PSP producing *Alexandrium* species). Therefore, the comparison between toxic diatoms and dinoflagellates is relevant when considering seasonal succession or interannual variability of species, but not in terms of absolute abundances.

Among the dinoflagellates producing DSP, PSP and NSP toxins, as mentioned above, the PSP group was the most abundant, with up to 10^3 cells L^{-1} detected on three occasions in spring (April, May). The DSP group achieved lower abundances (highest median 60 cells L^{-1}), although high values of several hundred cells L^{-1} or even above 10^3 cells L^{-1} were not uncommon, especially in the late summer and autumn months. YTXs producing species, *Lingulodinium polyedra* and *Protoceratium reticulatum*, were regularly found in seawater from spring to autumn, with peak levels in summer (maximum 890 cells L^{-1}). Potential producers of NSP toxins such as *Karenia* cf. *papilionacea* and other unidentified *Karenia* species occurred infrequently and in low numbers (average about 10 cells L^{-1}, maximum 320 cells L^{-1}). Because of the doubtful taxonomic classification and low abundances, the dynamics of the NSP group is not presented.

None of the species belonging to five toxin groups was always present in the seawater samples. In all cases, the lowest values were measured in the winter months. Since OA was the main toxin found in our study (see next section on toxins), we will focus on OA-producing dinoflagellates from the DSP group.

During the study period, 19 species of the genera *Dinophysis, Phalacroma*, and *Prorocentrum* associated with OA production were identified in the seawater samples (Table 1). However, the most common and abundant species were *D. caudata, D. fortii, D. sacculus, D. tripos* and *Phalacroma rotundatum*, which accounted for 85 % of total DSP abundance and each showed a distinct pattern of occurrence (Fig. 3). A pronounced species succession was observed in four *Dinophysis* species. First, *D. sacculus* began to increase in April, coinciding with a decrease in surface salinity from around 37.6 to around 36.8 (Fig. 3, blue line). *D. sacculus* reached the highest values in the late spring and summer months, while it was rarely detected in seawater after September. It was followed in second place by *D. caudata*, which significantly increased in July when we measured the highest seawater surface temperature (25.8 °C) and remained at a high level into November. *D. fortii* was a typical autumn species with high average abundances from September to November, which were statistically significantly different from the extremely low abundances in spring. A similar pattern was observed in *D. tripos*, but with an even narrower time window than in *D. fortii; D. tripos* peaked in September and October but may occur throughout the winter.

During the winter months (January-March), when the lowest temperatures and highest salinities were measured, *Dinophysis* species were virtually absent from seawater samples. Another characteristic of *Dinophysis* species was that medians and means were very low (< 50 cells L^{-1}), but high peaks (outliers in Fig. 3) were observed repeatedly during the study period for all species and ranged from 190 cells L^{-1} (*D. caudata*) to 950 cells L^{-1} (*D. fortii*) (mean values for all three sites).

Completely opposite was the dynamic of *P. rotundatum*. The species was almost always present in all months, and its abundances did not show much variation, as indicated by the similar medians and small outliers (maximum 80 cells L^{-1}) compared to *Dinophysis* species. However, the highest abundances were recorded from July to November.

Summary statistics for the five most abundant DSP species in each shellfish farm and cumulatively for all species responsible for five types of shellfish poisoning or toxins (DSP, ASP, PSP, NSP, YTXs) can be found

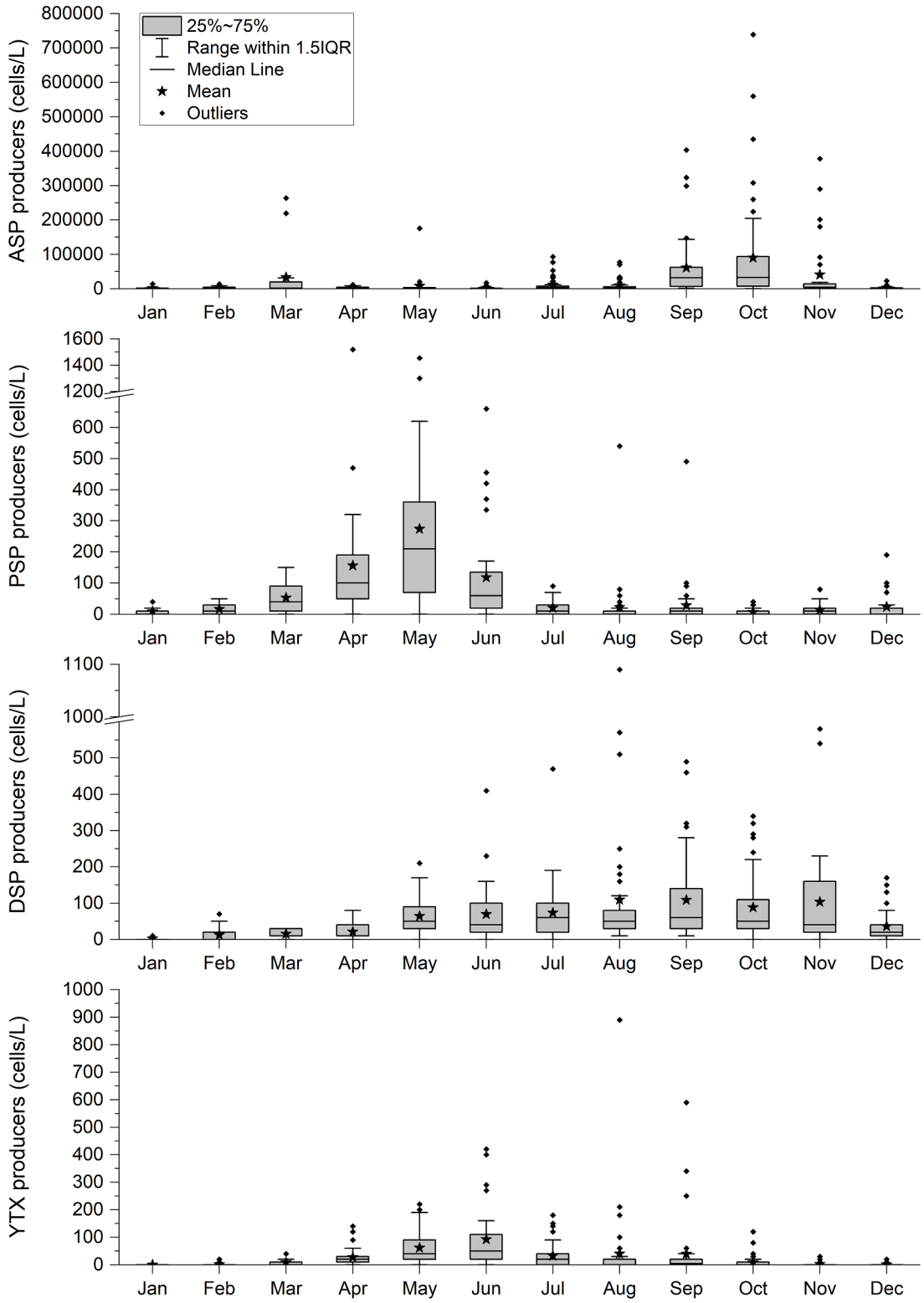

Fig. 2. The results of phytoplankton monitoring in Slovenian shellfish farms from 2014 to 2019. Box-plots represent the monthly distributions of toxic phytoplankton species from all three sampling sites, which were classified into four groups based on their toxins: ASP (amnesic shellfish poisoning), PSP (paralytic shellfish poisoning), DSP (diarrhetic shellfish poisoning), and YTXs (yessotoxins). Note the different scaling of the y-axis.

in the supplementary material (Tables S1-S3).

3.2. Toxins in mussels

During the study period 2014–2019, a total of 446 tests for toxins in mussels were performed for the group of lipophilic toxins (OA, DTX-1,2, PTX-1,2, YTX, 45-OH-YTX, homo-YTX, 45-OH-homo-YTX, AZA-1-3, SPX-C, GYM), 165 tests were performed for PSP (STX, NEO, dcSTX, GTX1-5, dcGTX2,3, C1,2) and 216 for ASP toxins (DA). None of the PSP and ASP toxins gave a positive result, while for lipophilic toxins 78 tests

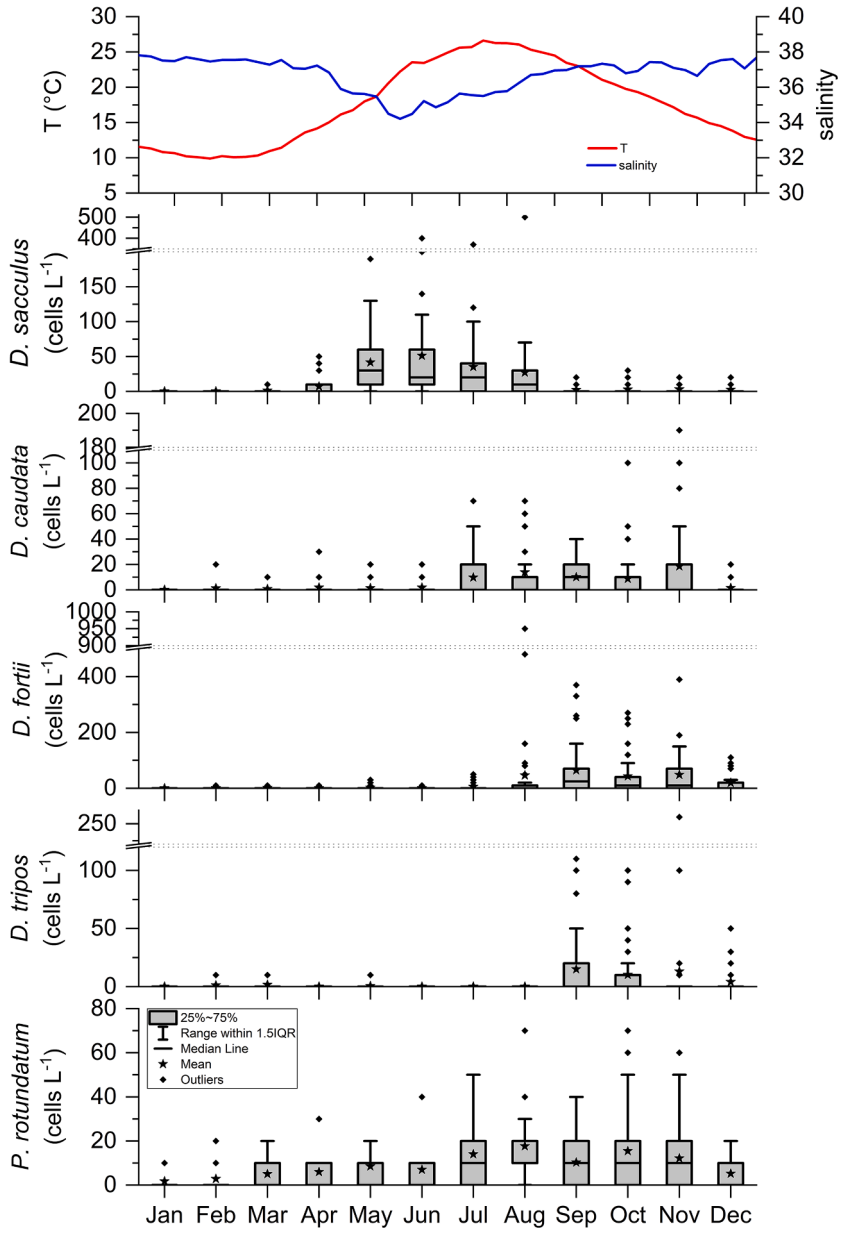

Fig. 3. Monthly distributions with respective abundances (in cells L^{-1}) of the five most abundant DSP species from all three sampling sites in the period 2014–2019. Note the different scaling of the y-axis. Upper panel: average annual distribution of sea surface temperature (red line) and salinity (blue line) measured at the oceanographic buoy 'Vida'.

were positive for the OA group (76 for OA and 2 for DTX-2) and three for YTXs (Table 2). However, only the concentrations of the OA group exceeded the regulatory limit of 160 μg kg^{-1} OA equivalents Regulation (EC) No 853/2004 (European Council, 2004) in 20 cases (4.5 % of the tests performed). Taking into account the measurement uncertainty of the method (10 %), the legal limit is exceeded at 176 μg kg^{-1} and the number of non-compliant samples was actually 17 or 3.8 % of all tests performed. The results of Debeli rtič from September 6, 2016 (sum of OA and DTX-2 using the toxicity conversion factor 0.6) are considered as one of the non-compliant samples.

In 2014, concentrations of OA up to 74 μg kg^{-1} were measured in Debeli rtič, Strunjan and in Seča, but in none of the harvesting areas did they exceed the limit value of 176 μg kg^{-1} (from here on only as a limit value). In addition to OA, YTXs were also detected on one sampling occasion (14 May) in all harvesting areas at very low concentrations (up to 0.34 mg kg^{-1}). In mid-September 2015, OA was detected in all harvesting areas and persisted until the end of December. However, the limit value was only exceeded in Seča (178 μg kg^{-1} in October and 301 μg kg^{-1} in November) and Strunjan (259 μg kg^{-1} and 266 μg kg^{-1} respectively), but not in Debeli rtič. The highest and therefore non-compliant OA concentrations of the entire study period were measured in all mussel farms in September 2016 (> 320 μg kg^{-1}). Concentrations also remained high in October, but the limit value was only exceeded in Debeli rtič and Strunjan (> 320 μg kg^{-1} and 230 μg kg^{-1}, respectively). 2016 was also the only time that we detected DTX-2 in mussels from Strunjan and Debeli rtič in addition to OA. However, the sum of the two values, corrected for the toxicity factor of DTX-2, was only above the limit value in Debeli rtič (219 μg kg^{-1}). In 2017, OA was detected in Debeli rtič (120 μg kg^{-1}) in August, almost a month before Seča and Strunjan. From mid-September to the first week of October, the limit value was exceeded in all areas, and then decreased until November, when it was below detectable levels. In 2018, no toxins were detected in any of the harvesting areas. In 2019, OA was detected in all areas from September to December, but the limit value was only

Table 2
Positive results of lipophilic toxins in mussels from the three Slovenian shellfish farms in the period 2014–2019: okadaic acid (OA), dinophysistoxin-2 (DTX-2) and yessotoxins (YTXs). Non-compliant samples (≥ 176 μg kg^{-1}) are in bold. < – concentration of toxin below the LOQ, NS – not sampled.

Shellfish farm	Seča			Strunjan			Debeli rtič		
Sampling date / toxin	OA μg kg^{-1}	DTX-2 μg kg^{-1}	YTXs mg kg^{-1}	OA μg kg^{-1}	DTX-2 μg kg^{-1}	YTXs mg kg^{-1}	OA μg kg^{-1}	DTX-2 μg kg^{-1}	YTXs mg kg^{-1}
10.04.2014	33	<	<	48	<	<	59	<	<
21.05.2014	<	<	0.34	<	<	0.29	<	<	0.26
29.05.2014	52	<	<	70	<	<	53	<	<
28.10.2014	NS	NS	NS	74	<	<	45	<	<
17.09.2015	43	<	<	65	<	<	57	<	<
6.10.2015	157	<	<	**259**	<	<	112	<	<
13.10.2015	104	<	<	88	<	<	58	<	<
21.10.2015	**178**	<	<	**266**	<	<	NS	<	<
5.11.2015	101	<	<	121	<	<	126	<	<
12.11.2015	61	<	<	48	<	<	48	<	<
25.11.2015	**301**	<	<	171	<	<	112	<	<
8.12.2015	86	<	<	120	<	<	38	<	<
17.12.2015	61	<	<	66	<	<	NS	<	<
6.09.2016	42	<	<	123	75	<	**140**	**86**	<
13.09.2016	**>320**	<	<	**>320**	<	<	**>320**	<	<
6.10.2016	109	<	<	**230**	<	<	**>320**	<	<
26.10.2016	51	<	<	78	<	<	<	<	<
24.08.2017	<	<	<	<	<	<	120	<	<
6.09.2017	<	<	<	<	<	<	72	<	<
19.09.2017	92	<	<	96	<	<	**183**	<	<
25.09.2017	163	<	<	**219**	<	<	NS	NS	NS
3.10.2017	**>320**	<	<	**239**	<	<	**203**	<	<
18.10.2017	<	<	<	78	<	<	63	<	<
25.10.2017	56	<	<	72	<	<	90	<	<
2.11.2017	58	<	<	85	<	<	NS	NS	NS
9.11.2017	NS	NS	NS	NS	NS	NS	61	<	<
16.09.2019	87	<	<	72	<	<	**274**	<	<
30.09.2019	66	<	<	57	<	<	**186**	<	<
11.10.2019	69	<	<	<	<	<	68	<	<
22.10.2019	50	<	<	44	<	<	51	<	<
25.10.2019	<	<	<	38	<	<	NS	NS	NS
26.11.2019	<	<	<	52	<	<	56	<	<

exceeded in Debeli rtič in September (274 μg kg^{-1} and 186 μg kg^{-1}).

These interannual variations in toxin concentrations were statistically significant (non-parametric post-hoc based on Kruskal-Wallis test, $p < 0.05$), while no statistically significant differences were found between sampling sites (non-parametric post-hoc based on Kruskal-Wallis test, $p > 0.05$). Two years in particular were responsible for the differences between years: 2018, which stood out statistically significantly from the rest of the years due to non-positive results, and to some extent 2015 with the highest incidence of toxicity events, followed by 2017 (non-parametric post-hoc based on Kruskal-Wallis test, $p < 0.05$).

There was a peculiar annual pattern of OA occurrence in mussels aggregated for three sampling sites, as shown in Fig. 4, also exemplified by statistically significant differences between months (non-parametric post-hoc based on Kruskal–Wallis test, $p < 0.05$). September, October, and November were the months with the highest probability of OA occurrence, with the highest median value of 53.50 μg kg^{-1} in October and were also the only months with non-compliant samples. These autumn months, especially October and September, were statistically significantly different from all others (non-parametric post-hoc based on Kruskal–Wallis test, $p < 0.05$), while November showed some similarity to December, in which OA occasionally occurred. On the contrary, the likelihood of OA occurring in the remaining months was minimal. In fact, only sporadic episodes of OA were detected in April, May and August, but not enough to highlight these months as statistically significantly different periods of OA occurrence.

3.3. Relationship between toxic phytoplankton, OA, and environmental factors

The results of the NMDS analysis performed on the abundances of the

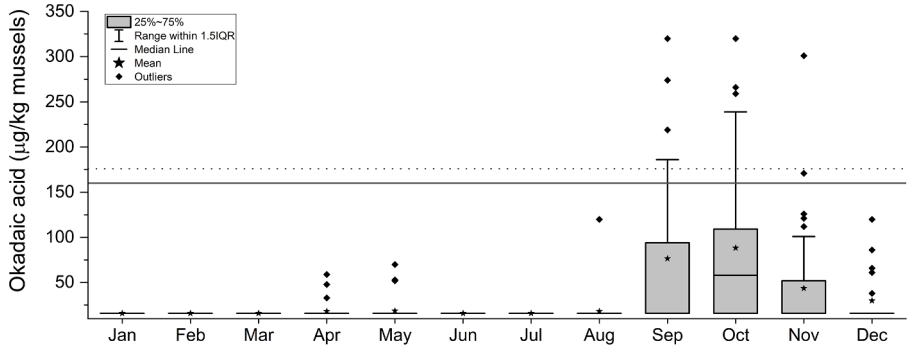

Fig. 4. Monthly distribution of okadaic acid concentrations in mussels from three Slovenian shellfish farms in the period 2014–2019. The solid line represents the regulatory limit of 160 μg kg^{-1} for bivalve molluscs laid down in Regulation (EC) No 853/2004, while the dashed line indicates the limit of 176 μg kg^{-1}, which considers the 10 % measurement uncertainty of the method.

five main DSP-producing species showed a clear separation of samples along the first axis, which correlates very well with surface salinity ($p < 0.001$) and temperature ($p < 0.001$) (Fig. 5). Along the salinity gradient, *D. sacculus* converges towards low salinities, while *D. tripos* and *D. fortii* converge towards high salinities. Temperature has an opposite effect on these species, as well as on *D. caudata*, with higher temperatures favoring *D. sacculus* and *D. caudata*. The distribution of *P. rotundatum* does not seem to be significantly influenced by any abiotic factor. The spatial homogeneity, i.e. the non-selective distribution of sampling sites, confirms the above-mentioned results of the non-parametric post-hoc test that there are no differences between stations. The size of the circles, i.e. samples, is scaled against OA concentrations in mussels. With a few exceptions, the size generally increases towards the group of samples centered on *D. fortii*.

These relationships are evaluated in more detail with the correlation coefficients in Table 3. The strongest and statistically highly significant correlation with OA concentrations was found for *D. fortii*. A moderate but still highly statistically significant correlation was found between the OA concentrations and the DSP group, while the correlations between the individual dinoflagellate species, except for *D. sacculus*, were statistically significant but weak.

Statistically significant correlations were found between the DSP group and selected species of the genera *Dinophysis* and *Phalacroma*, which was to be expected as they contribute most to the overall abundance of the DSP group. Most species and the DSP group were statistically significantly correlated with the environmental data, except for *D. tripos*. The strongest negative correlation was between salinity and *D. sacculus*, while the correlation with the DSP group, although still highly significant, was moderate. In contrast, the correlations between water temperature and toxic species were positive, but not as strong. Temperature was weakly correlated with *D. fortii* and *P. rotundatum* and moderately correlated with *D. caudata, D. sacculus* and the DSP group. OA was not correlated with either water temperature or salinity. Most species pairs were also statistically significantly correlated, but the correlation was weak.

4. Discussion

Our results show that so far only DSP toxins can pose a real and recurrent threat to food safety in Slovenian coastal waters in the northernmost part of the Adriatic Sea, which is consistent with the analysis of long-term trends of HAB species and toxic events in the Mediterranean Sea, where the most common toxins found above regulatory limits are DSP toxins (Arapov et al., 2015; Zingone et al., 2021). More specifically, among DSP toxins, OA was the predominant toxin (97 % positive results, with DTX-2 accounting for the remainder). During 2014 to 2019 it was found in concentrations over legal limits at 3.8 % of all tests performed and it was responsible for sales bans of shellfish in this period. This is the first chemical characterization of algal toxins in mussels farmed in the Slovenian sea, while in previous toxic events (prior to 2014), causation of DSP toxins could only be inferred from positive mouse bioassays for a mixture of lipophilic toxins with concomitant elevated *Dinophysis* and *Phalacroma* abundances in water samples (Francé et al., 2013). Apart from the OA group, the only other toxins found during the study period were YTXs.

This confirms previous results on the predominant positivity of OA

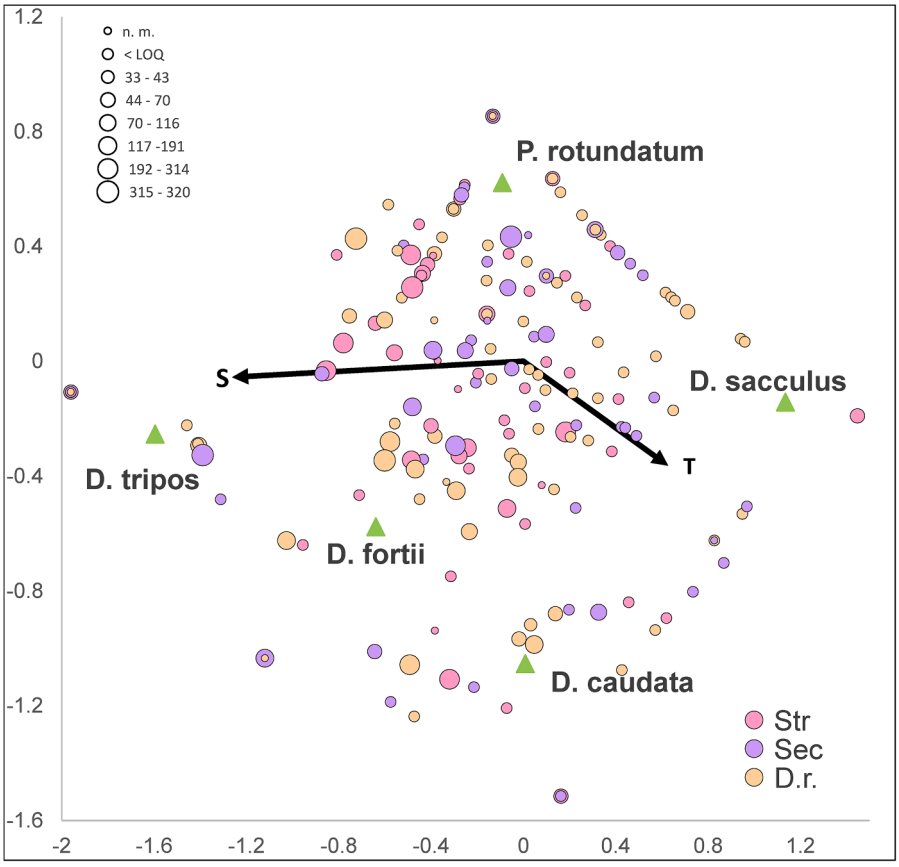

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination plot showing the distribution of samples based on the abundance of the main DSP-associated dinoflagellates at three shellfish farms (Strunjan (Str), Seča (Sec), Debeli rtič (D.r.)) in the period 2014–2019 ($n = 1700$). The size of the circles is scaled against OA concentrations in mussels (in μg kg^{-1}) on a modified ln scale. Centroids of DSP species are marked with triangles. Abiotic parameters are shown by vector arrows and their statistical significance for the ordination of samples indicated by p-values in brackets; temperature (T) ($p < 0.001$), salinity (S) ($p < 0.001$). Stress = 0.105. n.m. = toxins not measured.

Table 3
Spearman's correlation coefficient and adjusted p-values (Holm's method) between abundance of selected phytoplankton species and DSP group, the concentration of okadaic acid (OA) and sea surface temperature (T) and salinity (S). Numbers in bold indicate statistically significant moderate (0.40–0.59) to strong (0.60–0.79) correlations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	D. caudata	*D. fortii*	*P. rotundatum*	*D. sacculus*	*D. tripos*	DSP group	OA	S
D. fortii	0.394***	/						
P. rotundatum	0.331***	0.392***	/					
D. sacculus	0.218*	0.075	0.273**	/				
D. tripos	0.143	0.322***	0.042	-0.223*	/			
DSP group	**0.552*****	**0.649*****	**0.637*****	**0.597*****	0.202*	/		
OA	0.279**	**0.611*****	0.232*	-0.114	0.279*	**0.419*****	/	
S	-0.244**	-0.025	-0.268**	**-0.688*****	0.139	**-0.461*****	0.135	/
T	**0.440*****	0.245**	0.388***	**0.490*****	-0.017	**0.536*****	0.085	**-0.621*****

(and analogues) measured in bivalve molluscs from the northwestern (Accoroni et al., 2023) and eastern Adriatic coast (Ninčević Gladan et al., 2011), while the observed dominance of YTXs together with OAs in the same areas of the Adriatic (*ibid.*, Rubini et al., 2021) contrasts with our results. Despite the regular occurrence of the responsible species with outbreaks mainly of *Lingulodinium polyedra* (e.g. 890 cells L^{-1} in August 2015), traces of YTXs were detected only once in our study (0.26–0.34 mg kg^{-1}, May 2014). This may be in line with the recently observed negative trend (2015–2022) in the abundance of *L. polyedra* in the western part of the Gulf of Trieste (Tondelli, 2023). It is noteworthy that the number of positive YTX detections in certain Italian regions (e.g. Emilia-Romagna) has decreased since 2013 (Accoroni et al., 2023).

As for the other regularly monitored toxin groups - ASP and PSP - none of them have ever been detected in mussels from Slovenian waters, including this study. Nevertheless, caution should be exercised, especially regarding *Pseudo-nitzschia* spp. as potential producers of ASP, for several reasons. Firstly, *Pseudo-nitzschia* species in the Gulf of Trieste regularly form seasonal blooms with outbreaks sometimes exceeding several million cells L^{-1} (Cabrini et al., 2012). Secondly, the toxicity of certain species (*P. delicatissima, P. multistriata, P. calliantha, P. galaxiae*) isolated from the northwestern and central Adriatic Sea (Arapov et al., 2020; Penna et al., 2013; Pistocchi et al., 2012), as well as from the Slovenian sea (Turk Dermastia et al., 2022) was confirmed through the isolation of domoic acid from cultured strains. However, the toxin concentration per cell was found to be very low or highly variable among the different strains. Finally, the accumulation of ASP toxins has occasionally been measured in Adriatic mussels, but with concentrations consistently below regulatory limits (Arapov et al., 2016; Ciminiello et al., 2005; Ujević et al., 2010). Similarly, sporadic cases of PSP toxins in trace amounts have been documented (Ciminiello et al., 1995; Ujević et al., 2012) with only one instance in the northern Adriatic with surpassed regulatory limit, in 1994, which was associated with a bloom of *Alexandrium minutum* (Honsell et al., 1996). The discrepancy between the presence of the causative species and the absence of harmful events due to ASP and PSP toxins is not surprising and can also be observed on a global scale (e.g. ASP dominant on the west coast of North America, DSP in Europe) (Hallegraeff et al., 2021). This aspect requires ecological studies at local and species level, taking into account site-specific environmental characteristics and the consequences of various anthropogenic impacts, in particular climate change.

As for the group of potential OA producers, the most abundant (i.e. 85 % of the abundance of the DSP group) and recurrent species in Slovenian aquaculture areas during the study period were *Dinophysis fortii, D. caudata, D. sacculus, D. tripos* and *Phalacroma rotundatum*. The same dominant species and similar seasonal distribution and abundance ranges were found in a study carried out in the same area over 20 years ago (Francé and Mozetič, 2006). As then (*ibid.*), high densities of *D. sacculus* were found in spring and summer in the present study, while those of *D. caudata* and *D. fortii* were observed in summer and autumn, respectively. *P. rotundatum* was present throughout the year with no significant peaks in abundance. The only difference is that Francé and Mozetič (2006) did not find *D. tripos* as an important species. Instead, it only appeared regularly and in significant numbers from 2010 onwards (Francé, pers. observ.). The maximum abundances of the entire DSP group and in many cases of the individual causative species exceeded 200 cells L^{-1} (see Tables S1-S3), which in certain areas is considered the threshold cell concentration above which the species may pose a threat (Yasumoto et al., 1985). Among them, *D. fortii*, followed by *D. sacculus*, reached the highest abundances, i.e. 950 and 500 cells L^{-1} at Debeli rtič, respectively. *P. rotundatum* showed the least seasonality and was present at low densities in the water throughout the year (up to 80 cells L^{-1}), reflecting its heterotrophic nature. The five species mentioned above, with some local variations, were also found to be dominant along the eastern and western Adriatic coasts and show a similar seasonal pattern of occurrence (Bernardi Aubry et al., 2000; Caroppo et al., 2001; Ninčević-Gladan et al., 2008). They can therefore be considered as a typical assemblage of *Dinopyhsis* and *Phalacroma* species for the Adriatic.

A common feature of several ecological studies of *Dinophysis* is that increased growth of the species is associated with stable water column stratification, i.e., increases in seawater temperature and/or decreases in surface salinity following heavy precipitation or freshwater discharges in coastal waters, while local hydrodynamic features (upwelling/downwelling cycles, tides, coastal advection) promote dispersal, aggregation, or retention of established populations (Reguera et al., 2012). The statistically significant correlations found in our study between sea surface temperature ($r = 0.536$) and salinity ($r = -0.621$) and the DSP group (sum of abundance of all *Dinophysis* and *Phalacroma* species) (Table 3) are roughly consistent with these observations, although there are species-specific differences. For example, *D. sacculus* clearly preferred warm and less saline waters that supported strong stratification during the summer months, whereas *D. caudata* and, to some extent, *P. rotundatum* and *D. tripos* were more abundant in warm waters with no freshwater input. A similar seasonal pattern of toxic dinoflagellates in relation to environmental factors was found in shellfish farms on the eastern Adriatic coast, suggesting that high temperatures, water column stability, and precipitation determine species-specific seasonality (Ninčević Gladan et al., 2020).

The role of nutrients in *Dinophysis* dynamics, although not considered in our study, is more difficult to determine because *Dinophysis* species are obligate mixotrophs (Mitra et al., 2016) that switch from phototrophy to phagotrophy on ciliates such as *Mesodinium* spp. (Park et al., 2006; Reguera et al., 2012) in the absence of favorable conditions (light intensity, nutrient supply), which may be combined with acquired phototrophy (Anschütz et al., 2022). This nutritional strategy should be considered pro-competitive given the observed oligotrophication of the northern Adriatic (Grilli et al., 2020; Mozetič et al., 2010). The decrease in nutrients, especially phosphorus, and changes in the size structure of the phytoplankton community (increase in pico- and nano-sized taxa relative to microplankton) (Brush et al., 2021; Flander-Putrle et al., 2021), concomitant with meteorological and hydrological disturbances at the mesoscale could favor the growth of mixotrophic species such as *Dinophysis* and ultimately increase the risk of more harmful algal events in the future. If the current oligotrophy in the northern Adriatic continues or even intensifies in the future, this could lead to a relatively

higher proportion of *Dinophysis* biomass in the overall low biomass of the phytoplankton community and thus to increased toxicity of bivalves, in contrast to the lower toxicity observed when *Dinophysis* is a minor component of the phytoplankton bloom (Dahl and Johannessen, 2001).

The highest concentrations of OA were found in autumn (September–November; Fig. 4), statistically significantly higher than in other months. This corresponds to the period of highest abundance (in terms of median, third quartile, and most outliers) of DSP producers (Fig. 2) to which all species except *D. tripos* contributed significantly (pairwise correlation coefficients between DSP group and individual species, Table 3). These five most abundant species are also among those confirmed to contain OA group toxins and PTXs, as reported by Reguera et al. (2014), although there is great controversy about the toxigenic nature of *P. rotundatum*, as its toxicity is thought to be acquired through predation (González-Gil et al., 2011). Focusing on our area of interest, the Mediterranean Sea, which together with other European seas is the geographical area with the highest incidence of DSP events on a global scale (Hallegraeff et al., 2021), we can see that despite the similar species diversity of *Dinophysis* and *Phalacroma*, certain species are more clearly associated with this syndrome than others in the different sub-basins of the Mediterranean: the *D. acuminata* complex and *D. acuta* in the western Mediterranean along the Andalusian coast (Fernández et al., 2019), *D. ovum* during severe DSP outbreaks in the northwestern Aegean Sea in the eastern Mediterranean during the 2000s (Koukaras and Nikolaidinis, 2004) and the above mentioned five "typical" species in the Adriatic (Ninčević-Gladan et al., 2008).

In our study, only *D. fortii* was strongly associated with OA production, as indicated by the high correlation coefficient ($r = 0.611$, $p < 0.001$). However, without chemical analysis of the cultures (e.g. Park et al., 2006), we can only indirectly infer that *D. fortii* was the main contributor to OA production. *D. fortii* was also the first among *Dinophysis* species to be identified as a toxic agent in natural phytoplankton samples of Japan (Yasumoto et al., 1980), where it is also considered as the most toxigenic agent for DSP outbreaks. Since then, toxin analyses either on pooled picked cells or in cultures have shown different toxin profiles of *D. fortii*, ranging from dominance of PTX-2 followed by DTX-1 and moderate to only trace amounts of OA in Japanese strains (Nagai et al., 2011; Uchida et al., 2018) to a dominance of PTX-2 and moderate concentrations of OA in Adriatic strains (Draisci et al., 1996). The fact that PTX-2 was not detected in our study, while it was only sporadically present in mussels from the Italian coast (Accoroni et al., 2023), may indicate a faster transformation of PTX-2 into PTX-2-sa in mussels (Suzuki et al., 2001) and its rapid elimination in the environment (Moroño et al., 2003).

Several abiotic (e.g., temperature) or intrinsic (growth phase) factors may influence the production of toxins in *Dinophysis* species and their accumulation in mussels (Kamiyama et al., 2010), resulting, as in our study, in different toxicity in different years and locations, even at small spatial scales, although the latter were not statistically significant. This is not unusual, as large differences in toxin content per cell can be found even within the same species and at the same locality (in Reguera and Blanco, 2019). Furthermore, *Dinophysis* species can show different toxin variability even on the same day (Pizarro et al., 2009; Reguera et al., 2014). Some studies have found a negative correlation between population density and toxicity per cell (Lindahl et al., 2007). Consequently, the combination of cell abundance and cell toxicity influences shellfish toxicity (Lindahl et al., 2007). Filtration rate, toxin accumulation and the availability of alternative food sources for bivalve molluscs (Alves-de-Souza et al., 2014; Moroño et al., 2003) could also determine toxin levels in bivalve molluscs. The complexity of these mechanisms behind the ecological, biochemical, and physiological processes can only be addressed in food safety management by an effective and well-designed monitoring and early warning system, where a threshold cell concentration, either 200 cells L^{-1} (*sensu* Yasumoto et al., 1985) or another value, is only one of the warning categories.

With regard to how the introduction of analytical methods in toxin monitoring has affected the management of aquaculture activities, we compared phytoplankton and toxicity data with trade bans on mussels for the period 1989–2019 (Francé et al., 2013; Francé and Mozetič, 2006; unpubl. data). The example of the Strunjan mussel farm (Fig. 6) shows that although there were no significant differences in the temporal pattern of occurrence of toxic phytoplankton, the annual pattern of positive results for toxins and consequently trade bans changed after 2012, when the LC-MS/MS method for lipophilic toxins replaced the mouse bioassay due to its insufficient detection capability and limited specificity for control purposes (Commission Regulation (EU) No 15/2011, European Commission, 2011). Trade bans did not show a clear annual pattern until 2012 and were spread throughout the year, although more frequent in summer and autumn. In contrast, from 2013 onwards, trade bans were always limited to late summer-autumn.

This different pattern can be interpreted in two ways. Firstly, the introduction of the LC-MS/MS method made it possible to distinguish between OA and its derivatives and other lipophilic toxins, limiting trade bans to the autumn months (corresponding to the presence of the most likely causative species, *D. fortii*), while the mouse bioassay also gave positive toxicity results for other lipophilic toxins associated with the presence of species such as *L. polyedra* (Francé et al., 2013). With the introduction of the chemical method, YTXs are still analyzed within the group of lipophilic toxins, but they are chromatographically separated from the other lipophilic toxins. Secondly, specific environmental conditions in the earlier period, which were not repeated afterwards, may have led to an outbreak of DSP caused by other species and not only by *D. fortii*. For example, an unusually high abundance of *D. caudata* was detected in June and July 2010 (up to 630 cells L^{-1}), which was associated with very low salinity in the surface layer of the Gulf of Trieste from May to October (Francé et al., 2013). Nevertheless, some of the prolonged trade bans, such as the extension from autumn to winter in 1989–1990, 2010–2011 and 2011–2012, may reflect other reasons, such as the long biotransformation and detoxification requirements of bivalve molluscs under specific environmental and nutritional conditions (Botelho et al., 2018).

5. Conclusions

The study provides the first chemical characterization of algal toxins in Slovenian shellfish farms. It highlights that okadaic acid is the main DSP toxin posing a food safety risk in about 4 % of the mussel tests conducted between 2014 and 2019, mainly in the period from September to November. The study also shows a minimal occurrence of yessotoxins in contrast to other regions of the Adriatic Sea. The predominant DSP-producing species, including *Dinophysis fortii, D. caudata, D. sacculus, D. tripos* and *Phalacroma rotundatum*, form a *Dinophysis/Phalacroma* assemblage typical for the Adriatic Sea, with *D. fortii* showing a significant toxic potential. The introduction of the LC-MS/MS method has undoubtedly improved the management of aquaculture activities. The observed inter-annual variations in toxicity and phytoplankton dynamics, which may be exacerbated by the effects of climatic and other anthropogenic stressors in the future, may require even more efficient surveillance measures. Identified correlations between environmental factors, toxic species and OA in mussels could serve as a basis for predictive models that utilize advanced techniques such as machine learning to develop effective sampling plans for future shellfish production and ensure seafood safety in the fragile ecosystem of the northern Adriatic Sea.

Author declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work

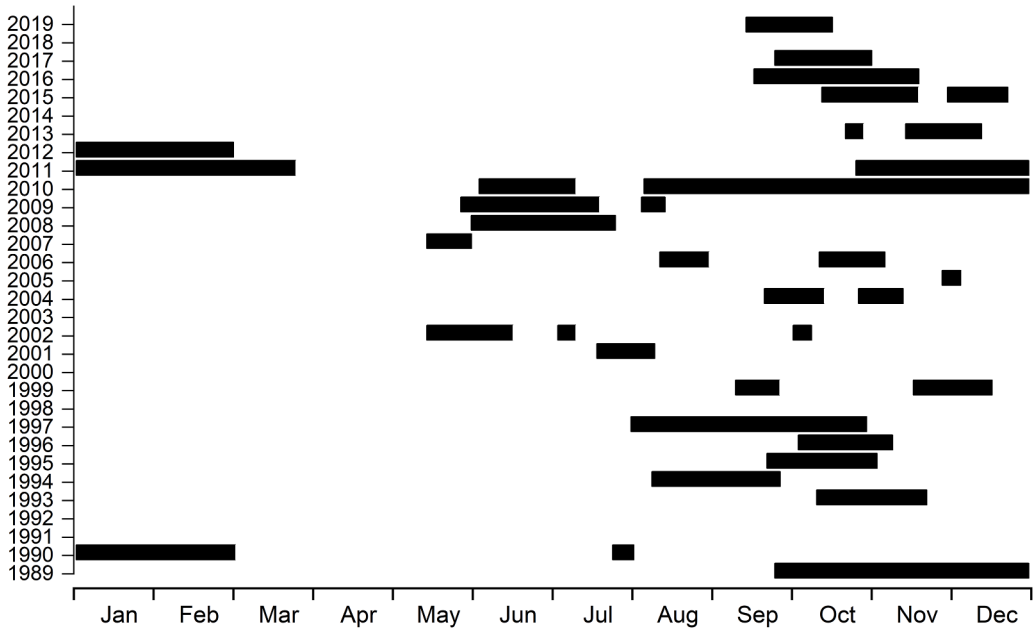

Fig. 6. Bans of shellfish trade at Strunjan mussel farm in the period 1989–2019 due to the exceeding the regulatory limit for lipophilic toxins. Detection methods: mouse bioassay until 2012, LC-MS/MS from 2013.

was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the copyright-holder.

By attaching this Declaration to the submission, the corresponding author certifies that:

- The manuscript represents original and valid work and that neither this manuscript nor one with substantially similar content under the same authorship has been published or is being considered for publication elsewhere.
- Every author has agreed to allow the corresponding author to serve as the primary correspondent with the editorial office, and to review the edited typescript and proof.
- Each author has given final approval of the submitted manuscript and order of authors. Any subsequent change to authorship will be approved by all authors.
- Each author has participated sufficiently in the work to take public responsibility for all the content.

CRediT authorship contribution statement

Urška Henigman: Writing – review & editing, Writing – original draft, Conceptualization. **Patricija Mozetič:** Investigation, Data curation, Writing – original draft, Writing – review & editing. **Janja Francé:** Writing – review & editing, Investigation, Data curation. **Tanja Knific:** Writing – review & editing, Formal analysis. **Stanka Vadnjal:** Writing – review & editing. **Jožica Dolenc:** Writing – review & editing, Investigation. **Andrej Kirbiš:** Project administration. **Majda Biasizzo:** Writing – review & editing, 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.

Data availability

Data will be made available on request.

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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.2024.102632.

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