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Long-term Trends in Pseudo-nitzschia Complex Blooms in the Black Sea - is there a Potential Risk for Ecological and Human Hazards

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Abstract. The potentially toxic genus *Pseudo-nitzschia* is constantly present in phytoplankton community in the Black Sea often proliferating to bloom concentrations. Production of domoic acid (DA) has been confirmed by local *P. calliantha* strain and the presence of the neurotoxin has been detected in cultured molluscs and plankton samples from the Bulgarian Black Sea coast. This study aims to provide an overview of the long-term trends of *Pseudo-nitzschia* blooms in the Black Sea based on inventory of the available information for the period 1959-2019 and assess the relation with key environmental variables. *Pseudo-nitzschia* species diversity and identification constraints are critically analyzed along with data about the presence of DA in the basin. The results demonstrate that *Pseudo-nitzschia* spp. may bloom at any time of the year under different environmental regimes. Application of advanced identification techniques, technologically adequate regular monitoring, complemented by toxin analysis is essential to improve our understanding of *Pseudo-nitzschia* bloom dynamics in order to protect public and ecosystem health hazards.

Key words: *Pseudo-nitzshia,* harmful algal blooms, long-term trends, Black Sea.

Introduction

The marine planktonic genus *Pseudo-nitzschia* became a global concern after the first documented Amnesic Shellfish Poisoning (ASP) event in Canada in 1987 resulted in three deaths and 105 confirmed cases of acute intoxications (Bates et al., 1989). The clinical signs include gastrointestinal or neurological symptoms like vomiting, abdominal cramps, diarrhea, headache, and loss of short-term memory, and were related to the ingestion of mussels contaminated by neurotoxin domoic acid (DA) produced by *Pseudo-nitzschia* (Perl et al., 1990). Later findings that these microalgae can induce mass mortality in sea birds, fish and mammals further raised the interest to these diatoms (Work et al., 1993; Beltran et al., 1997; Scholin et al., 2000). Among the 52 species described so far in the literature within this genus, 26 were listed as toxigenic - capable to produce DA (reviewed in Bates et al., 2018). Many *Pseudo-nitzschia* species (including toxigenic) are widely distributed and of cosmopolitan nature (reviewed by Bates et al., 2018). In addition, both toxic and non-toxic species worldwide were reported to cause dense blooms under different environmental conditions, such as diverse temperature and salinity regimes, nutrients, pH, photoperiod, meteorological phenomena (reviewed in Lelong et al., 2012). Precise identification of *Pseudo-nitzschia* species is crucial in monitoring programs and ecological investigations but the high cryptic and pseudo-cryptic diversity reported in the genus hampered the determination by light microscopy (Quijano-Scheggia et al., 2010). Many of the species have morphological features in common (e.g. identical valve shape, length and width, equal overlap of cell length in chain). Their delineation needs detailed morphological observation (e.g. the structure of the poroid hymen, the density of band striae and the structure of the band striae) using transmission electron microscope (TEM) or scanning electron microscopy (SEM) and molecular methods (Lundholm et al., 2002, 2003, 2012; Quijano-Scheggia et al., 2009; Lim et al., 2012).

Data about *Pseudo-nitzschia* in the Black Sea are fragmentary, but the constant presence of these potentially toxic diatoms has been documented (Terenko & Terenko, 2012; Dzhembekova & Moncheva, 2014). *Pseudo-nitzschia* blooms are often observed basinwide (Nesterova et al., 2008; Moncheva et al., 2019). Coastal waters are the main ''hot spots'', where *Pseudo-nitzschia* blooms occur (Moncheva et al., 1995, 2001; Velikova et al., 1999; Türkoğlu & Koray, 2002; Vershinin et al., 2005; Terenko & Terenko, 2012) but high abundances have been recorded offshore as well (Mikaelyan, 1995). Although the available data on *Pseudo-nitzschia* toxicity in the Black Sea are scarce and fragmentary, DA has been detected in culture (Besiktepe et al., 2008) as well as in natural mussel and plankton samples (Peneva et al. 2011; Peteva et al. 2018).

Globally, the increased awareness and monitoring programs implemented effectively protect human health from intoxication and fatalities (Trainer et al., 2012). A special attention and more extensive study of the dynamics of the genus is crucial at a regional level, including the Black Sea. The aim of this study is to review the occurrence (spatial and temporal distribution) of *Pseudo-nitzschia* blooms in the Black Sea since 1959 to present and to diagnose potential ecosystem and human risk. *Pseudo-nitzschia* species diversity and identification problems are critically analyzed along with data about the presence of DA in the Black Sea. The relation between some environmental factors and growth of potentially toxic *Pseudo-nitzschia* species along the Bulgarian Black Sea is also discussed.

Material and Methods

The results are based on long-term own (published and unpublished) and literature data for the period 1959-2019. Significant part of the records used was built on species identification and cell counts by light microscopy (LM) on fixed samples. Available literature data produced by scanning and transmission electron microscopy (SEM and TEM) and/or molecular analysis were reviewed to explore the current knowledge on species diversity. Detailed information about the material and methods for the published data can be found in the original articles referenced in the text and Table 1. The unpublished own data set includes 1283 records from 205 stations in Bulgarian and Romanian waters collected between 1999-2019 under different research and monitoring programs and projects. Niskin bottle samples attached to CTD – Sea Bird Electronics 911 have been collected from various depths (0-100m) and 1 L aliquots were fixed with formaldehyde solution, buffered to pH 8-8.2 with disodiumtetraborate (4% final concentration). Taxonomic identification and cell counting were done under inverted light microscope by the Utermöhl (1958) method in Sedgwick-Rafter counting chambers. 400 cells were counted from each sample, while rare and large species were checked in the whole counting chamber.

Temperature (T) and salinity (S) were measured *in situ* at each station during the cruises by the CTD sensors. Seawater samples for chemical analyses were collected in parallel with phytoplankton samples at the stations located in the Bulgarian waters (1999-2015). Nutrients (nitrates - NO_3, phosphates - PO_4, and silicates – SiO_4) were analysed using standard methods (Grasshoff et al., 1999).

A generalized additive model (GAM) was used as a flexible modeling technique, which is useful to model more complex ecological responses and capture nonlinearities in data, to examine the effects of T, S, NO_3, PO_4 and SiO_4 on *Pseudo-nitzschia* abundance dynamics. Due to the difficult taxonomic identification under LM *Pseudo-nitzschia* species were clustered into two groups on the base of cell width - *P. delicatissima* group (< 3 µm) and *P. seriata* group (> 3 µm) (according to Hasle & Syvertsen, 1997). The association between *Pseudo-nitzschia* species abundance (cells l^{-1}) and *in situ* environmental variables was assessed on a matrix of 141 records of *P. delicatissima* group and 102 data of *P. seriata* group collected across the Bulgarian Black Sea coastal, shelf and open sea waters during 1999-2015. The statistical analyses and graphic representations were undertaken in R version 4.0.3 (2020-10-10) (R Core Team, 2020), CRAN package: mgcv (Wood, 2003, 2004, 2011, 2017; Wood et al., 2016), mgcViz (Fasiolo et al., 2018), gratia (Gavin, 2021), available through the CRAN repository (www.r-pro ject.org). QGIS2.18.10 and ArcGIS software version 10.2.2 (ESRI 2011) were used for mapping the *Pseudo-nitzschia* species spatial distribution and the reported blooms in the Black Sea.

Results and Discussion

Pseudo-nitzschia blooms in the Black Sea

Pseudo-nitzschia blooms proved to be a common phenomenon for different areas in the Black Sea. However neither clear spatial pattern of distribution or species identity involved, nor a recurrent specific seasonal occurrence during the year cycle was inferred from the analysis and mapping of available long-term data (Fig. 1, Table 1).

Among the first described outbreaks of these potentially toxic diatoms was the proliferation of *P. seriata* ($1x10^6$ cells l^{-1}) in March 1959 in Ukrainian waters (Odessa Bay) (Ryabushko, 2003) and in Bulgarian coastal waters (Varna Bay) lasting from February to May 1959 with a cell density exceeding $4x10^6$ cells l^{-1} (Petrova & Skolka, 1963). In the first half of 1966 (from January to August), a massive, long-lasting *P. delicatissima* outburst affected the entire Bulgarian coast and the trend continued in the next three years with *P. delicatissima* blooms occurring in spring 1968 and 1970, and in autumn 1969 (Petrova-Karadzhova, 1973). Since then, *Pseudo-nitzschia* blooms continuously occurred in Bulgarian waters more often dominated by *P. delicatissima* (Moncheva and Krastev, 1997; Velikova et al, 1999; Moncheva et al., 2001; Petrova et al., 2006; Petrova & Gerdzhikov, 2007, 2009, 2011). In May 2014 and 2016 bloom concentrations of *P. delicatissima* (between $1x10^6$ and $1.3x10^6$ cells l^{-1}) were registered at a couple of sites along the Bulgarian coast. Interestingly high abundances of *Pseudo-nitzschia* spp. were observed also in the brackish Varna Lake in September 2000 (Petrova & Gerdzhikov, 2007).

Between 1960 and 1970 a dramatic outburst of *P. delicatissima* (21×10^6 cells l^{-1}) was observed in Romanian waters (Bodeanu, 2002). Actually, all *Pseudo-nitzschia* blooms reported along the Romanian coast between 1960 and 2014 were caused by *P. delicatissima* mainly in spring (Bodeanu, 2002; Moncheva et al., 2019). Recently, in 2019, a spring widespread growth of *Pseudo-nitzschia* was registered along the Romanian coast (97.5% of the samples) with co-presence of *P. delicatissima* and *P. seriata* and maximum abundance > 4.39×10^6 cells l^{-1} (unpublished own data).

In Ukrainian waters eight *Pseudo-nitzschia* outbreaks were recorded between 1959 and 2009 with *P. delicatissima* (in March 1959, June 1991, 1996, 2001 and May 2009, and in July 2006) and *P.*

pseudodelicatissima (in June 2008, max abundance 12x10^6 cells l^{-1}) and *Pseudo-nitszchia* spp. (December 2005, density 3x10^6 cells l^{-1}) cited as causal species, with the highest abundance found in Odessa Bay in the spring of 1996 when *P. delicatissima* reached 16.6×10^6 cells l^{-1} (Terenko & Terenko, 2012). *Pseudo-nitszchia* bloom in 2001 was found to cover a wide area of the Black Sea but the cell abundance exceeded 1x10^6 cells l^{-1} only in Ukraine (Bodeanu et al., 2004; Petrova & Velikova, 2004; Vershinin et al., 2004; Terenko & Terenko, 2008, 2012).

In 1996 two *Pseudo-nitzschia* blooms were reported in Turkish waters (Bay of Sinop) - in April of *P. delicatissima* (90×10^6 cells l^{-1}) and in July – of *P. pungens* (1.2×10^6 cells l^{-1}) (Turkoglu & Koray, 2002). Along the Georgian coast, a spring bloom of *P. delicatissima* in May 2009 was documented (Moncheva et al., 2019).

Interestingly, besides the numerous *Pseudo-nitszchia* outbreaks in the Black Sea coastal waters a massive spatially distributed winter bloom of *P. pseudodelicatissima* (4.19×10^6 cells l^{-1}) was detected in the open sea (Mikaelyan, 1995).

Generally, during the investigated period more bloom events have been registered in the coastal waters of Bulgaria, Romania and Ukraine with a large proportion reaching the highest cell abundances (> 5.5×10^6 cells l^{-1}) (Fig. 1). Blooms occurred in all seasons but more often they were registered in spring which is consistent with the general trend in the European waters (Hasle et al., 1996; Quiroga, 2006). On a decadal time scale, an increasing trend was observed in the second and third period in comparison with the pristine period (Table 1). However, it is difficult to generalize all these findings because as it was underlined the results are based on the accessible literature data for the different Black Sea regions.

Our long term data (1959-2016) in Varna Bay, Bulgarian waters show that even if the highest bloom density was reported in the period between 1959 and 1970 (13.5x10^6 cells l^{-1}) no clear trend in *Pseudo-nitzschia* blooms frequency during the decades was emerging (Fig. 2).

Out of the recounted 20 outburst events in Varna Bay, in the relatively pristine period for the Black sea region (up to 1970) there were 5 cases (4 caused by *P. delicatissima* and 1 - by *P. seriata).* During the highly eutrophic stage of the ecosystem (up to mid 1990) the number of these events was 8 (3 caused by *P. delicatissima* and 5 - by *P. seriata* with max density 5.3x10^6 cells l^{-1} in 1988) and thereafter (by 2016) – 7 blooms were registered with max density of 4.8x10^6 cells l^{-1} in 2009 due to *P. delicatissima*). After 1999 *P. seriata* group did not proliferate to bloom densities, even if some peaks were observed (frequently co-occuring with *P. delicatissima*).

Our dataset based on more frequent sampling campaigns between 2012 and 2018 in Varna Bay (Bulgarian waters) also confirmed the persistence of *Pseudo-nitzschia* throughout the year and in all seasons, except for spring 2017 when the genus was not detected (Figs. 3 and 4). As a common trend, *P. delicatissima* and *P. seriata* frequently co-occurred with the first species dominating in terms of frequency and abundance also reported for Ukrainian waters (Terenko & Terenko, 2012).

Pseudo-nitzschia species diversity and distribution

Presently, nine *Pseudo-nitzschia* species (*P. delicatissima*, *P. pseudodelicatissima*, *P. pungens*, *P. seriata*, *P. calliantha*, *P. inflatula*, *P. fraudulenta*, *P. prolongatoides*, *P. linea*) and one variety (*P. pungens* var. *aveirensis*) were reported in the Black Sea with eight of them precisely identified with electron microscopy and/or molecular methods (Fig. 5, Table 2).

One of the first records of *Pseudo-nitzschia* (*P. delicatissima* and *P. pungens*) in the Black Sea dates back to the middle of the 20th century (Morozova-Vodyanitskaya, 1954; Proshkina-Lavrenko, 1955). Spatially, among *Pseudo-nitzschia* species *P. delicatissima* and *P. seriata* were the most frequent, found in the coastal areas of all Black Sea countries. Both species occurred at many sites in Bulgarian waters as the most abundant too, while *P. pseudodelicatissima* and *P. pungens* appeared concurrently present along the Russian coast (Vershinin et al., 2005). *Pseudo-nitzschia pungens, P. pseudodelicatissima* and *P. delicatissima* were observed in Turkish waters in high concentrations (Turkoglu & Koray, 2002). In addition, *P. seriata* and *P. pseudodelicatissima* were found in open waters (Mikaelyan, 1995). Unlike the dominant *Pseudo-nitzschia*, some species have more restricted spatial distribution, spotted only at single areas - *P. inflatula* only in Ukrainian coast, *P. prolongatoides* only in Turkish waters, and *P. linea* only in Bulgarian waters (Fig. 5, Table 2). Interestingly, *P. delicatissima* was identified in surface sediment samples from the Black Sea via metabarcoding (Dzhembekova et al., 2018), although the information for the existence of resting stages in *Pseudo-nitzschia* spp. is rather contradictory and there is only one single report (Orlova & Morozova, 2009).

Generally, diversity within the genus is difficult to assess because of the complicated morphological identification under light microscopy, and the associated high uncertainty of the LM based data at a lower taxonomic level. Thus, the application of more sophisticated methods like scanning or transmission electron microscopy and/or molecular techniques is crucial for revealing the extant species diversity, including delineating cryptic and pseudo−cryptic species (Lelong et al., 2012). For example, on the basis of more advanced methods for identification (electron microscopy and molecular analyses) *P. calliantha* was defined in coastal waters of Turkey (Bargu et al., 2002), Romania (Lundholm et al., 2003) and Ukraine (Besiktepe et al., 2008; Ryabushko et al., 2008; Terenko & Terenko, 2012) (through the application of SEM and TEM) and in Bulgarian coast (via PCR amplification of LSU fragments with genus specific primers) (Dzhembekova et al., 2017a). The molecular technique has also allowed the determination of *P. pungens* var. *aveirensis* in Turkish (Baytut, 2013) and Bulgarian (Dzhembekova et al., 2017a) coastal waters. Worth to be noted, *P. calliantha* is comparatively newly described species within *P. pseudodelicatissima* complex (Lundholm et al., 2003). Altogether, the data reviewed suggest that the correct identification of the species reported earlier on the base of LM requires revision and re-examination as proved in other studies (e.g. McDonald et al., 2007; Terenko & Terenko, 2012; Rhodes et al., 2013; Ruggiero et al., 2015; Nagai et al., 2017).

Table 1. Number of *Pseudo-nitzschia* bloom events recorded within different periods and seasons in the Black Sea (1959-2019) (based on our own published and unpublished data and literature review data cited in the text).

Season	up to 1970	1971-1999	after 2000
Spring (April – June)	5	9	17
Summer (July – September)	0	7	1
Autumn (October - December)	1	1	1
Winter (January – March)	1	3	0

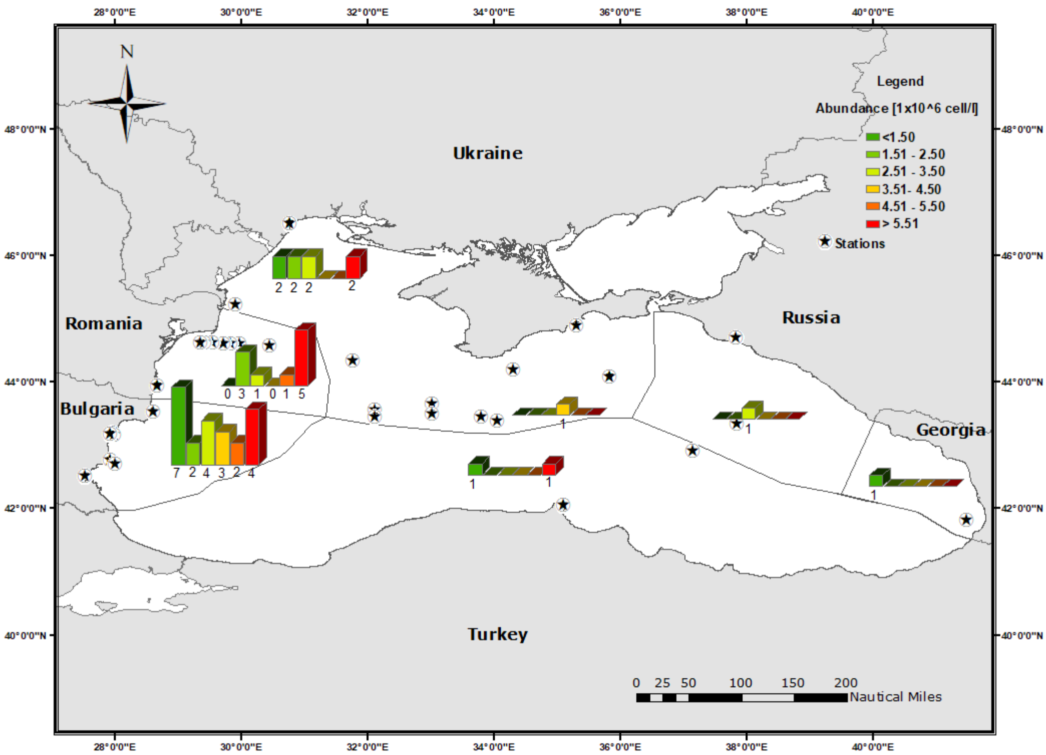

Fig. 1. Record of long-term *Pseudo-nitzschia* bloom events in the Black Sea countries marine waters (1959-2019) (based on our own published and unpublished data and literature review data cited in the text); the color denote cell abundance range ($1x10^6$ cells l^{-1}) and the columns - blooms frequency (number of events) within the corresponding abundance range; only the stations with bloom records are presented.

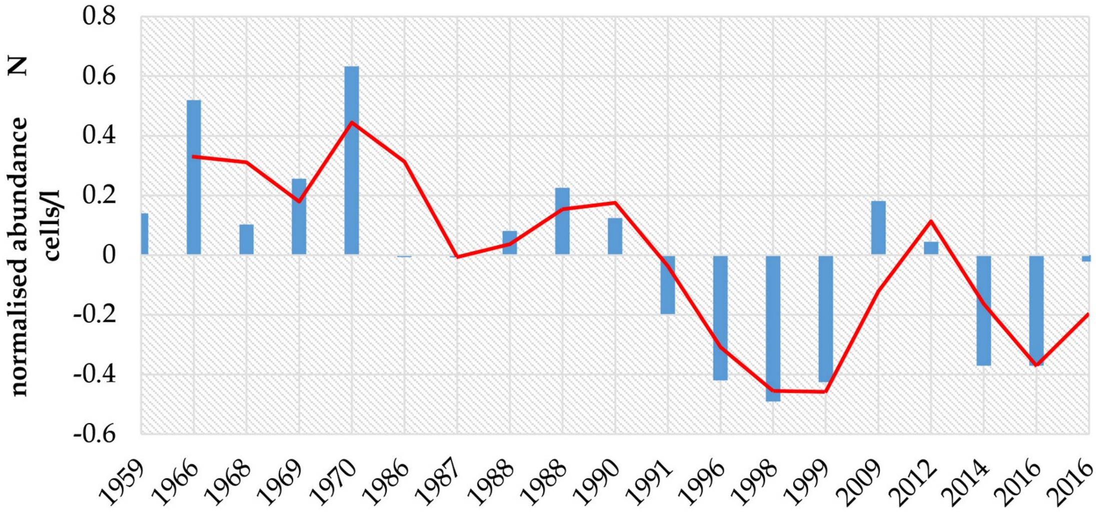

Fig. 2. *Pseudo-nitzchia* long-term (1959-2016) blooms variation (normalized abundance, N cells/l) in Varna Bay (Bulgarian waters); the red line denote the 2 period moving average trendline (own and literature data).

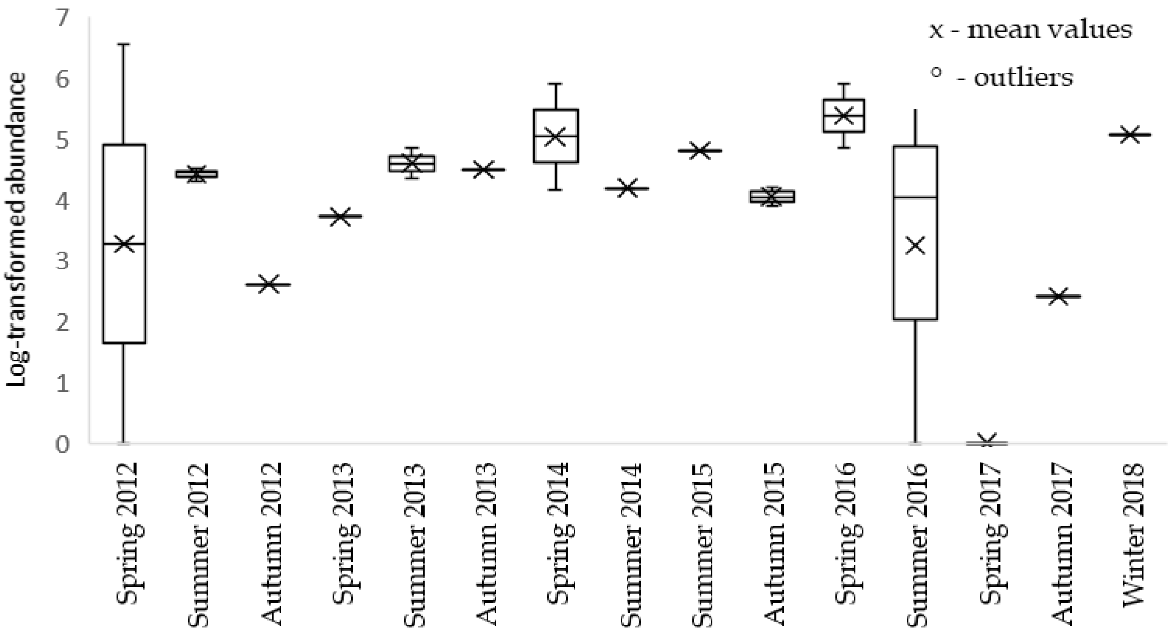

Fig. 3. Box plot - seasonal dynamics of *P. delicatissima* group abundance (cells/l, log-transformed) in Varna Bay (Bulgarian waters) in the period 2012-2018 (own data).

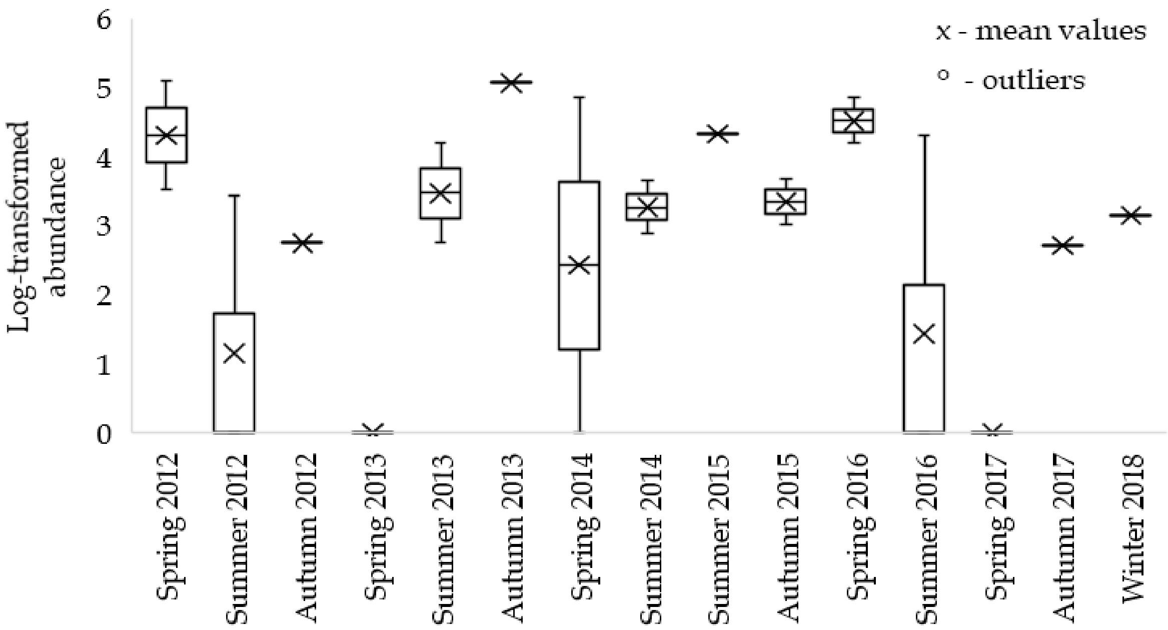

Fig. 4. Box plot - seasonal dynamics of *P. seriata* group abundance (cells/l, log-transformed) in Varna Bay (Bulgarian waters) in the period 2012-2018 (own data).

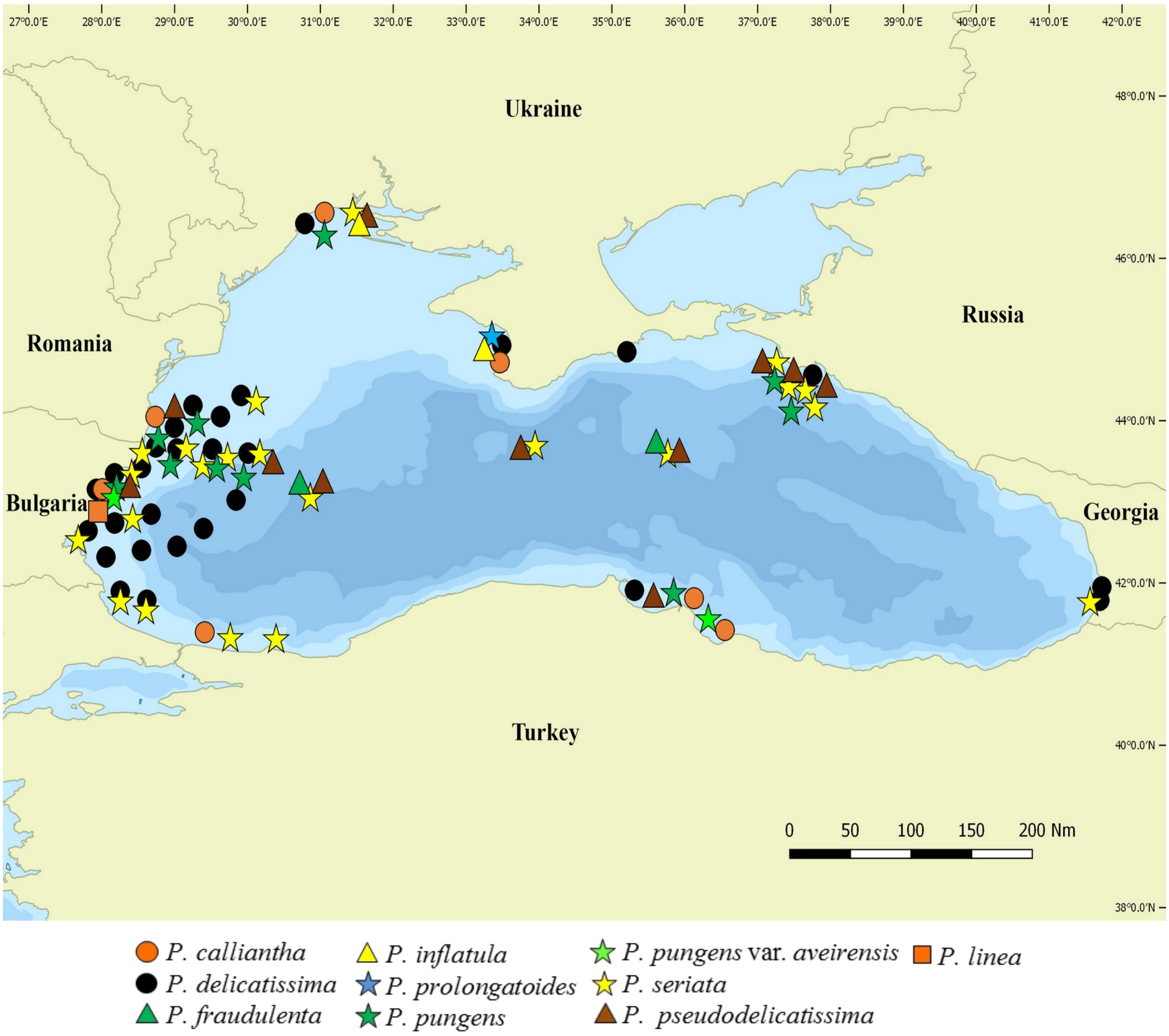

Fig. 5. *Pseudo-nitzschia* species distribution in the Black Sea.

Table 2. Inventory of Pseudo-nitzschia species, toxicity and distribution in the Black Sea. *Legend:* * Scanning Electron Microscopy (SEM); ** Molecular identification; [1] on the basis of cell width (according to Hasle & Syvertsen, 1997); BG – Bulgaria; GA – Georgia; RO – Romania; RU – Russia; TR – Turkey; UA – Ukraine.

Species	Group[1] (cell width)	Toxicity in the BS	Reference for toxicity	Distr.	Reference for distribution
P. calliantha **Lundholm, Moestrup and Hasle, 2003** ●	*delicatissima* (1.4–1.8 µm)			BG	Dzhembekova et al., 2017a**
				RO	Lundholm et al., 2003*
				TR	Bargu et al., 2002*; Baytut et al., 2013*

		Yes maximum value of 0.95 pg DA cell^{-1}	Ryabushko et al., 2008 Besiktepe et al., 2008	UA	Ryabushko et al., 2008*; Besiktepe et al., 2008*; Terenko & Terenko, 2012*
P. delicatissima **(Cleve) Heiden, 1928** ●	*delicatissima* (ca. 2 µm)	Not tested	-	BG	Petrova-Karadzhova, 1973; Moncheva et al., 1995; Moncheva et al., 2001; Petrova & Gerdzhikov, 2012
				GA	Gvarishvili et al., 2010
				RO	Bodeanu, 2002; Cărăuş, 2012
				RU	Yasakova, 2013
				TR	Turkoglu & Koray, 2002
				UA	Kuzmenko, 1995; Terenko & Terenko, 2012*
P. fraudulenta **(Cleve) Hasle, 1993** ▲	*seriata* (4.5-6.5 µm)	Not tested	-	UA	Ratkova et al., 1989
P. inflatula **(G.R.Hasle) G.R.Hasle, 1993** △	*delicatissima* (1.5-2.5 µm)	Not tested	-	UA	Senicheva, 2002; Terenko & Terenko, 2012*
P. prolongatoides **(G.R.Hasle) G.R.Hasle, 1993** ★	*delicatissima* (0.5-2.5 µm)	Not tested	-	TR	Ozturk, 1998
P. pseudodelicatissima **(Hasle) Hasle, 1993** ▲	*delicatissima* (1.5-2.5 µm)	No	Vershinin et al. 2005	BG	Dzhembekova & Moncheva, 2014; Dzhembekova et al., 2017b**
				RU	Mikaelyan, 1995*; Vershinin et al., 2005*
				TR	Baytut et al., 2010
				UA	Ratkova et al., 1989; Terenko & Terenko, 2012*
P. pungens **(Grunow ex Cleve) G.R.Hasle, 1993** ★	*seriata* (3.0-4.5 µm)	Not tested	-	BG	Dzhembekova & Moncheva, 2014; Dzhembekova et al., 2017b**
				RU	Vershinin et al., 2005
				TU	Turkoglu & Koray, 2002; Baytut et al., 2010
				UA	Terenko & Terenko, 2012*
P. pungens **var. *aveirensis* Lundholm, Churro, Carreira and Calado, 2009** ★	*seriata* (2.7-3.7 µm)	Not tested	-	BG	Dzhembekova et al., 2017a**
				TR	Baytut et al., 2013**
P. seriata **(Cleve) H.Peragallo, 1899** ☆	*seriata* (5.5-8.0 µm)	Not tested	-	BG	Petrova & Skolka, 1963; Petrova & Gerdzhikov, 2012; Dzhembekova & Moncheva, 2015
				RO	Cărăuş, 2012

				RU	Ratkova et al., 1989; Vershinin & Moruchkov, 2003; Mikaelyan, 1995*;Yasakova, 2013
				TR	Uysal, 2002
				UA	Ryabushko, 2003; Terenko & Terenko, 2012*
				GA	Komakhidze & Mazmanidi, 1998
***P. linea* Lundholm, Hasle & G.A.Fryxell, 2002**	*delicatissima* (1.8-2.2 µm)	Not tested	-	BG	Dzhembekova et al., 2017a**

Toxicity of Pseudo-nitzschia in the Black Sea

The available information on *Pseudo-nitzschia* toxicity in the Black Sea is very limited. DA detection was based either on single tests of *Pseudo-nitzschia* cultures (Besiktepe et al., 2008) or fragmentary studies on shellfish extracts and water samples (Peneva et al., 2011; Peteva et al., 2018). Toxic *P. calliantha* strains were isolated from Sevastopol Bay and DA was detected in batch culture with variable concentrations through the growth cycle and maximum (0.95 pg DA cell^{-1}) observed during the early exponential phase (Besiktepe et al., 2008). Among the other species, only *P. pseudodelicatissima* has been tested for toxicity but the examined strains proved non-toxic (Vershinin et al., 2005). The analysis of mussels tissue samples (*Mytilus galloprovincialis*) from aquaculture farms along the Bulgarian Black Sea coast (2009-2011) indicated that none of the tested 15 samples exceeded the regulatory level for DA of 20 mg kg^{-1} in shellfish (Peneva et al., 2011), the measured DA concentrations ranged from not detected to 0.55 mg kg^{-1}. During a later study (spring 2017) DA below the threshold was found in all tested samples of wild and farmed mussels, and plankton net samples (Peteva et al., 2018), however without targeting the source species. Globally, among all *Pseudo-nitzschia* species identified in the Black Sea, six (*P. delicatissima*, *P. pseudodelicatissima*, *P. calliantha*, *P. fraudulenta*, *P. pungens* and *P. seriata*) were reported as capable of DA production, two (*P. inflatula*, *P. linea* and *P. pungens* var. *aveirensis*) as non-toxic and one (*P. prolongatoides*) has not been tested yet. Some authors support the hypothesis that all species within the genus could be toxigenic under specific conditions (Parsons et al., 1999; Wells et al., 2005) and new reports confirmed the induction of DA production by different factors e.g, environmental conditions, zooplankton grazers, life cycle (Lema et al., 2017; Lundholm et al., 2018; Sauvey et al., 2019). Intraspecific diversity of toxin production requires more investigations to be carried out at a local level. Although globally the negative effects of *Pseudo-nitzschia* related toxicity have been prevented thanks to the increased research and effective implementation of monitoring programs and adequate management measures (Trainer et al., 2012; Bates et al., 2018) there is still limited institutional and medical awareness of shellfish poisoning events in the Black Sea countries (Vershinin & Moruchkov, 2003).

Environmental variables and Pseudo-nitzschia abundance

GAM was applied, to assess the significance of *in situ* environmental variables association with *Pseudo-nitzschia* group abundance (*P. delicatissima* group and *P. seriata* group) in the Bulgarian Black Sea waters. The *in situ* matrix was constructed based on data for temperature (T), salinity (S), nitrates (NO_3), phosphates (PO_4) and silicon (Si), covering the period 1999-2015 (statistical summary of the data is given in Table 3).

P. delicatissima group abundance was modeled first by the implementation of the following GAM:

$$P_{del} = \alpha + f_1(T) + f_2(S) + f_3(NO_3) + f_4(PO_4) + f_5(Si) + \varepsilon$$

GAM results (Table 4, Fig. 6) showed that nonlinear effects of salinity, temperature and nitrates concentrations have had statistically significant effects on *P. delicatissima* abundance. The model explained roughly 60% of total variations in abundance data.

Double penalty approach was applied to assess the terms effects on *P. dellicatissima* group abundance (by penalizing the null space), a process considered also as selection of model terms (Fig. 7). The results confirmed that PO_4 and SiO_4 have zero effect on *P. dellicatissima* group abundance.

Two-way interaction terms were also included as tensor products with the aim to study the interaction terms effects:

$$P_{del} s(T)+s(S)+s(NO_3)+s(PO_4)+s(Si)+ti(T,NO_3)+ti(Si,NO_3)+ti(Si,PO_4)+\epsilon$$

Their inclusion had not improved the model (deviance explained 56.2%), however underlined statistically significant interaction term effects (T in a combination with NO_3 concentrations, and SiO_4 interaction with NO_3 and PO_4 concentrations), which are of interest for further study and exploration.

PO_4 and SiO_4 concentrations were found to have statistically significant nonlinear association with *P. seriata* group abundance and linear association with salinity. The model:

$$P_{ser}=\alpha+f_1(T)+f_2(S)+f_3(NO_3)+f_4(PO_4)+f_5(Si)+\varepsilon$$

explained 31.5% of the deviance (i.e. the proportion of variance in *P. seriata* group abundance, explained by the predictor variables) (Table 4).

Stepwise model selection was implemented aiming at improving the model along with inclusion of linear terms for temperature salinity and nitrates, 2-way interaction terms were also included in the model, however neither of these model selection techniques provided better results. Double penalty approach was applied to provide insights on terms effects strength on *P. seriata* group abundance, showing that NO_3 concentration had zero effect and the effect of temperature remains unexplained by the model.

Worth noting, *Pseudo-nitzschia* abundance variations remained unexplained by the models could well be attributed to the uncertainty associated with the species identification, discussed above, as well as to the lack of systematic sampling frequency to follow the evolution of phytoplankton bloom dynamics during the outburst events. Unaccounted environmental noise could also have affected the results as it naturally contributes to data uncertainty, regardless of the robustness of GAM.

Worldwide, the growth and distribution of *Pseudo-nitzschia* has been linked to various environmental parameters and the relationship differs among species and regions. In Scottish waters, *P. seriata* group was linked to temperature, whereas *P. delicatissima* group was linked to salinity and the presence of ammonium (Bresnan et al., 2015) and seasonally *P. delicatissima* group dominate in spring, while *P. seriata* group dominate in the late summer/early autumn (Fehling et al., 2006). In the southern North Sea the highest abundance of *P. seriata* complex was observed at low nutrient levels (particularly DIN and $Si(OH)_4$), increasing surface solar irradiance and high pH, and the *P. seriata* complex seemed to tolerate higher variable environmental conditions (e.g. nutrients) as compared to the *P. delicatissima* complex (Delegrange et al., 2018). Ajani et al. (2013) found that in the coastal waters of south-eastern Australia *P. seriata* group abundance was temperature dependent, whereas *P. delicatissima* group development might be triggered by complex interactions between light availability and water temperature. In addition, the combination of factors related to *Pseudo-nitzschia* group abundance is species specific. For example, *P. pseudodelicatissima* growth was favored by high temperatures and salinities between 15 and 35‰ in Danish coastal waters (Lundholm et al., 1997). *P. calliantha*, *P. pseudodelicatissima* and *P.*

delicatissima abundance was positively correlated with nitrates in the Adriatic Sea and in the Bay of Fundy, and moreover in the Adriatic Sea a positive correlation was found between *P. delicatissima* abundance and salinity, whereas the densities of *P. calliantha* were negatively correlated with temperature (Caroppo et al., 2005; Kaczmarska et al., 2007). In the Marmara sea (Golden Horn estuary) for *P. calliantha* a significant negative correlation was found with temperature and a significant positive correlation with salinity, and in addition the species was correlated with NO_3 + NO_2 (Tas and Lundholm, 2017). *P. americana* and *P. australis* were positively correlated with high chlorophyll a, temperature and $Si(OH)_4$ concentrations, whereas *P. delicatissima* and *P. fraudulenta* were more abundant under high NO_3 and low $Si(OH)_4$ concentrations in samples from the Baie des Veys (Klein et al., 2010). *P. pungens* was more abundant in samples with higher concentrations of phosphates and lower concentrations of nitrates in the Bay of Fundy (Kaczmarska et al., 2007). In addition the association of any species to a single or a combination of environmental variables is further complicated by the reported intrinsic wide morphological and morphometrical variability as observed in the *P. pungens* clade I population from the Adriatic Sea (Accoroni et al., 2020). Although the relationships between environmental variables and *Pseudo-nitzschia* abundance are complex and vague, the knowledge at a regional scale is valuable allowing *Pseudo-nitzschia* bloom dynamics to be modelled and predicted (Anderson et al. 2009; Lane et al. 2009; Palma et al. 2010).

In the Black Sea, the exact mechanisms of *Pseudo-nitzschia* blooms are still uncertain and complex and *Pseudo-nitzschia* could proliferate under multiple environmental regimes (Terenko & Teremko, 2012; Dzhembekova & Moncheva, 2015). Suggesting that the *Pseudo-nitzschia* dynamics is controlled by a complex of environmental factors unique to a particular region (Trainer et al., 2012) future more comprehensive study could enable more robust conclusions for the effects of environmental parameters on growth and DA production at a local scale.

Table 3. Summary of descriptive statistics of environmental variables and *Pseudo-nitzschia* abundance (1999-2015) (own data).

Variable	Mean	SE Mean	StDev	Min	Median	Max
***P. delicatissima* group (cells l^{-1}), n=141**	87,643	30,297	359,751	5,101	27,549	3,495,680
T [C°]	22.07	0.34	4.03	7.67	23,005	26.27
S [‰]	16.87	0.08	0.96	13.34	17.02	18.53
NO_3 [µmol l^{-1}]	0.46	0.05	0.57	0	0.28	3.71
PO_4 [µmol l^{-1}]	0.14	0.02	0.19	0	0.09	1.76
Si [µmol l^{-1}]	3.27	0.38	4.5	0	2.48	46.4
Variable	**Mean**	**SE Mean**	**StDev**	**Min**	**Median**	**Max**
***P. seriata* group (cells l^{-1}), n=102**	19,152	3,307	33,397	1,031	9,311	211,355
T [C°]	22.1	0.39	3.91	8.21	22.76	26.27
S [‰]	16.9	0.09	0.89	14.17	17.04	18.15
NO_3 [µmol l^{-1}]	0.44	0.07	0.73	0	0.21	3.79
PO_4 [µmol l^{-1}]	0.15	0.02	0.2	0	0.09	1.56
Si [µmol l^{-1}]	3.38	0.22	2.2	0	2.93	9.26

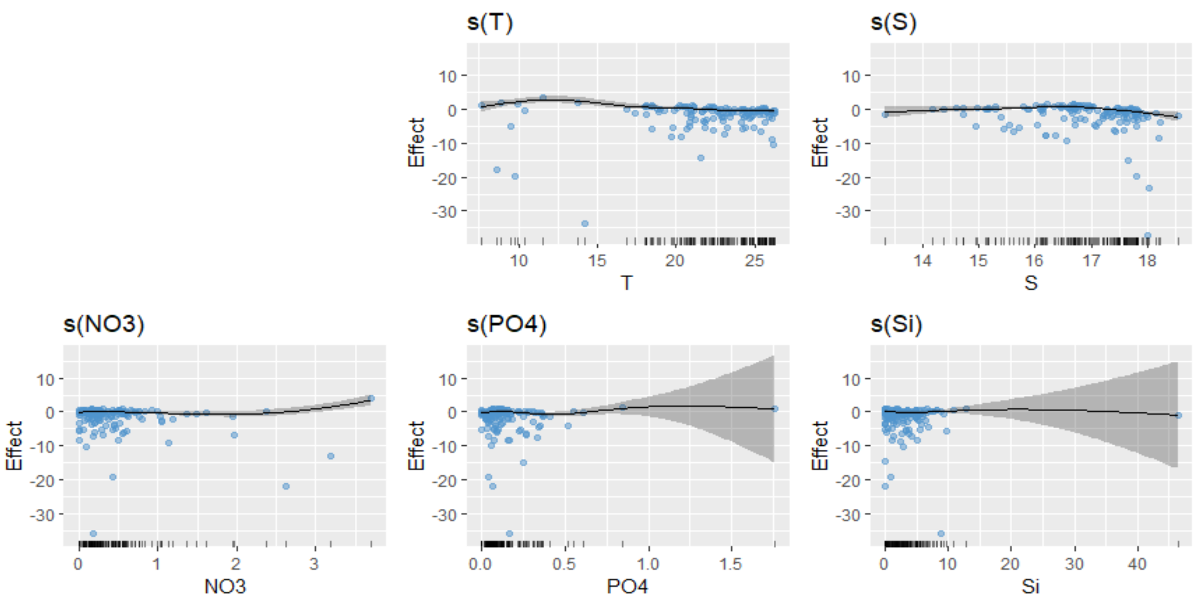

Fig. 6. Smooths of GAM terms showing the effect of various environmental variables on *P. dellicatissima* group abundance. Locations of observations are shown as vertical lines on the x-axes, the blue dots show the observations partial residuals. Solid lines are the estimates of the smooths; the dashed lines indicate 95% confidence intervals.

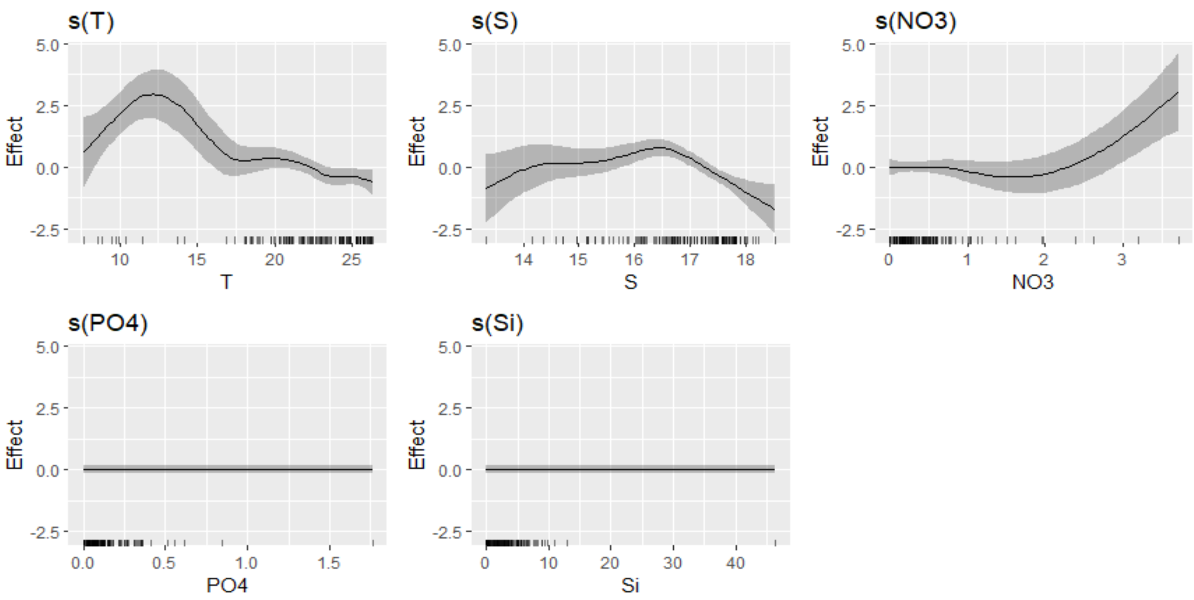

Fig. 7. *P. dellicatissima* group abundance GAM model terms effects evaluation after the implementation of double penalty approach.

Table 4. GAM model statistical summary (*P. delicatissima* group).

Family: Negative Binomial(1.096), Link function: log
Formula: Pdel ~ s(T) + s(S) + s(NO_3) + s(PO_4) + s(Si)

Parametric coefficients:
Estimate Std. Error z value Pr(>|z|)
(Intercept) 10.70098 0.08044 133 <2e-16 ***
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Approximate significance of smooth terms:

	edf	Ref.df	Chi.sq	p-value	
s(T)	4.888	5.899	39.117	3.77E-06	***
s(S)	4.118	5.09	32.012	7.48E-06	***
s(NO_3)	3.669	4.51	18.653	0.00165	**
s(PO_4)	4.003	4.818	10.027	0.09241	.
s(Si)	2.445	2.956	3.275	0.39121	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
R-sq.(adj) = 0.511 Deviance explained = 59.8%
-REML = 1681.3 Scale est. = 1 n = 141

Table 5. GAM model statistical summary (*P. seriata* group).

Family: Negative Binomial(1.096), Link function: log
Formula:Pser ~ s(T) + s(S) + s(NO3) + s(PO4) + s(Si)

Parametric coefficients:
Estimate Std. Error z value Pr(>|z|)
(Intercept) 9.64181 0.09489 101.6 <2e-16 ***
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Approximate significance of smooth terms:

	edf	Ref.df	Chi.sq	p-value	
s(T)	1.500	1.817	2.646	0.18244	
s(S)	1.001	1.001	4.777	0.02888	*
s(NO3)	1.000	1.001	1.339	0.24727	
s(PO4)	2.209	2.661	8.207	0.03978	*
s(Si)	4.339	5.333	18.347	0.00347	**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
R-sq.(adj) = 0.068 Deviance explained = 31.5%
-REML = 1100.6 Scale est. = 1 n = 102

Conclusions

In summary, *Pseudo-nitzschia* blooms are a common feature of Black Sea waters with four out of nine species proliferating in concentrations over 1×10^6 cells l^{-1}. *Pseudo-nitzschia* blooms are more likely to occur in spring but the genus representatives grow under different environmental conditions and may bloom at any time of the year. The regional differences in the *Pseudo-nitzschia* species diversity are likely to be related to the arduous morphological identification that could result in incorrect assessment of species composition. Despite the occurrence of high *Pseudo-nitzschia* spp. counts in some regions, no shellfish data exceeding the DA regulatory limit or ASP related health problems have been diagnosed. Given the uncertainties associated with the species identification, the scarcity of toxicity measurements during bloom events and the irregularity of environmental monitoring leave the answer to the question about the potential ecosystem and human risk pending. Hence, the application of advanced identification techniques, technologically adequate regular targeted monitoring of *Pseudo-nitzschia*, complemented by toxin analysis would be essential both to improve the understanding of *Pseudo-nitzschia* bloom dynamics in the Black Sea and for implementation of adequate HAB management to protect public and ecosystem hazards.

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