Temporal and spatial variability of the potentially toxic *Pseudo-nitzschia* spp. in a eutrophic estuary (Sea of Marmara)

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Spatial and temporal variability and bloom formation of the potentially toxic diatom Pseudo-nitzschia *spp. was investigated weekly to monthly from October 2009 to October 2010 in a eutrophic estuary, the Golden Horn.* Pseudo-nitzschia *spp. were detected in 195 of 512 samples (38%) collected throughout the year. Two species,* P. calliantha *and* P. pungens*, were identified based on the SEM examination. Blooms of* Pseudo-nitzschia *occurred in the lower and middle estuary in January and May. The bloom in January mainly comprised* P. calliantha. *In the bloom in early May,* P. calliantha *made up 72% of the* Pseudo-nitzschia *cells and* P. pungens *28%. However, the contribution of* P. pungens *increased to 83% in late May. The* Pseudo-nitzschia *blooms occurred at low temperature (9–15°C) and moderate salinity (17–18), and for* P. calliantha *a significant negative correlation was found with temperature and a significant positive correlation with salinity. The percentage of* Pseudo-nitzschia *cells decreased gradually from lower to upper estuary (59–14%), correlating with a decrease in Secchi depth (5.5–0.5 m). Principal components analyses (PCA) were used to explore the spatial and temporal variability of environmental factors in relation to* Pseudo-nitzschia *abundances, and showed that NH_4, pH, Secchi depth and DO values were the most important factors reflecting spatial differences, while temperature, salinity, Chl-a and Si:N were more important factors showing temporal differences. High abundances of* P. pungens *correlated mainly with pH, Secchi depth and DO values, whereas* P. calliantha *also correlated with $NO_3 + NO_2$. Low light availability due to high concentrations of suspended material and very variable environmental conditions (e.g. pH, DO and NH_4) may have limited growth of* Pseudo-nitzschia *in the upper estuary. Regular monitoring of* Pseudo-nitzschia *is important for improving the understanding of the influence of environmental parameters on bloom dynamics in the study area.*

Keywords: Diatom, *Pseudo-nitzschia*, bloom, light limitation, estuary, Sea of Marmara

INTRODUCTION

Estuaries are known as highly productive ecosystems, being often nutrient-rich and having multiple sources of organic carbon to sustain populations of heterotrophic organisms. Phytoplankton production may, however, be very low in river-dominated estuaries with high turbidity caused by river inputs of suspended particulate matter (SPM) and/or resuspension of bottom sediments. High SPM concentrations in estuaries result in rapid attenuation of light in the water column and phytoplankton photosynthesis is thus confined to a shallow photic zone. As a consequence, phytoplankton dynamics (including productivity and spatial and temporal changes in biomass) will be largely controlled by light availability (Cloern, 1987). Peterson & Festa (1984) concluded that estuarine productivity becomes strongly depressed as SPM concentration increases from 10 to 100 mg l⁻¹.

Under certain circumstances, some phytoplankton species can form high-biomass and/or toxic blooms, thereby causing harm to aquatic ecosystems (Kudela *et al.*, 2015). Marine diatoms of the genus *Pseudo-nitzschia* H. Peragallo have been confirmed as producers of domoic acid (DA), the toxin responsible for amnesic shellfish poisoning (ASP) (Trainer *et al.*, 2012). Domoic acid may enter the food chain via zooplankton or filter-feeding shellfish and subsequently accumulate in, for example, marine invertebrates, birds and mammals, and harmful effects have been documented in marine birds, mammals and humans (Trainer *et al.*, 2012; Lelong *et al.*, 2012). Presently, at least 16 *Pseudo-nitzschia* species and two *Nitzschia* species have been found to produce DA (Smida *et al.*, 2014; Teng *et al.*, 2014).

Pseudo-nitzschia species have been commonly observed in studies exploring phytoplankton distribution in Turkish coastal waters (Koray, 1995; Eker & Kıdeyş, 2000; Polat *et al.*, 2000; Türkoğlu & Koray, 2002; Balkis, 2003; Deniz & Tas, 2009; Tas *et al.*, 2009; Tas & Okus, 2011, 2014). Harmful algal blooms, including *Pseudo-nitzschia*, have been reported in Turkish coastal waters (Türkoğlu & Koray, 2002; Koray, 2004). Blooms of *P. delicatissima* (April) and *P. pungens* (late July to mid-August) were observed in Turkish coastal waters of the southern Black Sea (Türkoğlu & Koray, 2002). No negative impacts have been reported during the blooms, but no shellfish or fish farms presently exist in the area. The *Pseudo-nitzschia* species mentioned above have seldom been identified to species level, and not using

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state of the art methods like electron microscopy or molecular methods, except for *P. calliantha* Lundholm, Moestrup & Hasle which has been recorded from the Black Sea (Bargu *et al.*, 2002). *Pseudo-nitzschia calliantha* is a toxin-producing species (Lundholm *et al.*, 2003) which has been found widely from the Baltic Sea to the Adriatic Sea and Black Sea, indicating a cosmopolitan distribution (Lundholm *et al.*, 2003). In a previous study carried out in the Golden Horn Estuary (GHE), *P.* cf. *delicatissima* and *P.* cf. *pungens* were commonly observed (Tas *et al.*, 2009), but these identifications were based on light microscopy only.

Pseudo-nitzschia blooms have been associated with environmental conditions such as upwelling (Trainer *et al.*, 2000, 2002), coastal runoff or discharges (Smith *et al.*, 1990), low Si:N (Sommer, 1994) or submarine groundwater discharge (Liefer *et al.*, 2009), all indicating that *Pseudo-nitzschia* thrive at nutrient repletion, but that the nutrient ratios are also important (Trainer *et al.*, 2012). Anthropogenic eutrophication has in the last decades affected the ecosystem of GHE considerably. The effects of eutrophication on the GHE ecosystem including the phytoplankton should be considered to evaluate the ecosystem change.

The unplanned urbanization, increase in settlements and industrial facilities around the GHE since the 1950s have caused a high degree of pollution, particularly from wastewaters of pharmaceutical, detergent, dye and leather industries and domestic discharges. By the early 1990s, estuarine life was limited to the lower estuary, and the upper estuary had almost no eukaryotic phytoplankton due to anoxia and heavy sedimentation. The GHE became a severely polluted environment, where the water column was characterized by frequent anoxic episodes (Kıratlı & Balkıs, 2001). In 1997, the Rehabilitation Project in the GHE was initiated in order to improve water quality by reducing nutrient loading. For this purpose, surface discharges were gradually taken under control, and 4.25×10^6 m^3 of anoxic sediment was removed from the completely filled upper estuary, resulting in an increase of at least 5 m in depth in this region, and a bridge floating on pontoons was partially opened in order to provide possibilities for surface water circulation. These changes resulted in rapid renewal of water and oxygenation of the anoxic sediment of the highly eutrophic upper estuary (Yüksek *et al.*, 2006).

The present study aimed to investigate the spatial and temporal variability as well as bloom formation of potentially toxic *Pseudo-nitzschia* spp. in relation to environmental parameters and to understand better which factors are important for the presence of these organisms in the GHE.

MATERIALS AND METHODS

Location

The study area (GHE) is located south-west of the Strait of Istanbul extending in a north-west–south-east direction and is ∼7.5 km long and 700 m wide, with a surface area of 2.6 km^2 (Figure 1). The study area is divided in three parts based on the hydrographic structure: lower (LE), middle (ME) and upper estuary (UE). The LE is the deepest section (40 m) and it is strongly influenced by interaction with the Strait of Istanbul (Bosphorus). The depth rapidly decreases to 14 m in the ME, where a bridge operating on buoys
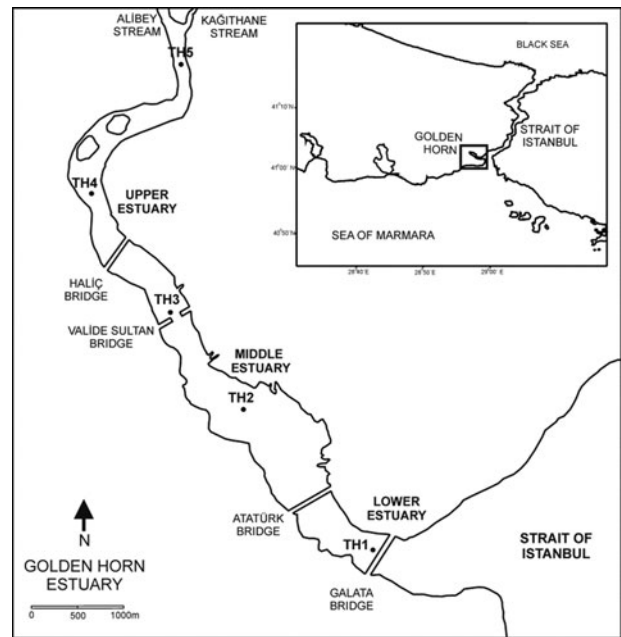

Fig. 1. The study area and sampling stations (TH1 to TH5). Insert shows the position of the GHE.

limits the upper layer circulation between the LE and the UE. The UE has a depth of 4 m due to a high degree of sedimentation (Figure 1). Although the two streams (Alibey and Kağıthane) carry fresh water to the estuary, the amount of the flow decreased remarkably by the end of 1990s following the construction of a series of dams. Therefore, the main source of the fresh water flowing into the GHE is rainfall (Sur *et al.*, 2002). The lower part of the GHE is characterized by a two-layered stratification similar to the neighbouring Strait of Istanbul, whose upper layer has a salinity of ∼18 originating from the Black Sea and lower layer, with a salinity of ∼38, originating from the Mediterranean Sea (Özsoy *et al.*, 1988).

Seawater sampling and analysis

Seawater samples were collected monthly (October 2009 to March 2010) or weekly (April 2010 to September 2010) at five sampling stations (Figure 1) from 0.5, 2.5 and 5 m depth using 5 l Niskin bottles. The parameters salinity, temperature, dissolved oxygen (DO) and pH were measured using a multi-parameter probe (YSI Incorporated Professional Pro Plus), and light transparency was measured using a Secchi disc. Chl-*a* analyses were carried out by an acetone extraction method according to Parsons *et al.* (1984). Inorganic nutrient ($NO_3 + NO_2$, NH_4, PO_4, SiO_2) analyses were measured using a Bran + Luebbe AA3 auto-analyser according to standard methods (APHA, 1999). All data were afterwards used as the average of values from 0.5, 2.5 and 5 metres.

Water samples (250 ml) for phytoplankton counts were taken from the Niskin bottle samples, and preserved with acidic Lugol's solution (2%). Aliquots of 20 ml were left to settle overnight in Utermöhl sedimentation chambers (Utermöhl, 1958). The cell counts were performed at 200 × magnification using a Leica DM IL LED inverted microscope equipped with phase contrast optics. At least 300 phytoplankton cells (>20 μm) or two or more transects in the settling chamber were counted.

Measurements of cell dimensions, and observations of cell shape and chain formation were mainly done using a Leica DM 2500 LM light microscope at needed magnifications. The percentage of *Pseudo-nitzschia* cells of total phytoplankton abundance was calculated. All data were used as the average of 0.5, 2.5 and 5 m.

A total of 64 net samples for qualitative analyses of phytoplankton were collected using a Nansen plankton net (0.57 m diameter, 55 μm mesh size) towed vertically from 10 m to the surface of the LE and ME and preserved with borax-buffered formalin (4%). All net samples were collected simultaneously with the water samples.

Species identification

For routine observations of *Pseudo-nitzschia* species, a Leica DM 2500 LM light microscope with brightfield optics was used. For ultrastructural identification of *Pseudo-nitzschia* species, organic material was removed using an acid-wash treatment prior to SEM observations as described by Bargu *et al.* (2002). A total of 10 net samples corresponding to the bloom periods in January and May were used for species identification. Samples were concentrated onto 0.2 μm pore size isopore polycarbonate membrane filters (Millipore). Salt was removed from samples by rinsing with deionized water under low vacuum (150 mm Hg). To remove organic material, saturated $KMnO_4$ was added until the filters were covered and the samples were allowed to digest for 15 min. Twelve M HCl (3 ml) was then added to the samples and held for a total of 30 min to complete the oxidation process. Samples were then vacuumed gently and rinsed with deionized water. This process was repeated twice. The filters were air-dried in a desiccator for 24 h and then mounted onto SEM stubs with double-sided tape and sputter coated with gold palladium. All micrographs were taken with a field-emission SEM (FEI-QUANTA FEG 450) at an accelerating voltage of 10 kV. Species identification of phytoplankton was based on

Cupp (1943), Hendey (1964), Drebes (1974), Delgado & Fortuna (1991) and Tomas (1997). *Pseudo-nitzschia* species were morphologically identified based on SEM examination of the frustules (Lundholm *et al.*, 2012). At least 20 valves were studied in detail in each sample. In SEM, the morphometric parameters measured were: width and length of valves, densities of interstriae, fibulae and poroids, and structure of the poroids.

Data analysis

Principal components analyses (PCA) were used to explore the spatial and temporal variability of environmental factors to relate *Pseudo-nitzschia* abundances to environmental variability. Prior to all PCAs, environmental data were transformed to fourth root to reduce the heterogeneity in the data and to normalize the data matrix using Primer v6 program. The relationships among abundance of *Pseudo-nitzschia* and environmental parameters were analysed by Spearman rank correlation following transformations to natural logarithms by using PASW v18 program.

RESULTS

Morphology of *Pseudo-nitzschia* cells

Ultrastructural examination by SEM revealed that the bloom-forming *Pseudo-nitzschia* species obtained from field material in January was mainly *P. calliantha*, while in May both *P. calliantha* and *P. pungens* were observed. The cells of *P. calliantha* were linear in valve view and overlapping in colonies. Apical axis of valves ranged from 45 to 50 μm, transapical axis of valves was between 1.5 and 2.0 μm. Overlap of cells in chains were short (about one-ninth of cell length) (Figures 2 & 3). The fibulae were regularly spaced, 18–20 in 10 μm, and interrupted by a central nodule in the middle of
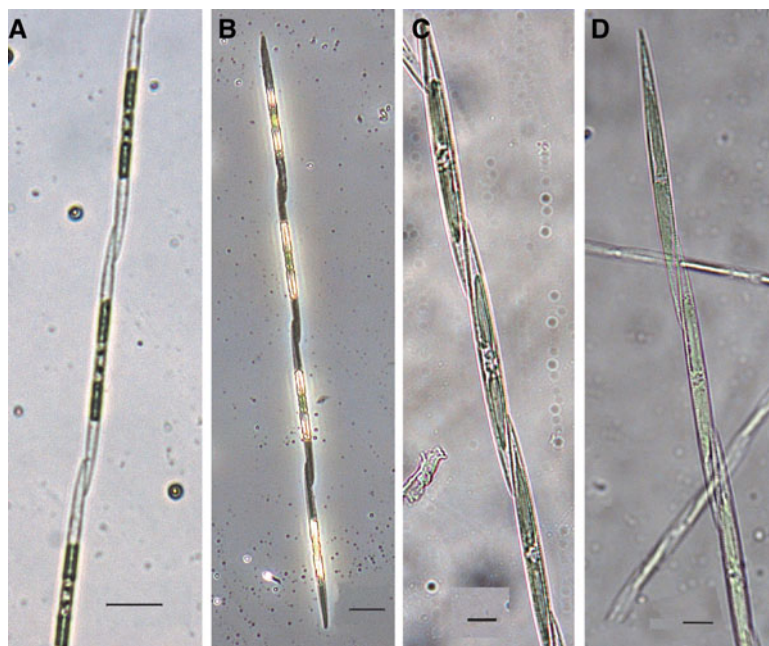

Fig. 2. Light micrographs of *P. calliantha* (A, B) and *P. pungens* (C, D) overlapping cells in colonies (Scale bars: 10 μm).
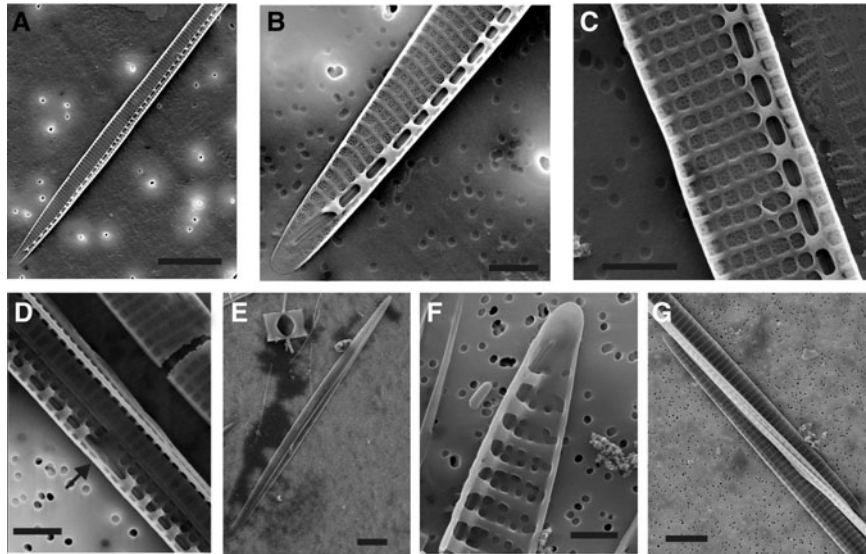
Fig. 3. Scanning electron micrographs of *P. calliantha* (A–D) and *P. pungens* (E–G) from the GHE. A large part of the valve (A), the tip of the valve (B), parts of the valve showing fibulae, interstriae and poroid structures (C) and a central nodule of *P. calliantha* (D). Whole valve (E), parts of the valve showing the ultrastructure (F) and overlapping valves of *P. pungens* (G). Scale bars: B, C, D and F: 1 μm; A, G: 5 μm; E: 10 μm.

the cell. The valves had 36–40 interstriae in 10 μm. The striae comprised one row of poroids, 5–6 poroids in 1 μm. Each poroid was divided in 4–10 sectors (Figure 3).

The cells of *P. pungens* were linear-lanceolate, and symmetrical about the apical axis. Overlap of cells in chains was considerable, close to one fourth of cell length (Figure 2). The apical axis ranged from 90 to 100 μm, and the transapical axis was 3.5–5.5 μm. The fibulae were regularly spaced with a density of 13–14 in 10 μm. The density of interstriae was 13–14 in 10 μm. The striae contained two rows of poroids, 4–5 poroids in 1 μm (Figure 3).

Cell abundance of *Pseudo-nitzschia* species

The major groups of phytoplankton in the study area were diatoms, dinoflagellates and other flagellates (silicoflagellates, chrysophytes, raphidophytes, cryptophytes, euglenophytes, prasinophytes and chlorophytes), constituting 54, 35.5 and 10.5%, respectively, of the total number of phytoplankton species. The most abundant species were the diatoms *Pseudo-nitzschia* spp., *Skeletonema marinoi*, *Ceratoneis closterium* (=*Cylindrotheca closterium*) and *Thalassiosira* sp., the dinoflagellates *Heterocapsa triquetra*, *Scrippsiella trochoidea* and *Prorocentrum cordatum* (=*Prorocentrum minimum*); the raphidophyte *Heterosigma akashiwo*, the euglenophytes *Euglena viridis* and *Eutreptiella marina*, the cryptophyte *Plagioselmis prolonga* and the prasinophyte *Pyramimonas grossii*.

Pseudo-nitzschia cells were detected in 195 of 512 water samples (38%) analysed. It was evident that *Pseudo-nitzschia* spp. were absent or occurred in low abundances in the UE, as seen in the abundance distribution over the entire study period (Figure 4). The frequency of *Pseudo-nitzschia* spp. in all water samples decreased considerably from LE (found in 59% of the examined samples) towards UE (14%), correlating with a decreasing Secchi depth (Figure 4).

Two *Pseudo-nitzschia* species, *P. calliantha* and *P. pungens*, were detected in the GHE during the study period. The two species were common in the LE and ME particularly during winter and spring. The abundance of *P. calliantha* started to

increase in December and reached bloom densities (average 1.2×10^6 cells l^{-1}) in January in the LE, and also high densities in the ME ($\sim 9 \times 10^5$ cells l^{-1}), whereas *P. pungens* was not observed in January (Figure 5). *Pseudo-nitzschia calliantha* made up 99% of the total phytoplankton in January in both the LE and ME (Figure 6). Only very low concentrations (20×10^3 cells l^{-1}) of *Pseudo-nitzschia* were observed in the UE in January. *Pseudo-nitzschia* spp. were almost absent in February and March, however in April, *P. calliantha* was detected again in the LE in sub-bloom densities (average 5.4×10^5 cells l^{-1}) (Figures 5 & 6). Concentrations were slightly lower in the ME and very low in the UE (Figure 5).

The second *Pseudo-nitzschia* bloom occurred in the ME during the first two weeks of May where cell densities reached an average of 1.1×10^6 cells l^{-1}. During the first week of this bloom, *P. calliantha* made up 72 and *P. pungens* 28% of the cell counts. An evident increase in the contribution of *P. pungens* took place during the third and fourth week of May, with *P. pungens* contributing with 40 and 83%, respectively, of the total *Pseudo-nitzschia* cell counts, with densities reaching 5.8×10^5 cells l^{-1} in late May (Figure 5). More or less the same appeared in May in LE although at lower cell densities and with a lower
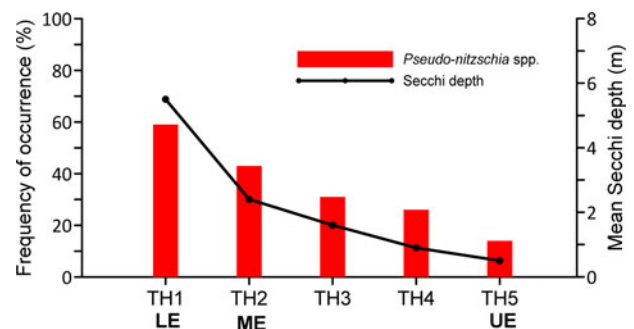

Fig. 4. The frequency of *Pseudo-nitzschia* occurrence and mean Secchi depths throughout the study area.
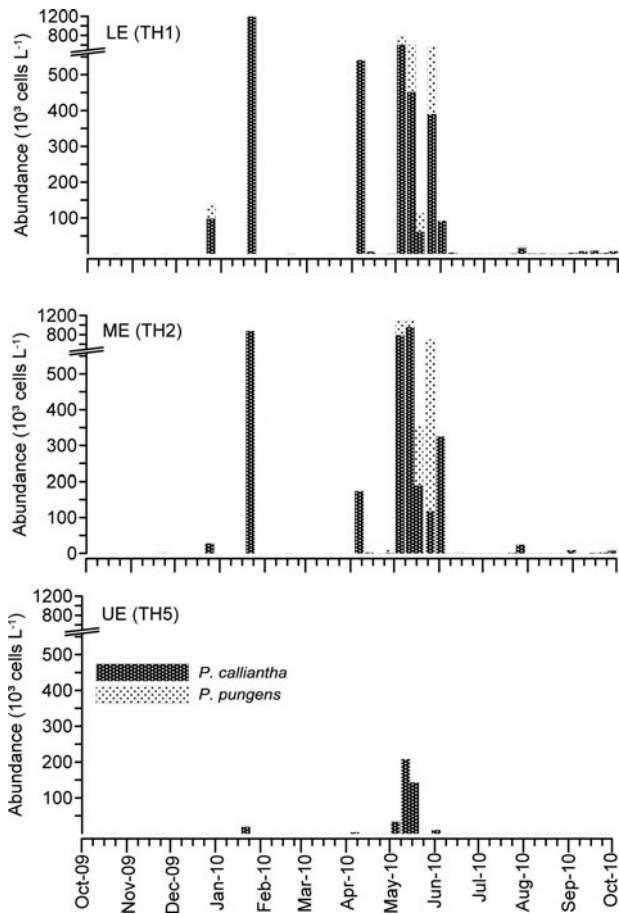
Fig. 5. The abundance of *Pseudo-nitzschia calliantha* and *P. pungens* throughout the study area.

Fig. 6. The contribution of *Pseudo-nitzschia* abundance to the total phytoplankton throughout the study area.

contribution of *P. pungens*. *Pseudo-nitzschia* cells were frequently observed until early June. The cell abundances decreased clearly from ME to UE and *Pseudo-nitzschia* spp. were observed in much lower densities in the UE than in the other parts of the estuary (Figure 5). During the winter bloom, cell densities were highest in the LE, while during the spring bloom they were highest in the ME (Figure 5).

The average annual contribution of diatoms to the total phytoplankton abundance was 81% in the LE and it decreased gradually towards the ME (65%) and was 37.7% in the UE (not illustrated). Similarly, the average annual contribution of *Pseudo-nitzschia* cells to the total phytoplankton abundance was on average higher in the LE (18.1%) than in the ME (11.6%) and it decreased markedly in the UE (0.4%) (Figure 6).

Abundance of *Pseudo-nitzschia* in relation to physical variables

Fluctuations in temperature, salinity and Secchi depth measured throughout the sampling period and the relationships between *Pseudo-nitzschia* densities in the three parts of the estuary are shown in Figure 7. Temperature showed a clear seasonal pattern with minor differences between the three parts of the estuary. Overall the temperature values ranged from 5.9°C (February) to 28.4°C (August) during the study period. The UE had a slightly relatively higher temperature
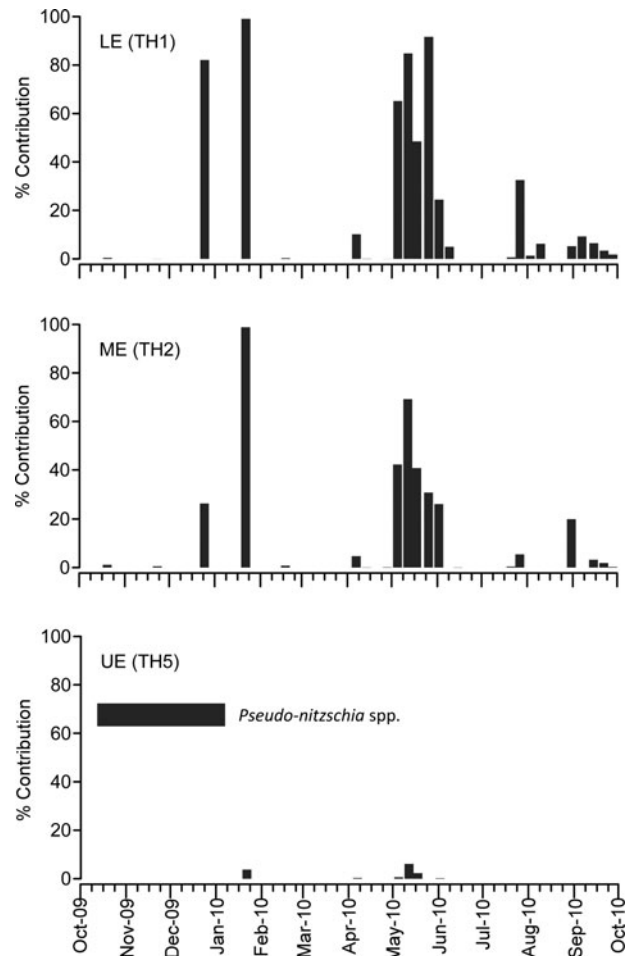
($\sim$2.2°C) than the LE. The mean annual temperatures and standard deviations were 17.11°C $\pm$ 5.73 for LE and 19.32°C $\pm$ 5.90 for UE.

Pseudo-nitzschia species were observed at a wide range of water temperatures from 9.3°C (January) to 23.5°C (August) in this study area, but the peaks in cell density appeared at a more narrow temperature between 9.3°C (January) and 15.5°C (early June). The mean abundance of *Pseudo-nitzschia* cells was 140 $\times$ 10^3 cells l^{-1} in December at 11°C, with 70% of the total density composed of *P. calliantha* and 30% *P. pungens*. The first bloom of *Pseudo-nitzschia*, comprising only *P. calliantha*, occurred at 9.3°C in January. In early April, the sub-bloom density of *P. calliantha* occurred at 9.8°C. The second bloom of *Pseudo-nitzschia* spp. in the first half of May happened at 14–15°C. Even though *P. calliantha* was found over a broad range of temperatures, it was more abundant at a temperature range between 9 and 15°C. There was a significant negative correlation (*P* < 0.001) between *Pseudo-nitzschia* spp. abundance and temperature (Table 1). This also agrees with *Pseudo-nitzschia* species being found only in low abundances during the warmer months, July–October, where the temperatures were between 19.5 and 23°C (Figure 7).

The surface salinity varied between 2.5 (December, UE) and 21.6 (May, LE) throughout the study period. Exceptionally, salinity decreased to less than 1.0 in the UE following a
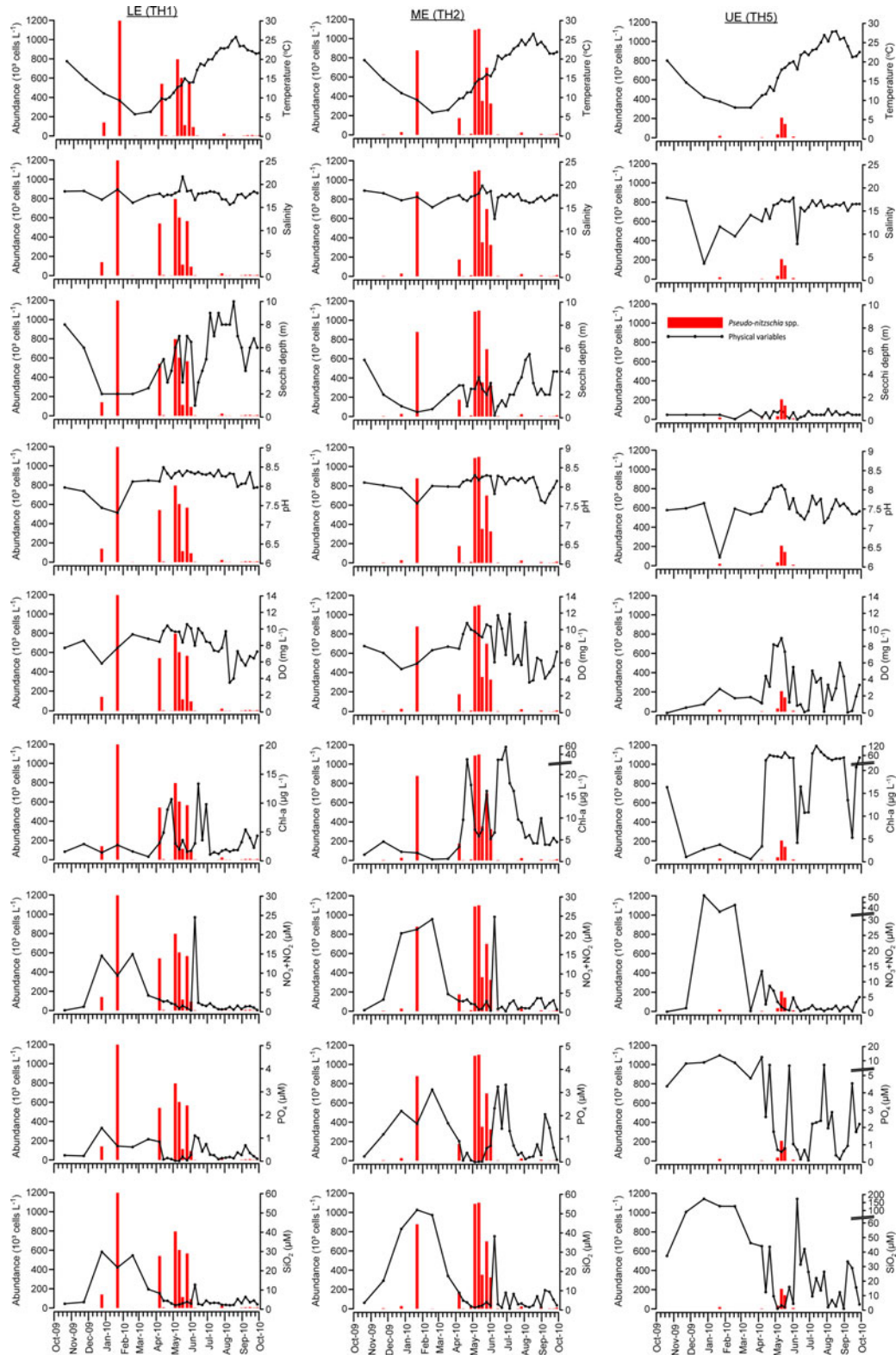
Fig. 7. Relationships between *Pseudo-nitzschia* abundances and physico-chemical variables during the study period.

Table 1. Spearman correlation coefficients (rho) calculated between *Pseudo-nitzschia* spp. and environmental factors.

	Secchi (m)	Temp. (°C)	Salinity	pH	DO (mg l⁻¹)	NO₃ + NO₂ (µM)	PO₄ (µM)	SiO₂ (µM)
P-nitzschia abundance	0.32‡	−0.33†	0.44†	0.36†	0.47†	0.05	−0.32†	−0.30‡

Statistically significant correlations are indicated by symbols: *$P < 0.05$; ‡$P < 0.01$; †$P < 0.001$ (N = 96).

heavy rainfall in early June. *Pseudo-nitzschia* spp. were found at salinities from 11.6 (TH5, January) to 21.6 (TH1, May), but the higher densities were found at salinities from 15.7 to 18.7. Salinity generally differed between the LE and the UE. The UE had a variable salinity (15.15 ± 3.05) which was always lower than the LE, which had relatively stable salinity (17.83 ± 1.08). Bloom densities of *P. calliantha* were detected at salinities of 18 and 19. There was a highly significant positive correlation ($P < 0.001$) between *Pseudo-nitzschia* abundance and salinity (Table 1, Figure 7), with *P. calliantha* in total making up the majority of the *Pseudo-nitzschia* abundance (average 80.2%) during the whole year.

Precipitation was the major freshwater input for the GHE. A high amount of suspended particulate material (SPM) carried by streams causes very low water transparency especially in the UE. Moreover, sludge dredging activities in the UE in combination with insufficient water circulations result in increased turbidity even in periods without rainfall. Water transparency based on Secchi depth decreased considerably during the rainy periods. It ranged from 0.1m (February, UE) to 10m (August, LE) and it was always much lower in the UE than the LE (Figure 7). The mean Secchi depths were 5.51 ± 2.34, 1.59 ± 0.22, 0.54 ± 0.22 in the LE, ME, UE, respectively. Spearman correlation coefficients showed a significant positive relationship ($P < 0.01$) between *Pseudo-nitzschia* spp. abundance and Secchi depth (Figure 4).

Abundance of *Pseudo-nitzschia* in relation to chemical variables

The mean annual pH values were 8.2 ± 0.26 in the LE and 7.5 ± 0.34 in the UE and pH generally decreased gradually from the LE to the UE. The minimum pH values in the surface were 6.3 in the UE in winter, while the maximum pH values were measured to 8.5 in the LE in spring. The fluctuations in pH showed a spatial and temporal variation (Figure 8). The pH values were more variable in the UE than ME and LE. In April and May, pH increased up to 8.5 in the LE and 8.1 in the UE. Even though the *Pseudo-nitzschia* spp. cells were observed at a wide range of pH values, the high cell densities were found at pH values over 8.0 and lower densities at the pH values below 8.0 particularly in the UE. There was a highly significant positive correlation ($P < 0.001$) between *Pseudo-nitzschia* spp. abundance and pH (Table 1).

The mean annual dissolved oxygen DO concentrations were $8.1 \pm 1.75 \, \text{mg} \, \text{l}^{-1}$ for LE and $3.0 \pm 2.57 \text{l}^{-1}$ for UE, and DO were generally very low ($< 3 \, \text{mg} \, \text{l}^{-1}$) in the UE except during bloom periods, while they were consistently high in the LE due to the strong hydrodynamic structure and interaction with the Strait of Istanbul. Although the UE has low DO values, the increase in DO values in May is based on phytoplankton abundance, and algal production is one of the main factors affecting DO level in the UE (Figure 8). Spearman correlation coefficients showed a highly significant positive relationship ($P < 0.001$) between *Pseudo-nitzschia* abundance and DO values (Table 1).

Chl-*a* concentrations increased considerably from the LE to the UE and ranged between 0.7 and $10.6 \, \mu\text{g} \, \text{l}^{-1}$ in the LE, 0.5 and $35.4 \, \mu\text{g} \, \text{l}^{-1}$ in the ME and 0.6 and $121.4 \, \mu\text{g} \, \text{l}^{-1}$ in the UE. Chl-*a* values showed two peaks in spring and

summer when flagellate blooms occurred. Chl-*a* measured as $2.8 \, \mu\text{g} \, \text{l}^{-1}$ in the LE in January when *Pseudo-nitzschia* dominated the phytoplankton with abundances reaching 1.2×10^6 cells l^{-1}. In May, chl-*a* measured as $7.3 \, \mu\text{g} \, \text{l}^{-1}$ in the ME when the blooms of *Pseudo-nitzschia* and cryptophyte *Plagioselmis prolonga* occurred. No clear correlation was observed between blooms of *Pseudo-nitzschia* spp. and the major chl-*a* peaks observed in spring and summer (Figure 7). At these periods flagellates such as euglenophyceans and cryptophyceans dominated.

Inorganic nutrient concentrations increased in winter due to the high amounts of terrestrial inputs from the streams and due to precipitation, while they decreased in spring because of phytoplankton activity. All nutrient values increased remarkably from lower to upper estuary (Figure 7). Mean concentrations of nutrients were $3.3 \pm 5.2 \, \mu\text{M}$ at LE and $6.4 \pm 12.4 \, \mu\text{M}$ at UE for $NO_3 + NO_2$ (NO_x); $0.39 \pm 0.36 \, \mu\text{M}$ at LE and $3.5 \pm 3.4 \, \mu\text{M}$ at UE for PO_4; $6.2 \pm 7.0 \, \mu\text{M}$ at LE and $37.1 \pm 47.2 \, \mu\text{M}$ at UE for SiO_2. Silicate concentrations decreased to $1.8 \, \mu\text{M}$ during the bloom in May, while the mean annual value was $17.7 \, \mu\text{M}$. There were significantly negative correlations between *Pseudo-nitzschia* abundance and PO_4 ($P < 0.001$) and SiO_2 concentration ($P < 0.01$). No relationship was found between *Pseudo-nitzschia* abundance and NO_x concentrations (Table 1, Figure 7). The mean annual N:P ratio based on DIN ($NO_3 + NO_2$-N and NH_4-N) and DIP (PO_4-P) varied between 26.2 ± 20.5 for the LE and 35.3 ± 35.6 for the UE, increasing towards the UE, and it was always higher than the Redfield ratio.

Data analysis

PCA analyses showed a significant spatial and temporal variation in environmental variables during the study period. The first two PCs explained 61.7% of the total variation (PC1: 41.2%, PC2: 20.5%). Projection of stations on the PCA ordination (Figure 8A) showed an upper to lower estuary separation of stations in the ordination plane along a diagonal transect, indicating that the highest variation between stations was in TH4 and TH5 (upper estuary) and that the lowest variation was in TH1 (lower estuary). Projection of sampling periods (months) on the PCA ordination (Figure 8B) provided a seasonal separation in the ordination plane along a diagonal transect, indicating the lowest variation between April and May, while the highest variation was observed in January and February depending on the effect of precipitation. Considering coefficients in the linear combinations of variables, it is seen that NH_4, pH, Secchi depth and DO (in the direction of grey arrow) have the greatest importance on the first PC (Figure 8A), while temperature, salinity, Chl-*a* and Si:N (in the direction of grey arrow) have most importance on the second PC (Figure 8B). The results of PCAs showed that the main factors causing spatial variation between stations were NH_4, Secchi depth, pH and DO values, indicating a very variable environmental condition in the upper estuary. According to PCAs, the factors causing temporal variation among months were temperature, salinity, Chl-*a* and Si:N. PCA analyses showed also a significant spatial and temporal variation in *Pseudo-nitzschia* abundances. The first two PCs explained 57.2% of the total variation (PC1: 37.6%, PC2: 19.6%). Projection of *P. calliantha* and *P. pungens* on the PCA ordination (Figure 8C & D) showed that the main factors causing
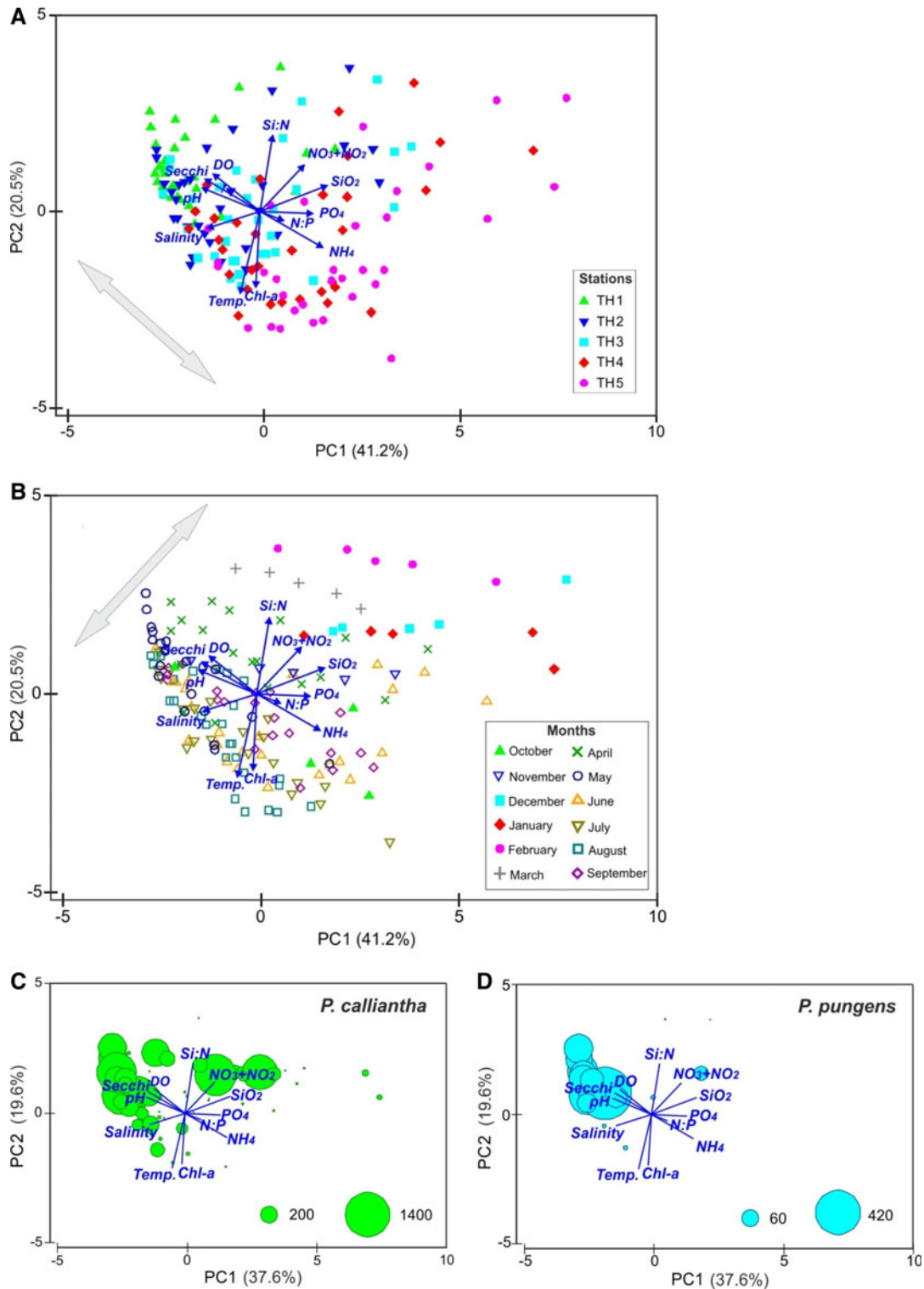
Fig. 8. PCA ordinations of environmental variables expressed as stations (A) and months (B) and superimposed abundances (cells l^{-1}) of *Pseudo-nitzschia calliantha* (C) and *P. pungens* (D).

spatial and temporal variation in both species were Secchi depth, DO, pH and NO$_x$. Notably *P. pungens* was clearly correlated with Secchi depth, DO and pH. These factors thus are important for the distribution of two *Pseudo-nitzschia* species. As can be seen in PCA plots, *P. calliantha* was more abundant at TH1 and TH2 in January and May, while *P. pungens* was more abundant at TH1 and TH2 particularly in May.

DISCUSSION

Domoic acid (DA) producing *Pseudo-nitzschia* species were commonly detected in the GHE, particularly two species, *P. calliantha* and *P. pungens*, which were found forming two blooms. Although *Pseudo-nitzschia* species were observed at a wide range of water temperatures from 9.3°C (January) to 23.5°C (August), peaks in abundance appeared between

9.3°C (January) and 15.5°C (early June) and abundances were very low, rarely exceeding 10^3 cells l^{-1}, in the summer months. A significant negative correlation between *Pseudo-nitzschia* spp. abundance and temperature indicates that low temperatures in nutrient-rich coastal areas and estuaries like the GHE may stimulate growth and bloom formation in different *Pseudo-nitzschia* species. Prior studies have shown a wide temperature range for presence of *Pseudo-nitzschia* spp. (Dortch *et al.*, 1997; Liefer *et al.*, 2009), consistent with the results of the present study. *Pseudo-nitzschia* spp. abundance also decreased considerably at high temperature (>20°C), which apparently limits the growth of *Pseudo-nitzschia* spp. in this area. *Pseudo-nitzschia calliantha* was found at temperatures between 9.3 and 21.7°C with maximum abundance at 9.3°C in January and at 14.7°C in May, whereas *P. pungens* was found at temperatures between 11.6°C and 22.8°C with maximum abundance at 16°C in late May. This is in agreement with several other studies with regard to *P. pungens* (Stonik *et al.*, 2001) and *P. calliantha* (Terenko & Terenko, 2012), but does not agree with reports of *P. pungens* showing a peak in abundance at a water temperature of 21.0°C (Terenko & Terenko, 2012). But *P. pungens* comprises several varieties, and the temperature preference of these varieties have been found to differ (Kim *et al.*, 2015), which may explain this disagreement. The contribution of *P. calliantha* to total phytoplankton was higher in January than in late May decreasing from 99% to 17%. During the same period, the contribution of *P. pungens* to total phytoplankton increased to 83%. This might reveal that these species shows different temperature responses as also indicated by Fryxell *et al.* (1990).

Pseudo-nitzschia spp. were found at a salinity range of 11.6 to 21.6, but the higher densities were detected at 15.7 to 18.7. *Pseudo-nitzschia* spp. were almost not observed during periods of low salinity (<10). A highly significant positive correlation between *Pseudo-nitzschia* abundance and salinity indicates that low salinities below 15 may not be suitable for growth and bloom formation of *Pseudo-nitzschia* spp. The same is seen in the Baltic Sea, where *Pseudo-nitzschia* decreases in abundance with a decrease in salinity (Hällfors, 2004). In general, *Pseudo-nitzschia* spp. demonstrate euryhaline characteristics, growing over a wide range of salinities (1 to >30) but they occur more frequently at higher rather than lower salinities (Thessen *et al.*, 2005). This general statement is supported by the present study, which found that *Pseudo-nitzschia* spp. was found to thrive in brackish waters, but preferred the higher salinities. Our results are in agreement with laboratory studies of *P. calliantha* and *P. pungens*. Salinity preference experiments on *P. calliantha* found growth at salinities from 5–>25 with a growth optimum >25 (Lundholm *et al.*, 1997), and *P. pungens* showed positive growth at salinities from 15 to >26, with optima at 20 or 26 (Cho *et al.*, 2001; Doan-Nhu *et al.*, 2008). On the contrary, other species like *P. americana* or *P. circumpora* showed no growth at salinities lower than 15 or 20, respectively (Miller & Kamykowski, 1986; Lim *et al.*, 2012).

Studies on harmful algal blooms carried out in the GHE showed that water discolouration with different types of colour depending on the causative species, decreasing light intensity and rapid changes in DO concentrations had major effects on the ecosystem (Tas and Okus, 2011; Tas, 2015; Tas & Yilmaz, 2015). Before the rehabilitation of the GHE, it was reported that high amounts of suspended solid matter, toxic gases and anoxic conditions limited phytoplankton growth and *Pseudo-nitzschia* species were observed at very low densities ($\sim 10^3$ cells l^{-1}) in the LE and ME (Tas & Okus, 2003). During the rehabilitation of the GHE, the abundance of *Pseudo-nitzschia* cells was very low ($\sim 10^3$ cells l^{-1}) and found in the LE and ME only. After the rehabilitation, *Pseudo-nitzschia* density increased considerably based on the improving water quality and cell densities exceeded 10^6 cells l^{-1} in the LE in January 2002. However, *Pseudo-nitzschia* cells were not observed in the UE which had a high turbidity (Tas *et al.*, 2009). The previous studies carried out in the GHE (Aslan-Yilmaz *et al.*, 2004; Yüksek *et al.*, 2006; Tas *et al.*, 2009) revealed that the most important problem for this area is the high concentration of terrestrial materials entering the ecosystem with rainwater and the streams, thereby affecting physico-chemical parameters and the phytoplankton community structure particularly in the upper estuary. In the present study, there was a significant positive relationship ($P < 0.01$) between *Pseudo-nitzschia* abundance and Secchi depth, and blooms of *Pseudo-nitzschia* spp. were not observed in the UE. Low light availability due to high amount of SPMs at the surface of the UE most likely inhibits the photosynthesis and limits the spatial distribution and growth of *Pseudo-nitzschia* spp. Thus, light limitation should be considered one of the most important factors determining growth of *Pseudo-nitzschia* in the UE.

It is well-known that high abundances of *Pseudo-nitzschia* are found in coastal areas enriched by nutrients (Parsons *et al.*, 2002; Lundholm *et al.*, 2004; Trainer *et al.*, 2012). High *Pseudo-nitzschia* abundances have also been associated with submarine ground discharge of nutrients (Liefer *et al.*, 2009) and their blooms can be stimulated by nutrients from several sources, including upwelling or mixing events and riverine inputs (Trainer *et al.*, 2000, 2012), suggesting a response to eutrophication (Parsons *et al.*, 2002). These findings agree with our observations of high nutrient concentrations supporting growth and blooms of *Pseudo-nitzschia* in the GHE. The increasing nutrient concentrations from the LE to the UE are the effects of two streams carrying high nutrient levels into GHE at the UE. Low silicate concentrations during summer due to lack of precipitation may have limited the growth of *Pseudo-nitzschia* cells.

The effect of pH on the growth of marine phytoplankton has been studied (Taraldsvik & Myklestad, 2000; Hansen, 2002; Lundholm *et al.*, 2004; Havskum & Hansen, 2006) and it has been suggested that high pH (8.7 to 9.1) inhibits growth of most *Pseudo-nitzschia* species (Lundholm *et al.*, 2004). Such high pH levels were never observed in the present study, where pH levels from 6.3 to 8.5 were found.

Multivariate analyses (PCA) performed on environmental factors reflected the deviating environmental conditions found in the upper estuary, where the abundance of *Pseudo-nitzschia* spp. was very low. The present study showed that the spatial variability was particularly structured along Secchi depth, pH, DO and NH_4, while the temporal variability was structured along temperature, salinity, Chl-*a* and Si:N. Our results agree with Tas & Yilmaz (2015), which suggested that a projection of stations and environmental variables provided an upper to lower estuary separation and that the variability was particularly structured along Secchi depth and temperature. Secchi depth, DO and pH were the main factors causing spatial and temporal variation in *Pseudo-nitzschia* and these factors have a significant positive relationship with *Pseudo-nitzschia* abundances as stated

in Spearman correlation coefficients. Light limitation and the very variable environmental conditions caused by several factors (e.g. Secchi depth, DO, pH and NH_4) particularly in the upper estuary may be the most important limiting factors for growth of *Pseudo-nitzschia* in the study area. The presence of the potentially toxic *Pseudo-nitzschia* blooms in GHE pose a risk for toxin accumulation in local fauna with associated problems for the ecosystem as well as for human consumers of marine products from GHE. As both *P. calliantha* and *P. pungens* have been found to produce toxins in other parts of the worlds (Bates *et al.*, 1989; Smith *et al.*, 1990; Bargu *et al.*, 2002; Hallegraeff, 2002; Besiktepe *et al.*, 2008; Moestrup *et al.*, 2009; Lundholm, 2011), they may, due to the high cell concentrations, pose a potential risk for toxic events at LE and ME. Future studies are needed to explore the toxic potential of the local strains at different environmental conditions. The cell concentrations found at the GHE suggest that accumulation of domoic acid in, for example, shellfish is a realistic scenario to consider, but as toxin production is affected by environmental and biological parameters in various ways and probably depends on the genetic potential of the local strains, a complete understanding of the potential risk requires monitoring of *Pseudo-nitzschia*, domoic acid, the ecosystem and the chemical-physical parameters.

In conclusion, available environmental conditions (i.e. high nutrient, low water circulations) in the GHE promote the potential toxic *Pseudo-nitzschia* blooms in the region. Therefore, rehabilitation efforts are very important to provide a healthy ecosystem in the region by reducing nutrient inputs, terrestrial runoffs and increasing upper layer circulation.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0025315416000837.

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