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Ecology and seasonality of *Pseudo-nitzschia* species (Bacillariophyceae) in the northwestern Adriatic Sea over a 30-year period (1988-2020)

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Abstract

The ecology and seasonality of *Pseudo-nitzschia* species and their contribution to the phytoplankton community were analysed for the first time at the coastal station of the LTER-Senigallia-Susak transect (north-western Adriatic Sea) from 1988 to 2020. Species composition was addressed using DNA sequence data obtained from 106 monoclonal strains isolated from January 2018 to January 2020.

The mean annual cycle of total phytoplankton in the study period (Feb. 1988-Jan. 2020) showed maximum abundances in winter followed by other peaks in spring and autumn. Diatoms were the main contributors in terms of abundance during the winter and the spring blooms. The autumn peak was due to phytoflagellates and diatoms. In summer phytoflagellates dominated the community, followed by diatoms and dinoflagellates, which in this season reached their annual maximum.

Pseudo-nitzschia spp. represented on average 0.4-17.6% of the diatom community, but during their blooms they could reach up to 90% of the total diatom abundances with 10^6 cells l^{-1}. By LM, six different taxa were recognized: *Pseudo-nitzschia* cf. *delicatissima* and *P.* cf. *pseudodelicatissima* were the most abundant, followed by *P.* cf. *fraudulenta*, *P. pungens*, *P. multistriata* and *P.* cf. *galaxiae. P.* cf. *fraudulenta* and *P. pungens* were indicator taxa of winter. *P.* cf. *delicatissima* and *P.* cf. *pseudodelicatissima* were spring and summer taxa, respectively. *P. galaxiae* showed maximum abundances in autumn. DNA sequences revealed the presence of two species belonging to the "*P. seriata* group" (i.e. *P. fraudulenta* and *P. pungens*) and four species belonging to the "*P. delicatissima* group" (*P. calliantha* and *P. mannii* within the *P. pseudodelicatissima* species complex, and *P. delicatissima* and *P.* cf. *arenysensis* within the *P. delicatissima* species complex). The presence of several cryptic and pseudo-cryptic species highlights the need to combine LM observations with DNA sequence data when the ecology of *Pseudo-nitzschia* is investigated.

Keywords: DNA sequence data; HAB diatoms; Time-series; Seasonal dynamics; Environmental parameters.

Introduction

The diatom genus *Pseudo-nitzschia* Peragallo is a common component of marine phytoplankton assemblages worldwide (Hasle, 2002). To date, among the 56 species of *Pseudo-nitzschia* taxonomically accepted (Guiry & Guiry, 2021), 26 are recognized as harmful (Lundholm, 2021), due to the production of a neurotoxin, i.e., domoic acid (DA). DA can bioaccumulate in shellfish and the consumption of DA-contaminated seafood may cause human intoxication known as Amnesic Shellfish Poisoning (ASP) (Lefebvre & Robertson, 2010), as well as mass mortality of mammals and marine birds, and economic losses for fisheries and aquaculture (Tasker, 2016). A number of studies have been conducted on the ecology, taxonomy and toxin production of *Pseudo-nitzschia* spp. in several regions around the world, highlighting the high variability and complexity of this genus (Ajani *et al*., 2020; Cho *et al*., 2001; Dong *et al*., 2020; Fehling *et al*., 2004; Lundholm *et al*., 2002, 2003; Marić *et al*., 2011; Orsini *et al*., 2004; Rhodes *et al*., 2013; Teng *et al*., 2016; Thessen *et al*., 2005; Trainer *et al*., 2009).

With the establishment of several new species, based on both minimal ultrastructural features and molecular differences, it became clear that this genus could hide more diversity than was originally thought (Lundholm *et al*., 2006; Quijano-Scheggia *et al*., 2009). Therefore, during routine monitoring performed by Light Microscopy (LM), identifications at species level are often unreliable and a conservative identification is generally preferred,

merging taxa that share similar morphology (e.g., valve width, frustule end shape, overlap in colony) in groups or complexes (Hasle, 1965): all the species wider than 3 µm have been combined in the "*P. seriata* group", while those less than 3 µm in the "*P. delicatissima* group". The *seriata* group includes several species (e.g., *P. fraudulenta*, *P. subfraudulenta*, *P. pungens*) that can be identified using morphological analyses and DNA sequence data. However, it was recently recognized that the paradigm of the cell width > 3 µm in the *seriata* group should be rejected, as narrower cells are common (e.g., Churro *et al*., 2009; Ljubešić *et al*., 2011; Moschandreou *et al*., 2012; Accoroni *et al*., 2020). Within the *delicatissima* group the rounded or pointed ends in girdle view have been used to identify *P. delicatissima* and *P. pseudodelicatissima* in LM, respectively, while in Electron Microscopy (EM) these species are characterized by biseriate and uniseriate striae, respectively, in valve view (Lundholm *et al.*, 2003, 2006). EM, however, is not always resolutive because there are several species within the same group that are morphologically identical or almost identical (cryptic and pseudo-cryptic species). In such cases, only a characterization based on DNA sequence data provides an unambiguous species delineation. The *P. delicatissima* and *P. pseudodelicatissima* complexes contain 11 and 25 species respectively, the majority of which are cryptic or pseudo-cryptic (Lundholm *et al.*, 2003, 2006, 2012; Amato & Montresor, 2008; Quijano-Scheggia *et al.*, 2009; Lim *et al.*, 2012; Teng *et al.*, 2015; Percopo *et al.*, 2016; Li *et al.*, 2017; Ajani *et al.*, 2018; Gai *et al.*, 2018; Huang *et al.*, 2019; Dong *et al.*, 2020; Chen *et al.*, 2021). The presence in such complexes of both toxic and non-toxic species complicates the monitoring efforts performed mainly using LM, and therefore not accurate enough to manage the DA contaminations of seafood as unable to discriminate potentially toxic and non-toxic species.

The Adriatic Sea is in the northernmost part of the central Mediterranean, and it is characterized by a general seasonal cyclonic circulation (Russo & Artegiani, 1996). In particular in the northern Adriatic basin, this circulation is quite complex and highly variable in response to wind and river forcing, both having small-scale structures and marked temporal variability (Giani *et al.*, 2012).

The main nutrient source in the northern Adriatic is the freshwater input provided mainly by the Po River outflow (Cozzi & Giani, 2011; Giani *et al.*, 2012). Nutrient input is therefore affected by the interaction between human activities and meteoclimatic events (Viaroli *et al.*, 2018).

There is an extensive literature concerning phytoplankton distribution and dynamics in the northern Adriatic Sea (e.g., Bernardi Aubry *et al.*, 2004, 2012; Cabrini *et al.*, 2012; Marić *et al.*, 2012; Mozetič *et al.*, 2012; Cerino *et al.*, 2019; Totti *et al.*, 2019) thanks to the Long-Term Ecological Research (LTER) sites, where multiparametric data (e.g., hydrographic, physical, chemical, biological) have been collected for decades. Consequently, these long-term data sets represent a powerful tool to understand, trace and predict ecosystem trends in response to local and global anthropogenic pressure (Totti *et al.*, 2019; Zingone *et al.*, 2019). In the coastal NW Adriatic, the phytoplankton annual cycle is typically characterized by an intense diatom winter bloom dominated by *Skeletonema marinoi*. Other multispecific diatom blooms occur in spring and autumn, mainly depending on rainfall regimes. In the inter-bloom periods, phytoplankton communities are dominated by heterogeneous assemblages of small phytoflagellates (Bernardi Aubry *et al.*, 2004, 2006; Totti *et al.*, 2005, 2019). In the last decade, this regular annual rhythm has been altered by exceptional meteorological events, resulting in anomalous freshwater discharges causing irregular intense blooms associated with water discoloration even in summer (Zingone *et al.*, 2020).

The composition of *Pseudo-nitzschia* assemblages has been resolved both in the NE (Turk Dermastia *et al.*, 2020), and in the NW Adriatic Sea (Penna *et al.*, 2013; Giulietti *et al.*, 2021). However, although the role of the main physico-chemical environmental variables on the seasonal behaviour of the main phytoplankton groups has been highlighted (Degobbis *et al.*, 2000; Bernardi Aubry *et al.*, 2012; Totti *et al.*, 2019), to date the role on the single *Pseudo-nitzschia* species was evaluated only in a study carried out in the NE sub-basin (Turk Dermastia *et al.*, 2020).

The phytoplankton trend in the LTER SG01 station (NW Adriatic Sea) over 30 years (1988-2016) has been previously discussed by Totti *et al*. (2019), showing that some changes occurred in the last decade in the community structure. The aim of this study was to focus on the *Pseudo-nitzschia* population of the same dataset (1988-2020), highlighting the seasonal variability of each species (or group of species) and their relationships with environmental factors. Moreover, to better characterize the *Pseudo-nitzschia* species composition in the LTER SG01 throughout the year, DNA sequence data were obtained from strains isolated from samples collected in the period 2018-2020.

Materials and Methods

Study area and sampling

The sampling station was located in the southern part of the northern Adriatic sub-basin, included in the LTER Italian sites (SG01, 43.75733° N, 13.2165° E, Fig. 1) at 1.2 nM from the Italian coastline. Temperature (°C) and salinity were measured with a multiparametric probe (Sea-Bird Electronics, SBE 19plus V2 SeaCAT Profiler CTD). Water samples for phytoplankton identification and enumeration were collected from sea surface (0.5 m) by Niskin bottles in 250 ml dark glass bottles and preserved by adding 0.8% formaldehyde, prefiltered and neutralized with hexamethylenetetramine (Throndsen, 1978) and stored at 4°C until analysis.

Water samples for chemical analysis were collected in 4 ml polyethylene bottles, filtered through GF/F Whatman filters, and stored at -21°C until analysis.

Seasons were subdivided as follows: winter (January-March), spring (April-June), summer (July-September), autumn (October-December).

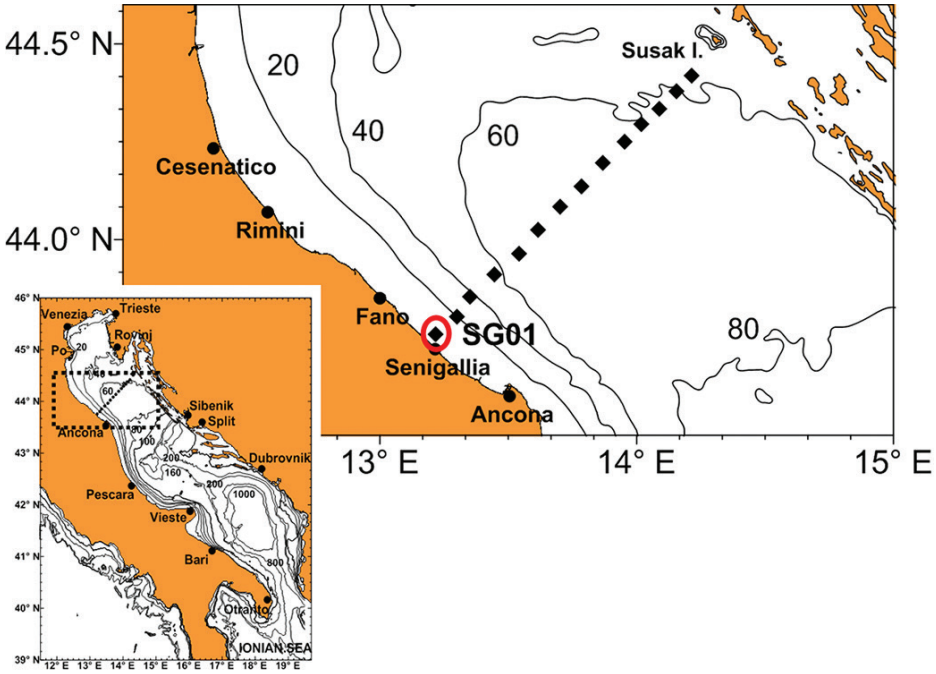

Fig. 1: The LTER Senigallia transect in the northern Adriatic Sea. The study station is highlighted by the circle.

Sampling was carried out with monthly frequency from February 1988 to January 2020, on board of several oceanographic vessels (S. Lo Bianco, Tecnopesca 2, G. Dallaporta, Tethis, Copernaut Franca, Urania, Alliance, Minerva, Bannock, D'Ancona, Actea). Moreover, net samples (20 µm mesh net) were collected dragging the net along the surface monthly from January 2018 to January 2020 for *Pseudo-nitzschia* cells isolation (below in detail).

Nutrient analysis

Nutrient concentrations were measured using a Perkin Elmer spectrophotometer 550A model in the period 1988-1998, an auto-analyzer TRAACS 800 BRAN+LUEBBE in 1999-2002 and then an auto analyzer QUAATRO Technicon, following Strickland & Parsons (1972). For both auto analyzer models, accuracy was ± 0.02 µmol L^{-1} for ± NO_2, ± NO_3, ± NH_4 and Si $(OH)_4$ and ± 0.03 µmol L^{-1} for PO_4. A calibration curve was made with 5 levels of Merck ® standards and the accuracy was tested using a standard as sample. The precision was tested on 10 replicates of the standard and were: ± 0.006 µmol L^{-1} (NO_2), ± 0.005 µmol L^{-1} (NO_3), ± 0.001 µmol L^{-1} (NH_4), ± 0.001 µmol L^{-1} (PO_4) and Si $(OH)_4$ ± 0.055 µmol L^{-1}. Dissolved Inorganic Nitrogen (DIN) concentration was calculated as the sum of NO_2, NO_3 and NH_4 concentrations.

Identification and enumeration

Morphological species identifications were obtained using an inverted microscope (ZEISS Axiovert 135) equipped with phase contrast, following the Utermöhl method (Edler & Elbrächter, 2010). Enumeration was carried out at 400× magnification along transects or in random visual fields, depending on cell abundance, to count a minimum of 200 cells. Moreover, half of the Utermöhl chamber was analysed at 200x magnification for a more precise estimation of less abundant microphytoplanktonic taxa. During enumeration, phytoplankton taxa were identified at the lowest possible taxonomic level based on the most updated taxonomic literature, and grouped into major groups (diatoms, dinoflagellates, coccolithophores, phytoflagellates and others). Abundances were expressed as cells l^{-1}. Dinoflagellates were considered as a whole taxonomic group and both autotrophic and heterotrophic species were included in counting. Phytoflagellates are an informal group that includes haptophytes (except for coccolithophores), cryptophytes, chrysophytes, dictyochophytes, raphidophytes, chlorophytes and euglenophytes. Others include cyanophytes and taxa *incertae sedis* (see for details Totti *et al*., 2019).

Regarding *Pseudo-nitzschia* identification, we proceeded as follows. Among the *delicatissima* group (TA< 3 µm) *P.* cf. *delicatissima* and *P.* cf. *pseudodelicatissima* were identified based on either truncated or pointed ends in girdle view, respectively (Hasle & Syvertsen, 1997). *P. multistriata* was identified based on the sigmoid shape in girdle view. Cells that were almost solitary presenting a thin fusiform shape with a dilated central portion and two long rostra were assigned to *P.* cf. *galaxiae*. Cells in colony with an overlap about one-eighth of the apical axis length, valves lanceolate and symmetrical in valve view, and linear to fusiform in girdle view in colony were assigned to *P.* cf. *fraudulenta* (composed of *P. fraudulenta* and *P. subfraudulenta*). Strongly silicified cells, with interstriae and fibulae discernible by LM, in colony with an overlap of one-third (or less) of the apical axis length, linear in valve, and fusiform in girdle view, were assigned to *P. pungens*.

Strain isolation and cultivation

The isolation of single cells of *Pseudo-nitzschia* was carried from 2018 to 2020 on net samples of phytoplankton into 24-well plates using the capillary pipette method (Hoshaw & Rosowski, 1973). Cultures were transferred at 21°C with a 12:12 h of L:D photoperiod and an irradiance of 100 µmol m^{-2} s^{-1}, in 50 ml flasks containing sterile filtered seawater enriched with f/2 nutrients (Guillard & Ryther, 1962) and maintained at these conditions until DNA extraction. Every month the algal cultures were checked for their purity and quality and refreshed with new culture medium.

DNA extraction from algal cultures, PCR amplification and sequencing

Of the total 138 strains set up, 106 were used for DNA extraction (Table S1). Algal cultures were harvested collecting up to 10 ml (depending on cell concentration to avoid having excess material that could lead to unsuccessful DNA extraction) during their late exponential phase and centrifuged at 4000 x g for 15 minutes in order to obtain the pellet. Pellets were extracted using CTAB buffer (2% CTAB, 1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, 5M NaCl, 1%) modified from Doyle & Doyle (1987).

The extracted DNA was used as template for PCR amplifications that were carried out using a SimpliAmp™ Thermal Cycler; amplification of the ITS rDNA region (ITS1–5.8S–ITS2) and LSU rDNA gene (region D1-D3) were performed as described in Accoroni *et al*. (2020). The PCR cycling profile for LSU and ITS was 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, annealing at 60 and 58°C (for LSU and ITS regions, respectively) for 45 sec, and elongation at 72°C for 1 min, followed by further elongation at 72°C for 5 min.

Sequences were compared to sequences publicly available in the NCBI databases by BLAST search with default settings (Altschul *et al.*, 1990), using the megablast option.

Statistical analyses

Abundances of *Pseudo-nitzschia* and physico-chemical data were checked for normal distribution and homogeneity with Shapiro-Wilk test and the Bartlett's test, respectively. Data were not normally distributed, nor homogeneity of variances were respected, therefore Kruskal-Wallis test was performed to test species seasonality and influence of physical and chemical parameters. When significant differences were detected ($p < 0.05$), a Pairwise t-test comparison was performed.

To determine which species can be used as indicators of a season, the Indicator Value (IndVal) index was applied (Dufrêne & Legendre, 1997) using INDSPANA 1.1. This index combines the relative abundance of a species with its relative frequency of occurrence in a given period (in this case in each season). The significance of the relationships between species and seasons was tested using a permutation test.

In order to test correlations between environmental parameters and *Pseudo-nitzschia* abundances, Spearman-Rank order correlation and Principal Component Analysis (PCA) were carried out. PCA was performed on a Euclidean distance matrix of ranked physical and chemical variables (i.e., DIN, orthophosphate, silicate, salinity, and temperature) and abundances of each *Pseudo-nitzschia* species were fitted as supplementary variables.

Results

Seasonal variability of chemical and physical parameters

Temperature, salinity, and DIN concentrations varied significantly among seasons. In detail, temperature showed higher mean values in summer (24.8 ± 0.23°C, n=28 $p < 0.001$) and lower in winter (8.6 ± 0.4°C, n=26) than in the other seasons (Table 1), reaching the highest mean value in July (25.5 ± 0.25°C) and the lowest in January (7.4 ± 0.45°C) (Fig. 2A). Higher values of salinity

Table 1. Summary statistics of physical and chemical environmental variables collected in the LTER-SG01 station (February 1988-January 2020). Max-min range, mean ± standard error, "n" indicates the number of sampling.

Season	Temperature (°C)	Salinity	Silicate (µM)	DIN (µM)	PO_4 (µM)
Winter	4.4–11.8 8.6 ± 0.4 n=26	26.2–37.2 34.0 ± 0.4 n=26	0.0–20.6 5.5 ± 1.2 n=20	2.1–45.3 14.2 ± 2.4 n=20	0.00–0.30 0.11 ± 0.02 n=20
Spring	15.4–23.4 18.9 ± 0.4 n=23	30.7–36.9 34.0 ± 0.4 n=23	2.1–8.6 3.3 ± 0.5 n=17	0.3–19.9 6.3 ± 1.4 n=17	0.01–0.83 0.10 ± 0.05 n=17
Summer	21.5–27.1 24.8 ± 0.23 n=28	30.8–37.4 35.0 ± 0.3 n=28	2.0–8.5 2.8 ± 0.4 n=23	0.06–4.91 1.96 ± 0.30 n=23	0.02–0.68 15 ± 0.03 n=23
Autumn	8.6–20.4 14.8 ± 0.7 n=25	24.1–37.6 32.6 ± 0.8 n=25	6.9–28.6 8.9 ± 1.5 n=21	14.7–64.6 16.5 ± 3.2 n=21	0.02–0.58 0.17 ± 0.03 n=21

were recorded in summer (35.0 ± 0.3, n=28 $p < 0.05$) and lower values were recorded in autumn (32.6 ± 0.8, n=25) than in the other seasons (Table 1). Salinity showed an irregular trend reaching the highest mean value in August (35.9 ± 0.39) and minimum in December (30.9 ± 1.1) (Fig. 2B). Higher concentrations of DIN were recorded in autumn (16.5 ± 3.2 µM, n=21 $p < 0.001$) and winter (14.2 ± 2.4 µM, n=20 $p < 0.001$) than in the other seasons. Conversely, this parameter had lower values in summer (2.0 ± 0.3 µM, n=23) than in the other seasons (Table 1). A general decrease in DIN concentrations was observed from winter to summer (when the mean minimum concentration was recorded in August, i.e., 1.1 ± 0.26 µM), followed by an increase until December, when the mean maximum concentration of 26.1 ± 5.26 µM was recorded (Fig. 2C). A clear seasonal trend was not observed for PO_4 concentrations, with the highest value in December (0.25 ± 0.07 µM) and the lowest in April (0.04 ± 0.01 µM) (Fig. 2D). The highest concentrations of silicate were recorded in late autumn-early winter, with mean maximum concentrations in December (12.1 ± 2.60 µM) and mean minimum concentrations in April (1.4 ± 0.50 µM) (Fig. 2E).

Phytoplankton trend and seasonality with a focus on Pseudo-nitzschia *spp.*

The mean annual cycle of total phytoplankton in the whole study period (Feb. 1988-Jan. 2020) showed maximum abundances in winter (January-March) followed by other peaks in spring and autumn (Fig. 3). Diatoms were the main contributors in terms of abundance during the winter and the spring blooms (from 75 to 59% of the phytoplankton community, Fig. 4). Whereas in winter the diatom community was dominated by *Skeletonema marinoi*, in spring there was no prevailing species, and the community was multispecific. The autumn peak was characterized by phytoflagellates and diatoms, which represented 55-65% and 31-35% of the phytoplankton community, respectively. In summer, minimum values of diatoms were recorded; phytoflagellates dominated the community (65-81%) followed by diatoms (13-27%) and dinoflagellates (1-4%), which in summer reached their annual peak.

In the annual trend of *Pseudo-nitzschia*, species of this genus represented 0.4-17.6% of the diatom community (i.e. minimum and maximum in October and May, respectively) (Fig. 4). However, during the diatom blooms, the abundance of *Pseudo-nitzschia* spp. could increase up to 90% of the total diatom community, as happened in May 2018 with 1.5 x 10^6 cells l^{-1} (the highest value in the whole dataset). Considering the mean abundances through the year calculated on monthly basis, the main peak was observed in April (2.6 x 10^5 ± 1.0 x 10^5 cells l^{-1}). On the contrary, during the rest of the year, *Pseudo-nitzschia* spp. never exceeded an abundance of 10^4 cells l^{-1}. The lowest average abundance was recorded in November (2.5 x 10^4 ± 1.0 x 10^4 cells l^{-1}) (Fig. 5).

Using LM, 6 different groupings or taxa were distinguished: *P.* cf. *delicatissima*, *P.* cf. *pseudodelicatissima*, *P.* cf. *fraudulenta*, *P. pungens*, *P. multistriata*, and *P.* cf. *galaxiae*. *P. multistriata* has been recorded since September 2012. In general, the most abundant taxa were *P.* cf.

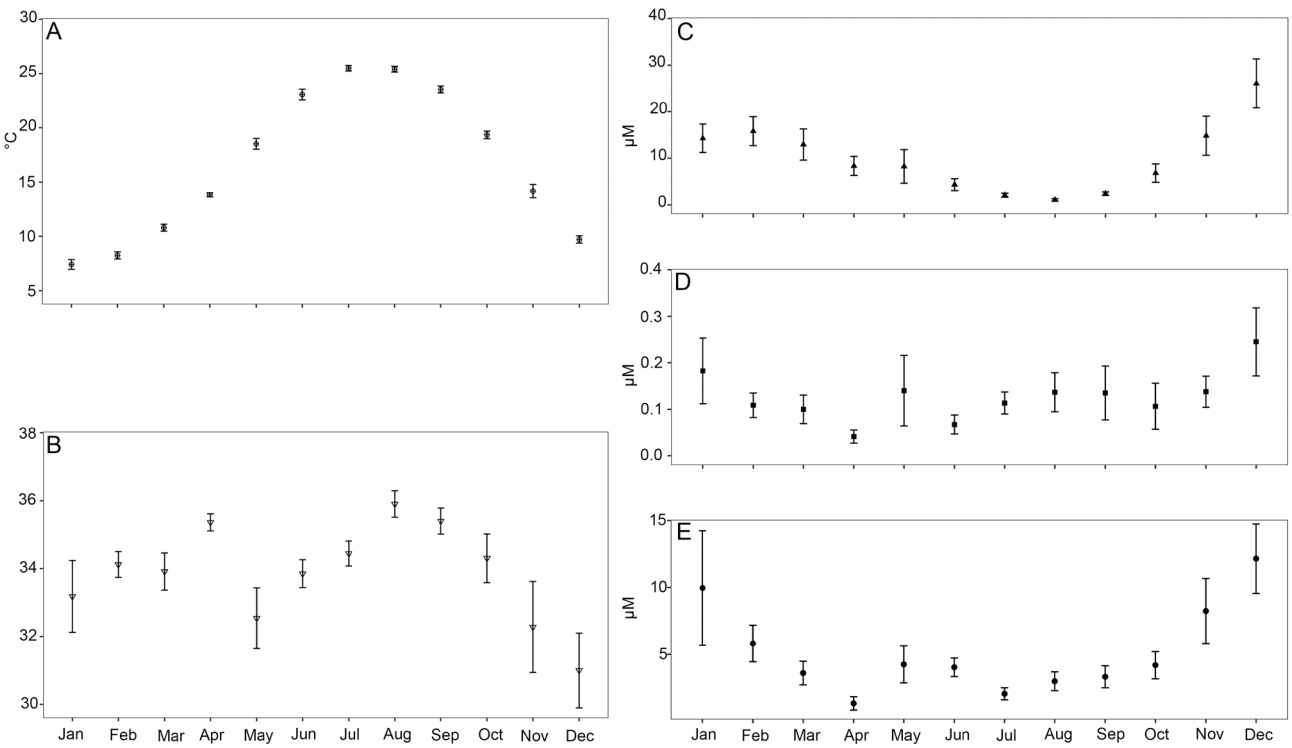

Fig. 2: Mean annual trend ± standard error of physico-chemical parameters in surface waters of at the LTER-SG01 station calculated monthly from February 1988 to January 2020: (A) water temperature, (B) salinity, and concentrations of (C) Dissolved Inorganic Nitrogen (DIN), (D) orthophosphates and (E) silicates.

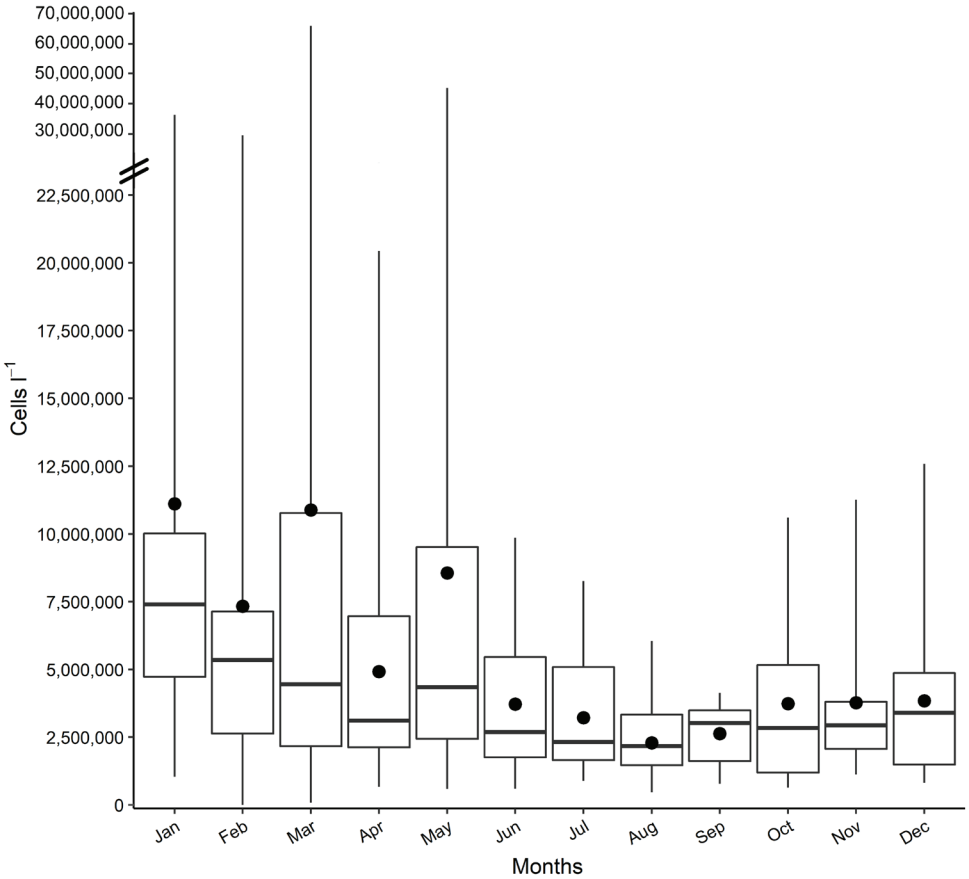

Fig. 3: Boxplot reporting the mean annual cycle of the phytoplankton abundances in the LTER-SG01 station (February 1988-January 2020), with the mean (circle), median (bold horizontal line), the interquartile range (boxes) and the min-max range (vertical line). The extremes and outliers are not reported.

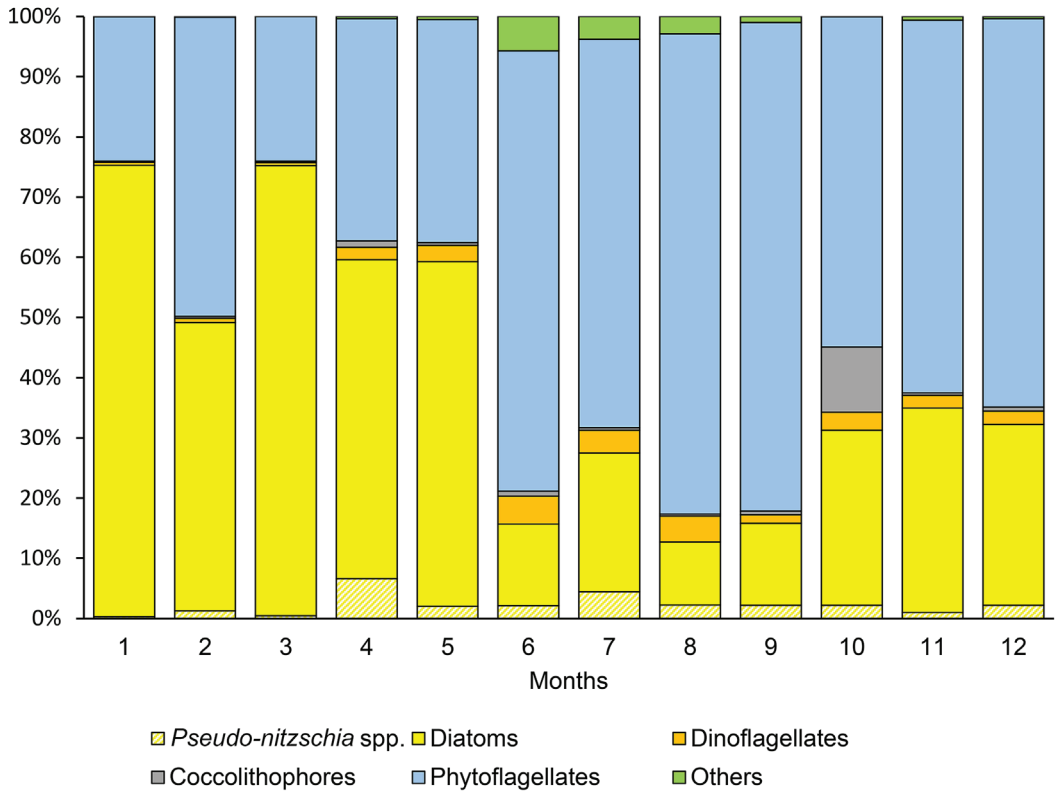

Fig. 4: Mean percent composition of phytoplankton community in the LTER-SG01 station (February 1988-January 2020) in terms of abundance monthly. Among diatoms (yellow bars), *Pseudo-nitzschia* spp. (crossed yellow bars) are highlighted.

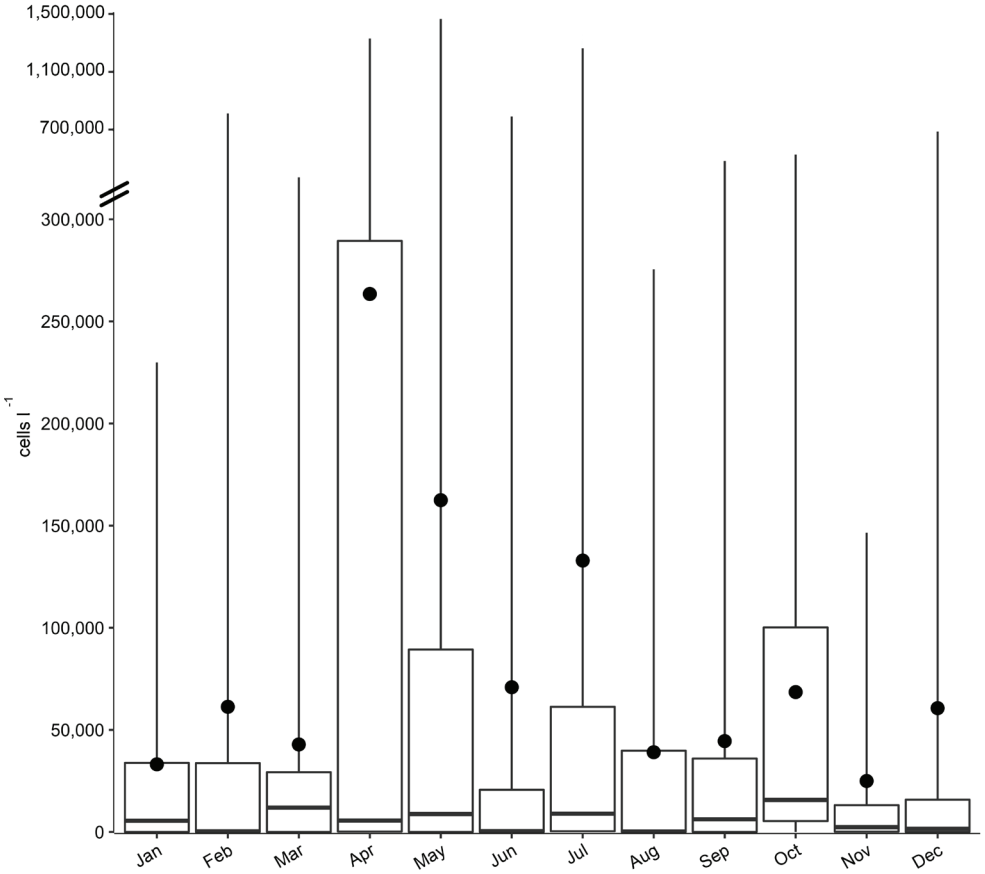

Fig. 5: Boxplot reporting the mean annual cycle of the total *Pseudo-nitzschia* species in the LTER-SG01 station (February 1988-January 2020), with the mean (circle), median (bold horizontal line), the interquartile range (boxes) and the min-max range (vertical line). The extremes and outliers are not reported.

delicatissima and *P.* cf. *pseudodelicatissima*, representing on average 36 and 53% of the whole *Pseudo-nitzschia* community, respectively.

Seasonal variation of *P.* cf. *delicatissima* was not significant. The peak of abundance was recorded in May ($1.2 \times 10^5 \pm 7.7 \times 10^4$ cells l^{-1}) and the minimum in December ($4.4 \times 10^3 \pm 3.4 \times 10^3$ cells l^{-1}) (Fig. 6A). In the whole study period, the highest abundance was observed in May 2018 (2.6×10^6 cells$\cdot l^{-1}$).

P. cf. *pseudodelicatissima* showed a significant seasonal variation ($p < 0.05$), with the peak in April ($1.3 \times 10^5 \pm 8.7 \times 10^4$ cells l^{-1}) and the minimum in February ($5.4 \times 10^3 \pm 2.9 \times 10^3$ cells l^{-1}) (Fig. 6B). The highest abundance throughout the study period was recorded in April 1991 (1.3×10^6 cells$\cdot l^{-1}$).

P. cf. *fraudulenta* represented on average 1.9% of the entire *Pseudo-nitzschia* community, showing a significant seasonality ($p < 0.05$), with the highest mean abundance in February ($1.7 \times 10^3 \pm 1.4 \times 10^4$) (Fig. 6C). During the rest of the year *P.* cf. *fraudulenta* was rarely recorded from May to November and never exceeded 1000 cells l^{-1}, with the lowest values in December (10 ± 10 cells l^{-1}). The highest abundance throughout the study period was recorded in February 2001 (3.3×10^4 cells$\cdot l^{-1}$).

P. pungens represented on average 3.5% of the entire *Pseudo-nitzschia* community, showing a significant seasonal variation ($p < 0.001$). This species was recorded from late autumn to early spring, with peaks in January ($1.3 \times 10^4 \pm 8.0 \times 10^3$ cells l^{-1}) and in April ($1.8 \times 10^4 \pm 1.8 \times 10^4$ cells l^{-1}), while it was not recorded in late spring/summer (Fig. 6D). The highest abundance in the course of the study was recorded in April 1988 (3.4×10^5 cells$\cdot l^{-1}$).

P. multistriata represented on average 0.4% of the *Pseudo-nitzschia* community. Although the seasonal variation was not significant, the highest mean abundances were recorded from September to January (maximum in October, 182.1 ± 163.5 cells l^{-1}) (Fig. 6E). This species was rarely recorded in spring and summer. The highest abundance throughout the study period was observed in October 2016 (3.4×10^3 cells$\cdot l^{-1}$).

P. cf. *galaxiae* represented on average 2.3% of the entire *Pseudo-nitzschia* community and did not show a marked seasonality. This species was never recorded from July to September and had the highest mean abundance in October ($9.9 \times 10^3 \pm 9.7 \times 10^3$ cells l^{-1}) (Fig. 6F). Its abundance peak was recorded in October 1990 (2.0×10^5 cells$\cdot l^{-1}$).

The mean contribution of each species to the *Pseudo-nitzschia* community is shown in Fig. 7. In terms of composition of the *Pseudo-nitzschia* assemblage throughout the year, *P.* cf. *delicatissima* and *P.* cf. *pseudodelicatissima* were always present, representing up to 85% (in February) and 91% (in December) of the *Pseudo-nitzschia* community, respectively. The maximum contribution of *P. pungens* to the community was re-

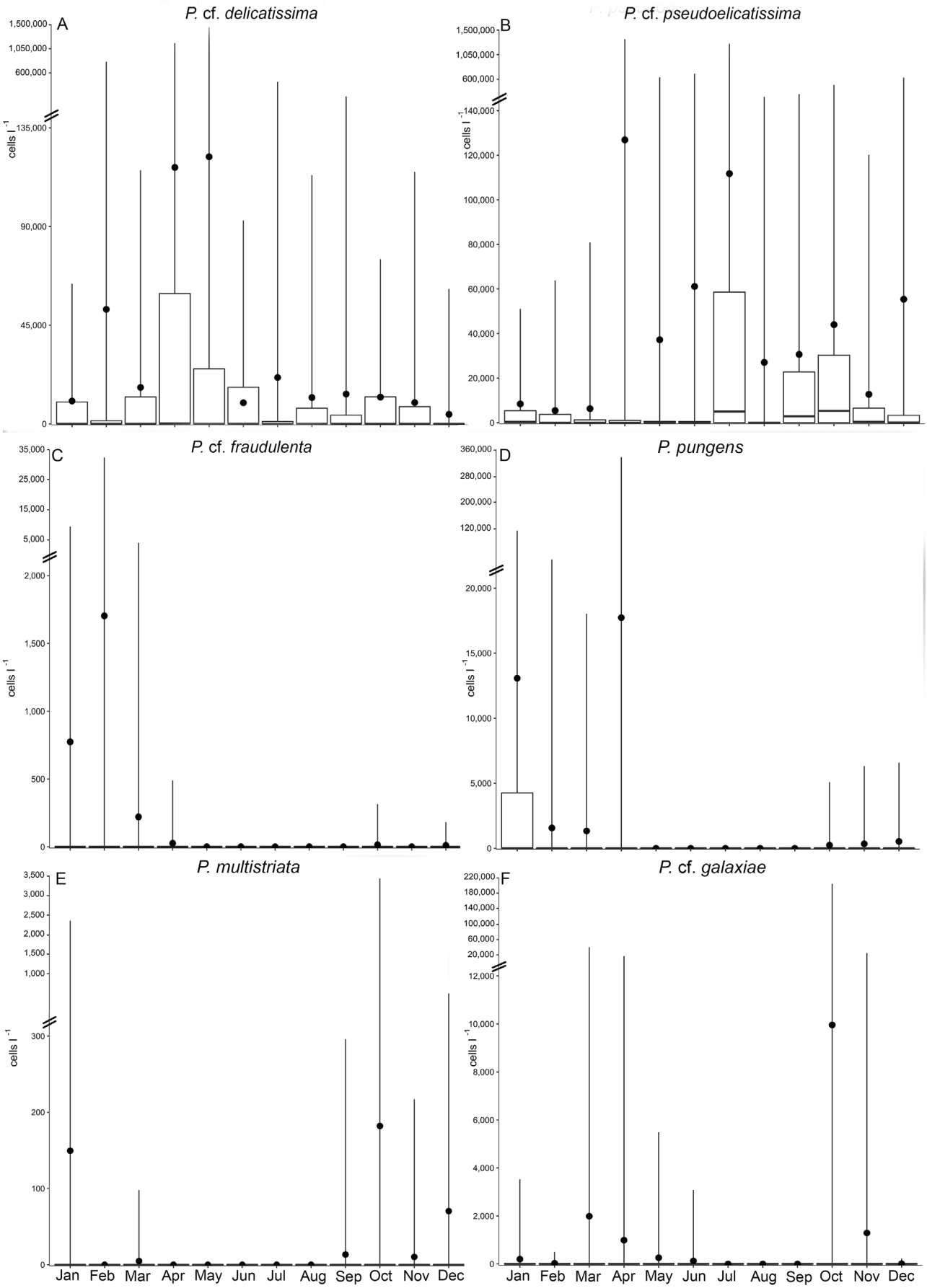

Fig. 6: Abundances of (A) *Pseudo-nitzschia* cf. *delicatissima*, (B) *P*. cf. *pseudodelicatissima*, (C) *P*. cf. *fraudulenta*, (D) *P. pungens*, (E) *P. multistriata* and (F) *P*. cf. *galaxiae* mean values (cells l^{-1}) in the LTER-SG01 station (February 1988-January 2020). Boxplot report the data distribution of the genus *Pseudo-nitzschia* with the mean (circle), median (bold horizontal line), the inter-quartile range (boxes) and the min-max range (vertical line). The extremes and outliers are not reported.

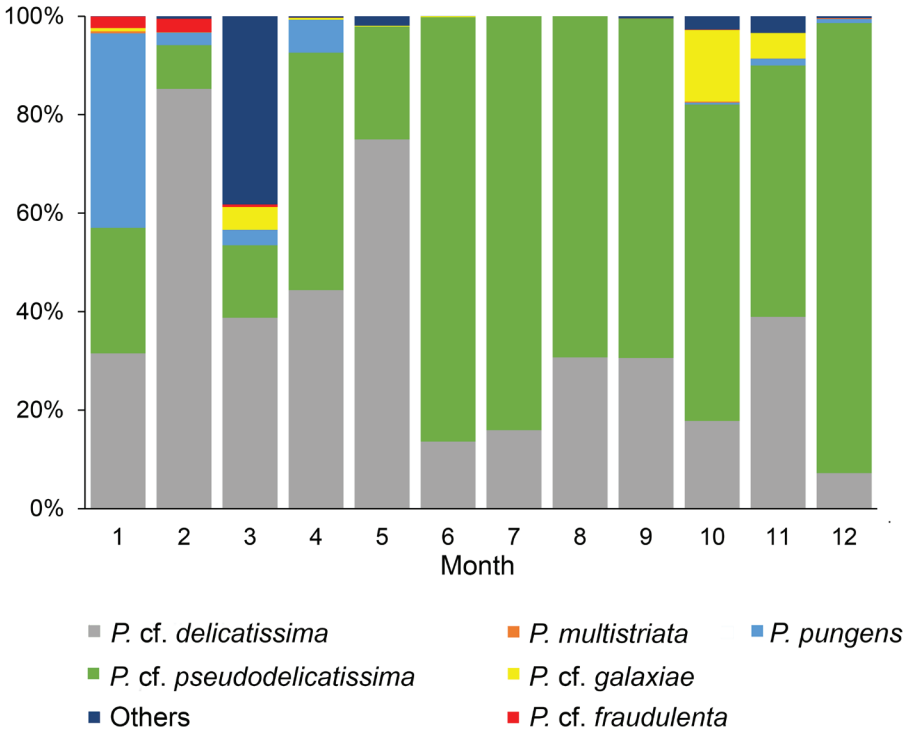

Fig. 7: Mean percent composition of *Pseudo-nitzschia* population in the LTER-SG01 station in terms of abundance monthly during the period February 1988-January 2020.

corded in January, when this species represented 39%. *P.* cf. *fraudulenta* never exceeded 3% (in February) of the *Pseudo-nitzschia* community. *P. multistriata* represented a small proportion of the community, reaching barely 0.5% in January. The maximum contribution of *P.* cf. *galaxiae* to the assemblage (15% of the *Pseudo-nitzschia* community) was recorded in October.

The composition of the seasonal assemblages in terms of significant species revealed by IndVal analysis is shown in Table 2. In winter, the highest IndVal was observed for *P.* cf. *fraudulenta* (14.05, $p < 0.001$) and *P. pungens* (11.90, ns) while in spring, the most significant species were found for *P.* cf. *delicatissima* (30.65, $p < 0.01$). Although not significant, in summer high IndVal values were observed for *P.* cf. *pseudodelicatissima*.

The PCA analysis showed the percentages of explained variance of the first two components (PC1 and PC2) of 73% (Fig. 8).

The first component (PC1) shows the DIN and silicate gradient opposite to those of salinity and temperature. On the contrary, the second axis (PC2) shows the PO_4 gradient associated with water temperature. The PCA analysis clearly separates winter and summer based mainly on a temperature gradient, but no clear separation is recovered for spring and autumn. Although total *Pseudo-nitzschia* spp. abundances were negatively correlated with PO_4, this correlation was not apparent in any of the single taxon analysed, indicating that each taxon has its own environmental requirements (Table 3). *P.* cf. *pseudodelicatissima* showed an opposite pattern compared to *P. pungens* and *P.* cf. *fraudulenta*. *Pseudo-nitzschia pungens* and *P.* cf. *fraudulenta* abundances were negatively correlated with water temperature and positively correlated with DIN (and DIN:PO_4), a typical condition characterizing most winters in the study area.

P. cf. *galaxiae* and *P. multistriata* were correlated with

Table 2. *Pseudo-nitzschia* species in the LTER-SG01 station (February 1988-January 2020) characterized by the highest IndVal for each season. IndVal values indicated in bold italic are significant at $p < 0.01$, those in bold italic and underlined are significant at $p < 0.001$.

	Winter	Spring	Summer	Autumn
P. cf. *fraudulenta*	***14.05***	0.01	0.00	0.03
P. pungens	11.90	1.55	0.00	0.46
P. cf. *delicatissima*	8.19	***30.65***	4.59	2.50
P. cf. *pseudodelicatissima*	1.48	14.76	18.39	12.00
P. cf. *galaxiae*	1.10	0.53	0.00	7.77
P. multistriata	1.59	0.00	0.05	6.33

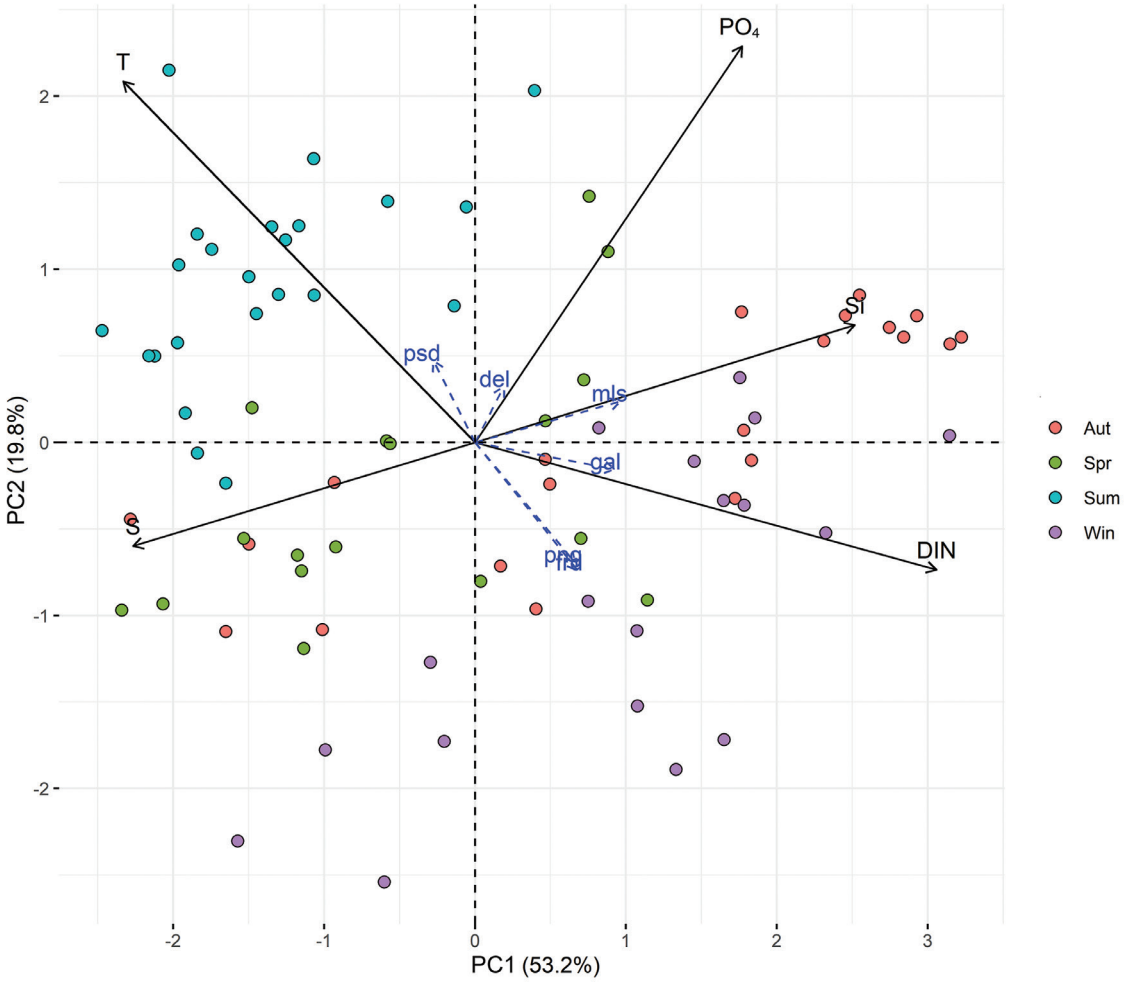

Fig. 8: Principal Component Analyses (PCA) based on correlation matrix of ranked environmental variables (active variables, black lines) and *Pseudo-nitzschia* abundances (supplementary variables, blue dotted lines) in the LTER-SG01 station (February 1988-January 2020). Data are plotted with individuals representing seasonal samples (Aut = autumn, Spr = spring, Sum = summer, Win = winter). T = temperature; S = salinity. PO_4 = orthophosphate; DIN = Dissolved Inorganic Nitrogen; Si = Silicate; "psd" *P.* cf. *pseudodelicatissima*; "del" *P.* cf. *delicatissima*; "mls" *P. multistriata*; "frd" *P.* cf. *fraudulenta*; "png" *P. pungens*; "gal" *P.* cf. *galaxiae*.

DIN, and the latter showed a significant positive correlation with PO_4 values (Table 3).

DNA sequence analysis

The trend of the *Pseudo-nitzschia* assemblage in the period January 2018-January 2020, when the species composition was resolved with DNA sequence data, is shown in Figure 9. In the year 2018, the temporal trend reflected the mean annual trend recorded in the last 30 years, while in 2019 *Pseudo-nitzschia* abundances were lower without any recorded blooms.

DNA sequences revealed the occurrence of six species of *Pseudo-nitzschia*, i.e., *P. delicatissima*, *P.* cf. *arenysensis*, *P. mannii*, *P. calliantha*, *P. pungens* and *P. fraudulenta*, identified from 106 monoclonal strains isolated in the LTER Senigallia transect (N Adriatic Sea) (Table S1). BLAST matches of the LSU and ITS rDNA sequences (Table S1) confirmed the identification of the above *Pseudo-nitzschia* spp. (see Giulietti *et al*., 2021 for details of the phylogenetic and EM characterization of some strains collected in this study).

Among species of the *seriata* group, *P. pungens* was identified by LM and its identification was confirmed by DNA sequences (Table S1). The presence of *P.* cf. *fraudulenta*, among the isolated strains was revealed only by DNA sequences (Table S1). The maximum abundance was recorded in January 2020 (1.0×10^4 cells l^{-1}) and January 2018 (9.8×10^3 cells l^{-1}) for *P. pungens* and *P.* cf. *fraudulenta*, respectively, as expected based on the seasonal trend recorded in the 30-year time series. Strains of *P. pungens* were isolated in January and March 2018, when this species was also detected by LM. Conversely, although no cells of *P. pungens* were detected using LM in April and May 2019, some *P. pungens* strains were isolated from net samples (Fig. 9A). Strains of *P. fraudulenta* were isolated only in March 2018, when *P.* cf. *fraudulenta* was detected using LM as well.

For the *delicatissima* group, LM observations allowed only identification at the 'complex' level (i.e., *P.* cf. *pseudodelicatissima* and *P.* cf. *delicatissima*). DNA sequences of isolates allowed identification of 4 species: *P. calliantha* and *P. mannii* within the *P. pseudodelicatissima* com-

Table 3. Spearman-rank order correlation between water temperature (°C), salinity, nutrients (silicate, Dissolved Inorganic Nitrogen (DIN), orthophosphate (PO_4) and inorganic Nitrogen:Phosphorus ratio (DIN:PO_4) and abundances of *Pseudo-nitzschia* cells, *P.* cf. *delicatissima*, *P.* cf. *pseudodelicatissima*, *P.* cf. *fraudulenta*, *P. pungens*, *P. multistriata* and *P.* cf. *galaxiae*) in the LTER-SG01 station (February 1988-January 2020). Values indicated in italic are significant at $p < 0.05$, those in bold italic are significant at $p < 0.01$, those in bold italic and underlined are significant at $p < 0.001$.

	P. cf. *delicatissima*	*P.* cf. *pseudodelicatissima*	*P.* cf. *fraudulenta*	*P. pungens*	*P. multistriata*	*P.* cf. *galaxiae*	*Pseudo-nitzschia* TOTAL
Temperature (°C)	0.06	*0.23*	***-0.28***	***-0.34***	-0.11	-0.19	0.13
Salinity	-0.11	-0.07	-0.02	-0.13	-0.17	-0.15	-0.11
Silicate	0.07	0.01	0.07	0.05	*0.24*	0.19	-0.13
DIN	0.05	-0.11	*0.27*	*0.23*	***0.31***	*0.24*	-0.10
PO_4	-0.05	-0.08	0.03	0.03	0.17	0.13	*-0.24*
DIN:PO_4	0.13	-0.01	***0.31***	***0.34***	0.21	0.17	0.11

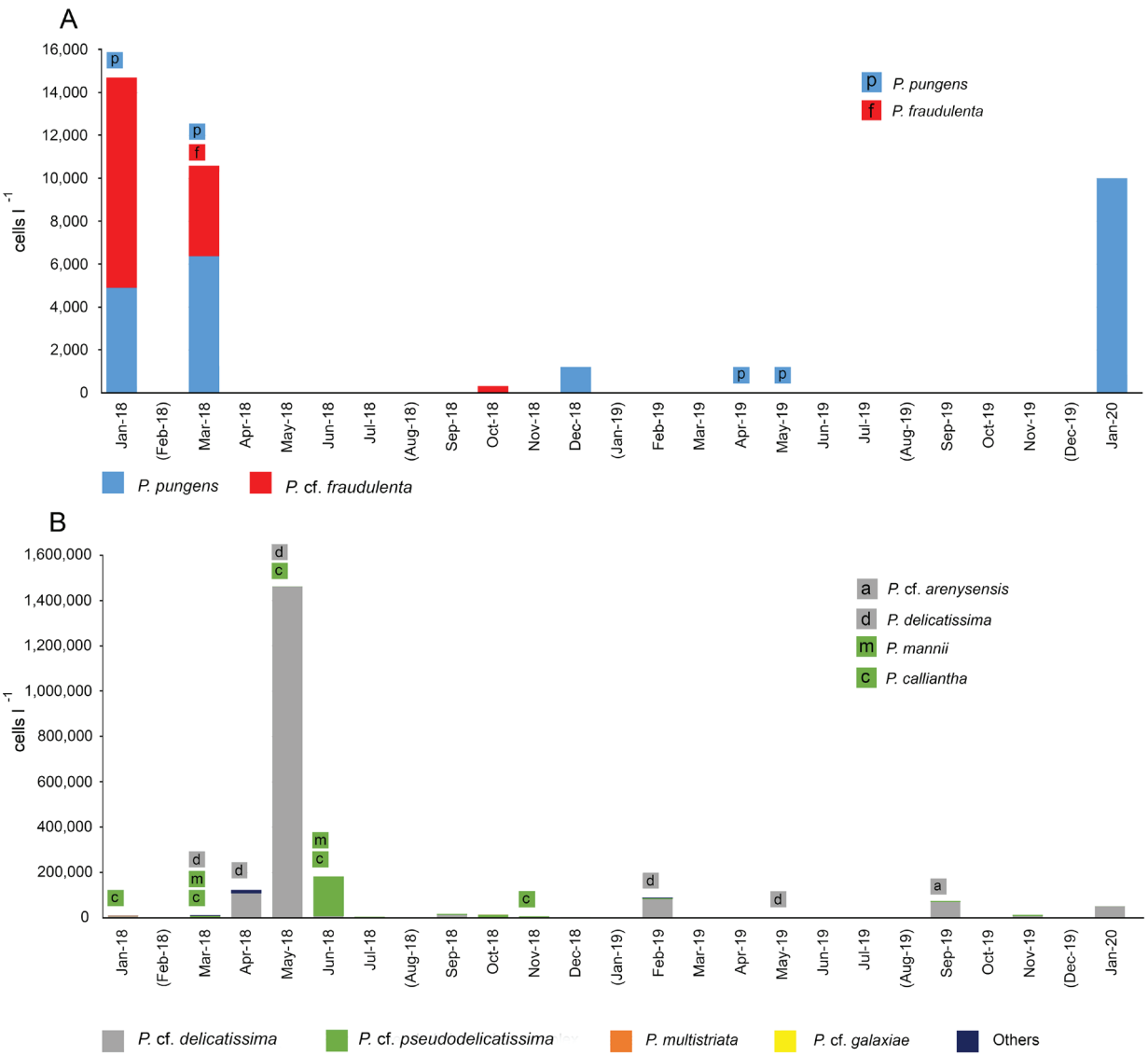

Fig. 9: Temporal trend of the abundances (cells l^{-1}) of (A) *Pseudo-nitzschia* seriata group and (B) *Pseudo-nitzschia delicatissima* group (divided in *P.* cf. *delicatissima* and *P.* cf. *pseudodelicatissima*) identified by LM (bars) and presence of species isolated in LTER-SG01 station during January 2018-Janyary 2020 period and identified by molecular analyses (letters within boxes above bars). No sampling was carried out in months in brackets.

plex, and *P. delicatissima* and *P.* cf. *arenysensis* within the *P. delicatissima* complex (Fig. 9B).

The maximum abundance was recorded in June 2018 (1.8×10^5 cells l^{-1}) and in May (2.6×10^6 cells l^{-1}) for *P.* cf. *pseudodelicatissima* and *P.* cf. *delicatissima*, respectively. When *P.* cf. *pseudodelicatissima* was detected in LM, strains of *P. calliantha* were isolated with higher frequency (i.e., in January, March, May, June and November 2018) than strains of *P. mannii* (isolated only in March and June 2018).

Among the isolated strains of the *P. delicatissima* complex, *P. delicatissima* was the only species recorded in March, April, May 2018, and February and May 2019, while *P.* cf. *arenysensis* was isolated only once in September 2019 (Fig. 9B). Neither species were isolated in the same month.

P. cf. *galaxiae* was neither recorded in these two years by LM nor in any of the net samples analysed.

Discussion

The analysis of time series in the coastal station of the LTER Senigallia-Susak transect in northwestern Adriatic Sea during the last three decades highlighted the specific composition and the seasonal trend of *Pseudo-nitzschia* spp. Such genus produced consistent blooms in spring and summer, with abundances up to 10^6 cells l^{-1}, contributing up to 90% of the total diatom community. The observed seasonal trend differed from that reported both in the Gulf of Trieste, NE Adriatic Sea (where blooms occurred in autumn and only occasionally in spring, Turk Dermastia *et al.*, 2020), and in an estuarine area of the eastern Adriatic Sea (where *Pseudo-nitzschia* showed the highest abundance in late-summer, representing the bulk of diatom communities, Arapov *et al.*, 2020). These differences may be explained by considering that in these distinct areas of the N Adriatic Sea environmental conditions differ markedly, particularly in terms of trophic status (the eastern Adriatic Sea is characterized by oligotrophic waters less influenced by river discharges compared to the western and northern coastal areas (Giani *et al.*, 2012)), and therefore seasons are not necessarily characterized by the same conditions.

The temporal trend of environmental parameters during the period considered (1988-2020) showed the typical seasonal trend of temperature, with cold winters and warm summers (Grilli *et al.*, 2020). The pattern of nutrients was driven by both the mixing/stratification regime in the water column and the rhythm of freshwater discharges, as well as by the high concentration of inorganic nitrogen. The low concentrations of phosphates reflected the typical P-limited condition of northern Adriatic Sea (Cozzi & Giani, 2011; Giani *et al.*, 2012). The phytoplankton mean annual cycle from 1988 to 2020 is very similar to that observed in almost the same period (1988-2016) as discussed by Totti *et al*. (2019), in agreement with what has already been reported for the N Adriatic (Bernardi Aubry *et al.*, 2004, 2006; Totti *et al.*, 2005, 2019).

Several studies have highlighted correlations between the abundances of *Pseudo-nitzschia* spp. and some environmental factors, such as water temperature, salinity, photoperiod, rainfall and nutrient concentrations (Caroppo *et al.*, 2005; Quijano-Scheggia *et al.*, 2008; Thorel *et al.*, 2017; Turk Dermastia *et al.*, 2020). A positive correlation with water temperature and salinity and a negative correlation with nutrients was detected for the whole genus *Pseudo-nitzschia* in an estuarine area of the eastern Adriatic Sea (Arapov *et al.*, 2020). In the Gulf of Trieste, the amount of *Pseudo-nitzschia* cells was positively correlated with water temperature and negatively with nutrients (Turk Dermastia *et al.*, 2020), while in this study only a negative correlation with PO_4 was detected. In the same way, species of the *Pseudo-nitzschia delicatissima* group (*P*. cf. *delicatissima* and *P*. cf. *pseudodelicatissima*) correlated with high nitrate and low silicate concentrations in some areas (e.g., Bay of Seine, Bay of Fundy, Eastern English Channel, and Alfacs and Fangar Bay (Kaczmarska *et al.*, 2007; Quijano-Scheggia *et al.*, 2008; Downes Klein *et al.*, 2010; Thorel *et al.*, 2017) or to low nitrate concentrations in others, e.g., western English Channel (Downes-Tettmar *et al.*, 2013). In this study, a clear positive correlation with water temperature was recognizable only in the *P. pseudodelicatissima* complex. The IndVal analysis revealed an association of this complex with summer, while the *P*. *delicatissima* complex was associated with spring. Such variability highlights the heterogeneity of this group, which includes several very different species, as well as the complexity of their interactions with several environmental factors.

Unfortunately, knowledge about species-specific correlation with environmental parameters is scarce and based mainly on strains kept in controlled, experimental conditions (Thessen *et al.*, 2009; Lema *et al.*, 2017). In the field, southern Adriatic *P. calliantha* correlated negatively with water temperature and positively with nutrient concentrations during a winter bloom (Caroppo *et al.*, 2005). Conversely, in the northeastern Adriatic Sea *P. calliantha* correlated with nutrients (phosphate and ammonium) and water temperature during a summer/autumn bloom (Ljubešić *et al.*, 2011).

In this study, *P. pungens* and *P.* cf. *fraudulenta* occurred mostly in winter, and therefore correlated positively with DIN and negatively with temperature. Our results for *P. fraudulenta* are in agreement with results from previous studies reporting this species almost exclusively in winter in NE Adriatic Sea (Turk Dermastia *et al.*, 2020) and in early-spring of cold waters in other Mediterranean areas (Quijano-Scheggia *et al.*, 2008) and the English Channel (Downes-Tettmar *et al.*, 2013). *P. multistriata* is an autumn species, as already reported by Totti *et al*. (2019), and showed a positive correlation with DIN and silicates, as reported by Turk Dermastia *et al*. (2020) for the NE Adriatic Sea.

Despite our efforts to identify *Pseudo-nitzschia* at the lowest possible taxonomic level, significant seasonal variability may have been missed in our study, because of the presence of species that are difficult to discriminate using only LM. The importance of species discrimina-

tion becomes even more important when both potentially toxic and non-harmful species exist in the same group. In this regard, species revealed by DNA sequence data, such as *P. delicatissima*, *P.* cf. *arenysensis*, *P. calliantha*, *P. mannii*, *P. pungens* clade I and *P. fraudulenta* spp. have already been reported from the Adriatic Sea in studies focusing on this genus (Lundholm *et al.*, 2003; Caroppo *et al.*, 2005; Burić *et al.*, 2008; Marić *et al.*, 2011; Penna *et al.*, 2013; Arapov *et al.*, 2016, 2017). *P. galaxiae* and *P. subfraudulenta* were also frequently reported from this area. Unfortunately, neither *P. galaxiae, P. subfraudulenta* or *P. multistriata* were successfully isolated, even though *P*. cf. *galaxiae* and *P. multistriata* were identified using LM. This suggests that, although numerous strains were collected in the last two years of sampling (i.e., 138), further cryptic and/or pseudo-cryptic species could be present in the area.

Among the *P. pseudodelicatissima* complex, *P. calliantha* and *P. mannii* were the only two species often co-occurring. In the this complex, however, *P. delicatissima* was often the only isolated member, except for *P.* cf. *arenysensis* that was isolated once in late summer (see also Lamari *et al.,* 2013 reported as *P.* cf. *delicatissima*). However, *P.* cf. *arenysensis* is not considered a strictly summer species, as it has already been recorded in NW Adriatic Sea in winter (Pugliese *et al.* 2017).

Remarkably *Pseudo-nitzschia multistriata*, an allochthonous species (Corriero *et al.*, 2016; Zenetos *et al.*, 2010), became a regular inhabitant of phytoplankton communities of this area since its first record in September 2012, although with low abundances. In the northern Adriatic Sea it has been recorded mainly in autumn-winter (Totti *et al.*, 2019; Turk Dermastia *et al.*, 2020), while in the Gulf of Naples (Tyrrhenian Sea) it has a widespread annual distribution (Ribera d'Alcalà *et al.*, 2004; Ruggiero *et al.*, 2015) including summer, autumn and winter.

In the Adriatic Sea, DA was recorded for the first time by Ciminiello *et al.* (2005) in *Mytilus galloprovincialis* with a toxin content ranging from 63 to 190 ng/g, i.e., well below the regulatory limit of DA in tissue (20 mg/kg) (EU, 2004). Since then, DA was detected several times, but rarely reaching 2 mg/kg in tissue (Ljubešić *et al.*, 2011; Marić *et al.*, 2012; Arapov *et al.*, 2017). Therefore, finding a DA producer in NW Adriatic Sea was not expected.

Among the five species confirmed by DNA sequence data in the SG01-LTER station during the present study, *P. pungens*, *P. fraudulenta*, *P. delicatissima* and *P. calliantha* were reported to be toxic elsewhere (Hasle, 2002; Lundholm *et al.*, 2003; Moschandreou *et al.*, 2012). However, none of the strains from this study produced DA in detectable amounts (Giulietti *et al.*, 2021). Indeed, several of these potentially toxic species have been reported to be non-toxic (Trainer *et al.*, 2012), in particular in the Adriatic, where DA has been detected in low amounts only in *P. delicatissima* (Penna *et al.*, 2013), and in *P. multistriata* (Turk Dermastia *et al.*, 2018).

In conclusion, in this study the species composition and the seasonal variability of the *Pseudo-nitzschia* populations were investigated in detail in the coastal station of the LTER Senigallia-Susak transect, highlighting that these diatoms may produce intense blooms. *P.* cf. *fraudulenta* and *P. pungens* were associated with winter, *P.* cf. *delicatissima* and *P.* cf. *pseudodelicatissima* with spring and summer respectively, and *P. galaxiae* had a peak of abundance in autumn. This study unravelled several cryptic and pseudo-cryptic species, leading to marked differences in seasonality even in geographically close areas. We suggest that in future studies focusing on the ecology of *Pseudo-nitzschia* microscopy and DNA sequence data should be combined.

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Supplementary data

The following supplementary information is available online for the article:

Table S1. List of *Pseudo-nitzschia* spp. strains isolated in the LTER Senigallia transect (N Adriatic Sea) in January 2018-January 2020. The three columns on the right, i.e. cover%, identity% and best match, are referred to the BLAST results.