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Vertical distribution of Pseudo-nitzschia in the Gulf of Naples across the seasons

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Research Article

Vertical distribution of *Pseudo-nitzschia* in the Gulf of Naples across the seasons

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Abstract

Pseudo-nitzschia is a widespread genus of marine pennate diatoms comprising 58 species of which 28 can produce the neurotoxin domoic acid, causative of Amnesic Shellfish Poisoning (ASP). Species of the genus are found in both oceanic and coastal waters where they can form large blooms. The timing and magnitude of blooms is generally described based on water samples collected at surface, while limited information is available on cell distribution within the water column. To address this knowledge gap, we investigated the vertical (0-50 m) distribution of *Pseudo-nitzschia* species identified in light microscopy using weekly samples collected in the Gulf of Naples (Italy) across an annual cycle (April 2006-April 2007). Physical variables and nutrient concentrations were also monitored. The genus is primarily abundant in surface waters, where spring blooms of *P.* cf. *delicatissima* and two morphotypes of *P. galaxiae* were related to the presence of a water layer with low salinity and high concentration of inorganic nutrients. Blooms of *P.* cf. *pseudodelicatissima, P. allochrona* and *P. multistriata* were recorded at surface during the summer-early autumn, when the water column was stratified and nutrient concentrations were low. Our observations confirmed the marked seasonality of *Pseudo-nitzschia* species described at our study site with light microscopy and molecular approaches, with species virtually absent from surface waters for long periods of the year. However, the high quantitative resolution applied in this study allowed to reveal low concentrations of *Pseudo-nitzschia* species/species complexes throughout the water column over the year. Since *Pseudo-nitzschia* do not produce benthic resting stages, their presence in deeper waters suggests that these species rely on rare overwintering cells that can function as bloom inocula.

Keywords: Diatoms; HABs; LTER-MC; diversity; *Pseudo-nitzschia.*

Introduction

Pseudo-nitzschia is a genus of planktonic pennate diatoms that includes 58 species (Guiry and Guiry, 2021) among which at least 28 are known to produce the neurotoxin domoic acid (DA) (Lundholm, 2021). DA accumulates in the tissues of filter feeders and enters the food web killing seabirds and mammals (e.g., Work *et al.*, 1993; Scholin *et al.*, 2000; Soliño *et al.*, 2019) and causing the Amnesic Shellfish Poisoning (ASP) syndrome in humans (Jeffery *et al.*, 2004). Blooms of *Pseudo-nitzschia* are a common feature in coastal areas over a broad latitudinal range and also in open oceanic waters (Lelong *et al.*, 2012; Trainer *et al.*, 2012; Bates *et al.*, 2018). When toxic species are involved, these blooms can cause severe economic losses due to prolonged closure of aquaculture plans and/or ban on wild mussel and crustacean collection (e.g., Díaz *et al.*, 2019; Trainer *et al.*, 2020).

In the Mediterranean Sea, seasonal blooms of *Pseudo-nitzschia* spp. are frequently reported (e.g., Cerino *et al.*, 2005; Quijano-Scheggia *et al.*, 2008; Marić *et al.*, 2011). However, impacts on health and economy by these diatoms are rare in the basin (Zingone *et al.*, 2020). At the Long-Term Ecological Research station MareChiara (LTER-MC) in the Gulf of Naples (GoN), *Pseudo-nitzschia* species can form a substantial fraction of the phytoplankton assemblage with the most recurrent and abundant species being species of the *P.* cf. *delicatissima* group (mostly *P. arenysensis*), *P. galaxiae* and *P. multistriata* (Zingone *et al.*, 2006; Ruggiero *et al.*, 2015).

In routine light microscopy (LM) observations, the distinction of *Pseudo-nitzschia* species is possible in a limited number of cases. Species identification in this genus is often based on ultrastructural features of the siliceous frustule, only visible in acid-cleaned material, better seen in electron microscopy, while in many cas-

es sequences of the ribosomal genomic regions (e.g., ITS, LSU) need to be used to uncover cryptic and pseudo-cryptic diversity within complexes of taxa that appear morphologically identical (e.g., Amato *et al.*, 2007; Bates *et al.*, 2018; Lim *et al.*, 2018).

As in all diatoms, cell division in *Pseudo-nitzschia* causes a progressive decrease in the average cell size of the population (Davidovich and Bates, 1998; D'Alelio *et al.*, 2009). This miniaturization process is counterbalanced by sexual reproduction by which cells of the maximum size are formed within the auxospore produced by gamete mating (Montresor *et al.*, 2016). The sexual phase of various *Pseudo-nitzschia* species has been widely studied in culture (Bates *et al.*, 2018), while reports of sexual events in the natural environment are extremely rare. A massive sexual event of two *Pseudo-nitzschia* species at the same time (*P.* cf. *delicatissima* and *P.* cf. *calliantha*) was reported in the Gulf of Naples in September 2006 (Sarno *et al.*, 2010). Another sexual event, involving *P. pungens* and *P. australis*, was recorded in June 2006 along the North Pacific coast of the U.S. (Holtermann *et al.*, 2010).

Molecular studies in the Gulf of Naples have allowed the improvement of species circumscription and refined the seasonal patterns of cryptic and pseudo-cryptic taxa with 12 different *Pseudo-nitzschia* species identified at our study site to date (McDonald *et al.*, 2007; Amato and Montresor, 2008; Ruggiero *et al.*, 2015; Percopo *et al.*, 2021). Two studies based on clone libraries of the LSU rDNA marker region (McDonald *et al.*, 2007; Ruggiero *et al.*, 2015) demonstrated that spring blooms of the *P. delicatissima* complex are mainly driven by two distinct species, i.e., *P arenysensis* (= *P. delicatissima* in McDonald *et al.*, 2007) and *P. delicatissima sensu stricto* (=*P. delicatissima*2 in McDonald *et al.*, 2007), while the recurring summer/early autumn blooms should be attributed to a different genotype recently described as a new species, *P. allochrona* (Percopo *et al.*, 2021). Cryptic diversity was detected also within the *P. pseudodelicatissima* complex (*P. pseudodelicatissima, P. mannii, P. hasleana* and *P. calliantha*) and different genotypes were also recorded within *P. galaxiae*: ribogroups I and II, corresponding to the small morphotype and to the medium-large morphotypes, respectively (Cerino *et al.*, 2005; McDonald *et al.*, 2007; Ruggiero *et al.*, 2015).

Blooms of *Pseudo-nitzschia* often show remarkable seasonality, which cannot be always explained by specific environmental factors (e.g., Zingone *et al.*, 2003; Fehling *et al.*, 2006, Smith *et al.*, 2018). In other diatom species, benthic spores may act as reservoirs in the time window between distinct blooms and seed the population in the water column upon germination (McQuoid and Godhe, 2004; Montresor *et al.*, 2013; Piredda *et al.*, 2017). However, *Pseudo-nitzschia* resting stages have never been found in sediments (Belmonte and Rubino, 2019). Bloom inocula for *Pseudo-nitzschia* could hence be provided by vegetative cells that persist at low abundance in deeper layers of the water column. However, monitoring data are generally gathered only in surface layers, while quantitative information on the vertical distribution of *Pseu-do-nitzschia* species is sparse.

Patterns of vertical distribution of phytoplankton species depend on both environmental factors – e.g., irradiance, physical structure of the water column, nutrient availability - and biological characteristics of the species – e.g., species equipped with flagella, capable to regulate their buoyancy or adapted to a low light environment. The development of blooms requires the availability of light and inorganic nutrients and generally occurs in surface layers. However, microalgae can accumulate and even grow in subsurface chlorophyll maxima, and/or in correspondence to density gradients, where they can form thin phytoplankton layers (e.g., Durham and Stocker, 2012).

In the present study we used weekly sampling to determine the vertical distribution (0-50 m) of *Pseudo-nitzschia* taxa in the water column using light microscopy at LTER-MC across an annual cycle (April 2006-April 2007). This detailed quantitative analysis aimed at assessing the persistence of cells at low concentration in the deeper layers of the water column and testing their possible accumulation in correspondence to the pycnocline (Velo-Suarez *et al.*, 2008). Light microscopy observation of the samples was also aimed at assessing the possible presence of sexual stages of *Pseudo-nitzschia* in subsurface layers.

Material and Methods

Study site

The Gulf of Naples (GoN, Tyrrhenian Sea, Western Mediterranean) (Fig. 1) is a coastal embayment with an average depth of 170 m covering an area of approximately 870 km^2. The densely populated region surrounding the Gulf heavily influences the coastal area by land runoff. However, riverine inputs are limited and intermittent throughout the year, giving a rather stable salinity to the basin. The relatively eutrophic inner coastal area is coupled with the oligotrophic offshore Tyrrhenian waters (Cianelli *et al.*, 2015). These two subsystems coexist separated by a boundary whose width and location vary at the seasonal scale (D'Alelio *et al.*, 2015; Cianelli *et al.*, 2017).

Salinity in the water column is primarily governed by runoff and lateral advection of freshwater from the Sarno River to the south and from Volturno River to the north. The frequency of these events may vary across the seasons (Iermano *et al.*, 2012). At seasonal scale, water column density, and thus stratification, are primarily governed by temperature, although lateral advection of coastal waters frequently determines a decrease in surface salinity that affects the dynamics of the mixed layer (Ribera d'Alcalà *et al*., 2004).

Sampling and analyses

Sampling was carried out weekly, from April 12th 2006 to April 11th 2007, at the LTER-MC site (40.81 N,

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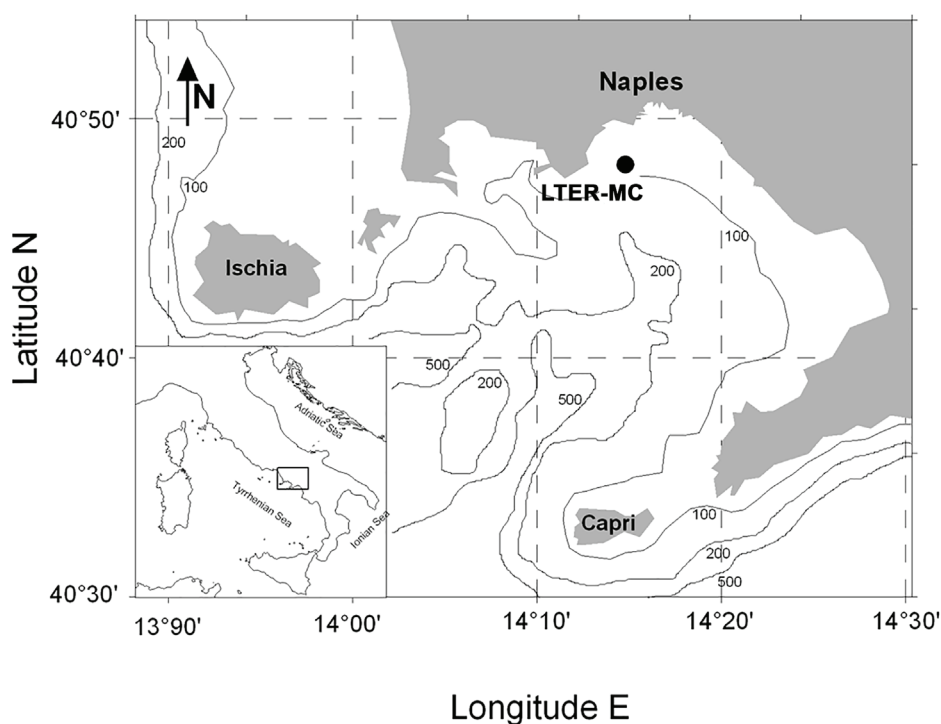*Medit. Mar. Sci., 23/3 2022, 525-535*

Fig. 1: Study area with the location of the sampling site LTER-MC in the Gulf of Naples.

14°15 E) located two nautical miles offshore from the coastline in the proximity of the 75-m isobath. Temperature, conductivity, dissolved oxygen concentration and fluorescence profiles were acquired by means of a SBE 19plus (Sea-Bird Scientific, U.S.) multi-parametric probe, connected to a Sea-Bird Electronics automatic Carousel sampler equipped with 12 Niskin bottles (capacity 10 L). Data were processed with the SeaSave Data Processing software. The processing included measurement alignment, binning to standard depths and computation of salinity and potential density (kg m^{-3}) based on EOS-80 equations. Samples for dissolved inorganic nutrient analyses (NH_4, NO_3, NO_2, PO_4 and SiO_4 concentrations) were collected in 20 mL high-density polyethylene vials from the Niskin bottles at eight depths (0.5, 2, 5, 10, 20, 30, 40 and 50 m) and immediately stored at –20 °C. Analyses were carried out with a five-channel continuous flow autoanalyzer (Flow-Sys, Systea, Italy), according to Hansen and Grasshoff (1983). The detection limits were 0.1 mmol m^{-3} for SiO_4, 0.05 mmol m^{-3} for NH_4 and 0.01 mmol m^{-3} for NO_3, NO_2 and PO_4. Dissolved inorganic nitrogen (DIN) was calculated as the sum of NH_4, NO_2 and NO_3 concentrations.

Samples for chlorophyll *a* (chl *a*) determination were collected in dark plastic containers from Niskin bottles at 0.5, 2, 5, 10, 20 and 40 m depths. After careful mixing, subsamples (300-540 mL) were filtered onto GF/F filters and immediately stored in liquid nitrogen until the analysis. Chl *a* and phaeopigments were analysed according to Holm-Hansen *et al.* (1965) with a Shimadzu RF-5301 PC (Shimadzu Scientific Instruments, Japan) spectrofluorometer. The instrument was daily calibrated with a chl *a* standard solution (from *Anacystis nidulans*, Sigma). All biogeochemical variables were subjected to quality control and flagged according to Sabia *et al.* (2019).

A volume of 250 mL of seawater for phytoplankton analyses was collected at 0.5, 10, 20, 30, 40 and 50 m depths and immediately fixed with neutralized formaldehyde (0.8% final concentration). Diatoms of the genus *Pseudo-nitzschia* were identified to the species or group level. In the latter category the *Pseudo-nitzschia delicatissima* complex (*sensu* Lundholm *et al.*, 2006) and the *P. pseudodelicatissima* complex (*sensu* Lundholm *et al.*, 2003) were included; two morphotypes of *P. galaxiae* were considered, '*P. galaxiae* small', including cells ≤20 μm, and '*P. galaxiae* large'*,* including both medium and larger morphotypes, respectively (Cerino *et al.*, 2005). *Pseudo-nitzschia* cells that could not be identified at the species or species complex level were listed as *Pseudo-nitzschia* spp. Only alive cells i.e., cells with cytoplasmic content, were counted. Species concentration was determined according to the Utermöhl method (Edler and Elbrächter, 2010) with an inverted light microscope Zeiss Axiovert 200 (Zeiss, Germany) at 400X magnification. Cell counts were performed after sedimentation of variable sample volumes (10–100 mL), depending on cell concentration, on 1-8 transects, representing ca 1/68-1/8 of the whole bottom area of the sedimentation chamber, with a detection limit ranging from 1647 to 82 cells L^{-1} (82 cells L^{-1} in 75% of the 297 samples).

Statistical analyses

Canonical correspondence analysis (CCA) was carried out using the vegan package (Oksanen *et al.*, 2019) under R environment (R Core Team, 2021) to investigate the relationship between standardized environmental variables (temperature, salinity, sigma theta, daylength, chl *a*, DIN, phosphate, silicate) and log transformed *Pseu-*

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do-nitzschia species abundances using data collected at the surface layer (0.5 m). Daylength values (hours) were computed from the R package geosphere (Forsythe *et al.*, 1995). The original abundance dataset was reduced, discarding counts of unidentified *Pseudo-nitzschia* species. Based on the results of Percopo *et al.* (2021), cell concentrations of *P.* cf. *delicatissima* occurring from July to December were attributed to *P. allochrona*.

Results

Physical and chemical structure of the water column

During the sampling period, temperature ranged between 13.43 °C (at 50 m on May 5th) and 27.50 °C (at surface on August 1st) (Fig. 2A). It is noteworthy to point out the high differences observed in early spring between 2006 (which followed a severe winter, F. Margiotta unpublished data) and 2007 (extremely warm winter), resulting in about 1.5 °C of difference in the layer below 30 m between the two years. Highest temperatures (>24 °C) were recorded at the surface from the end of June through August. Salinity values were in the range 36.65-38.36 (Fig. 2B). Frequent pulses of low salinity waters, particularly in the surface layer (<10 m), were recorded

in spring (minimum values: 36.65 in 2006 and 37.07 in 2007) and in summer, when they caused a sharp halocline. From October until mid-January high salinity values (>38) were recorded throughout the water column. The vertical structure of the water column, in terms of potential density, was mainly driven by temperature, although the pulses of low salinity water in the upper layer enhanced the stratification in some cases (Fig. 2, Suppl. Fig. 1A). Water column stratification started in mid-April and peaked during summer between July and August, when the thermocline was located around 20 m depth. The thermocline started to deepen in late September until it was completely disrupted in November (Fig. 2A).

All nutrient concentrations displayed the same vertical distribution characterized by higher and more variable values in the 0-10 m layer as compared to the layer below (Suppl. Fig. 1B-D). Dissolved inorganic nitrogen (DIN) concentrations were highest (up to 10.07 mmol m^{-3} in 2006 and up to 6.36 mmol m^{-3} in 2007) in spring. A DIN increase was observed in autumn (<8.05 mmol m^{-3}) while the lowest values were recorded in summer, although marked depletion never occurred (values always >0.3 mmol m^{-3}). In winter, DIN vertical gradients were less pronounced. Phosphate concentrations reached their maximum values in late spring 2006 (up to 0.55 mmol m^{-3}) while the lowest values were recorded in summer
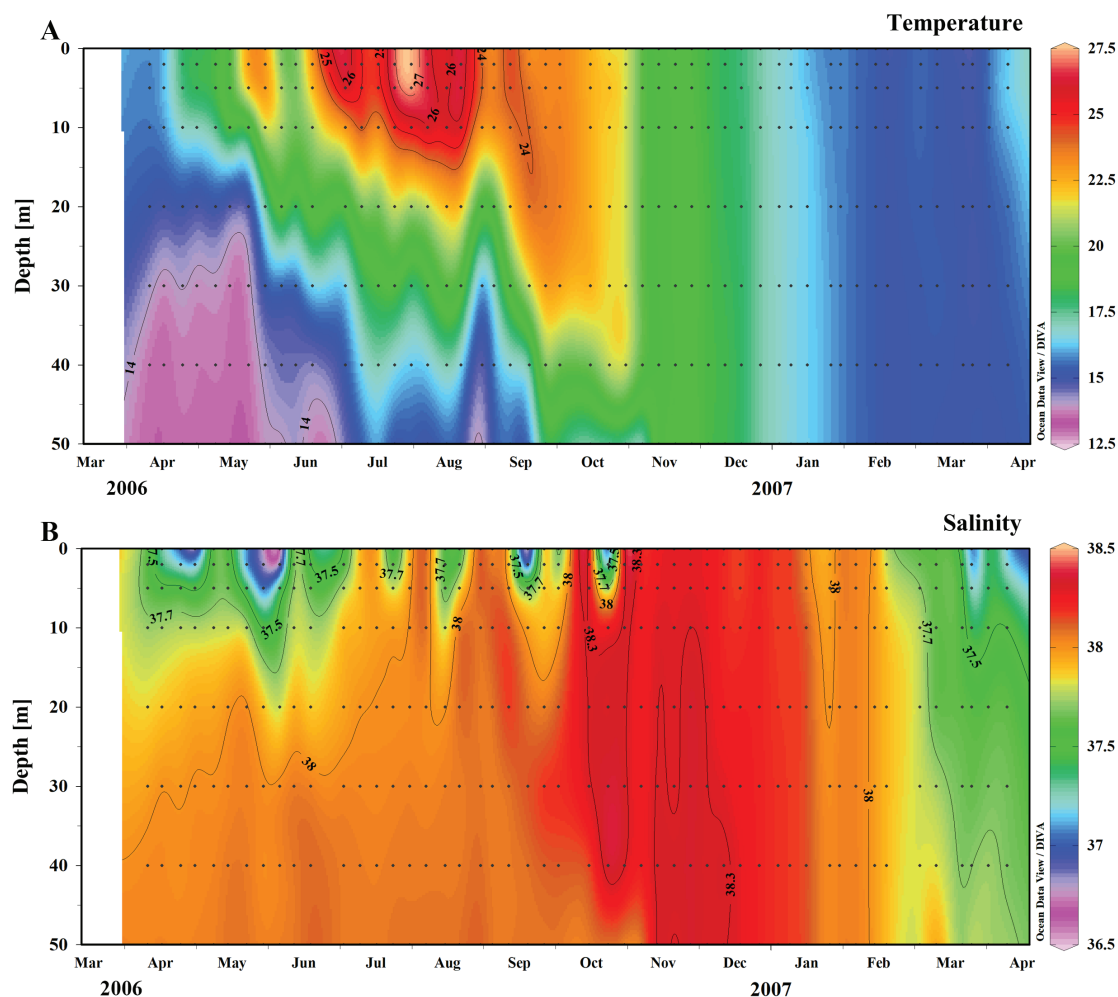

Fig. 2: Temporal and vertical distribution of (A) temperature (°C) and (B) salinity along the water column at LTER-MC (plotted with Ocean Data View; Schlitzer, 2018).

and late winter (Suppl. Fig. 1C). Silicate concentrations showed the highest peak (6.45 mmol m^{-3}) in the surface layer on April 11th 2007; high concentrations (>2 mmol m^{-3}) were also observed in May, October and December (Suppl. Fig. 1D).

Chl a concentrations showed several peaks between spring and late summer/autumn that reached the highest values of 7.6 mg m^{-3} on May 2nd and 6.6 mg m^{-3} on August 22nd (Fig. 3A). The highest chl a concentrations were observed concomitant with the lowest salinity values. The lowest chl a values (<1 mg m^{-3}) were recorded in winter from December to the end of February, when the water column was fully mixed. Vertical distribution of chl a often displayed strong vertical gradients and only on two occasions, during the stratification of the water column, i.e., May 24th at 40 m depth and July 4th at 20 m depth, subsurface peaks of chl a were detected (1.1 mg m^{-3} and 0.9 mg m^{-3}, respectively).

Vertical distribution of Pseudo-nitzschia *species over the annual cycle*

A total of six species/species complexes of the genus *Pseudo-nitzschia* were identified in LM: *Pseudo-nitzschia delicatissima* complex (from here onwards named *Pseudo-nitzschia* cf. *delicatissima*), *P. fraudulenta*, *P.*

multistriata, *P. pseudodelicatissima* complex (from here onwards named *Pseudo-nitzschia* cf. *pseudodelicatissima*), *P. galaxiae* large morphotype (including medium and large morphotypes) and *P. galaxiae* small morphotype (Fig. 3B, Fig. 4A-F). The annual distribution of *Pseudo-nitzschia* spp. (Fig. 3B) showed four major peaks that were mainly concentrated in the surface layer: in April 2006 and in April 2007 (both due to blooms of *P.* cf. *delicatissima*, *P. galaxiae* large and *P. galaxiae* small), in July 2006 (mainly *P.* cf. *delicatissima* and *P.* cf. *pseudodelicatissima*) and in the second half of September 2006 (mainly *P.* cf. *delicatissima*) (Fig. 4A-F, Suppl. Fig. 2).

In the following, we describe the temporal succession of the *Pseudo-nitzschia* species throughout the water column, based on both Figures 4A-F and Supplementary Figure 2 in which the vertical distribution of the species is reported for each sampling point. In April 2006, when the thermocline started to develop, *P.* cf. *delicatissima* and *P. galaxiae* large were present at relatively low concentrations throughout the water column (<2 x 10^5 cells L^{-1}) but with higher abundances at surface, where the two taxa showed comparable cell concentrations of ~4.5 x 10^5 cells L^{-1} on April 12th and 18th, corresponding to lower salinity values. From May to the first half of June, extremely low concentrations of *Pseudo-nitzschia* were recorded. However, *P. galaxiae* large, *P. galaxiae* small and *P.* cf. *delicatissima* persisted in the water column below
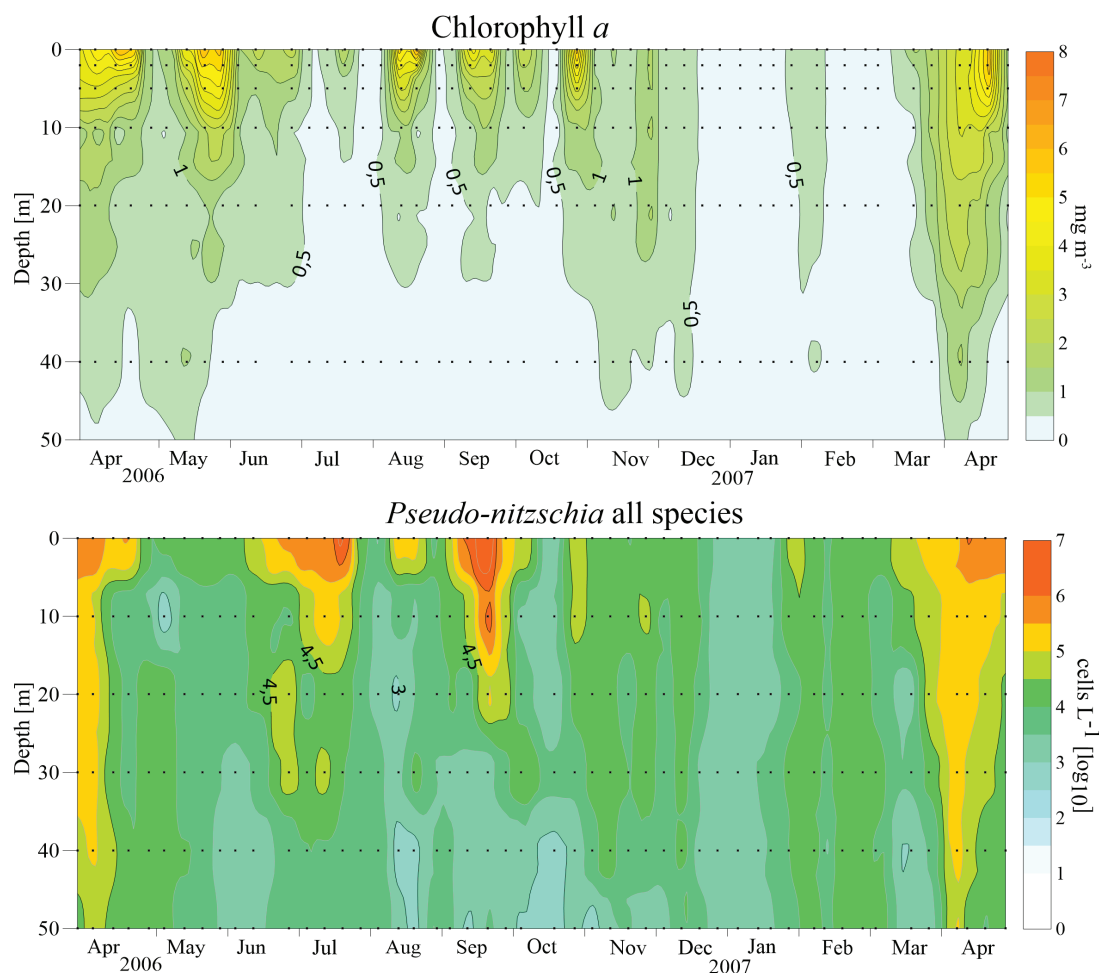

Fig. 3: Temporal and vertical distribution of (A) total chlorophyll a and (B) total abundance of *Pseudo-nitzschia* species throughout the water column at LTER-MC (plotted with Surfer® from Golden Software, LLC).
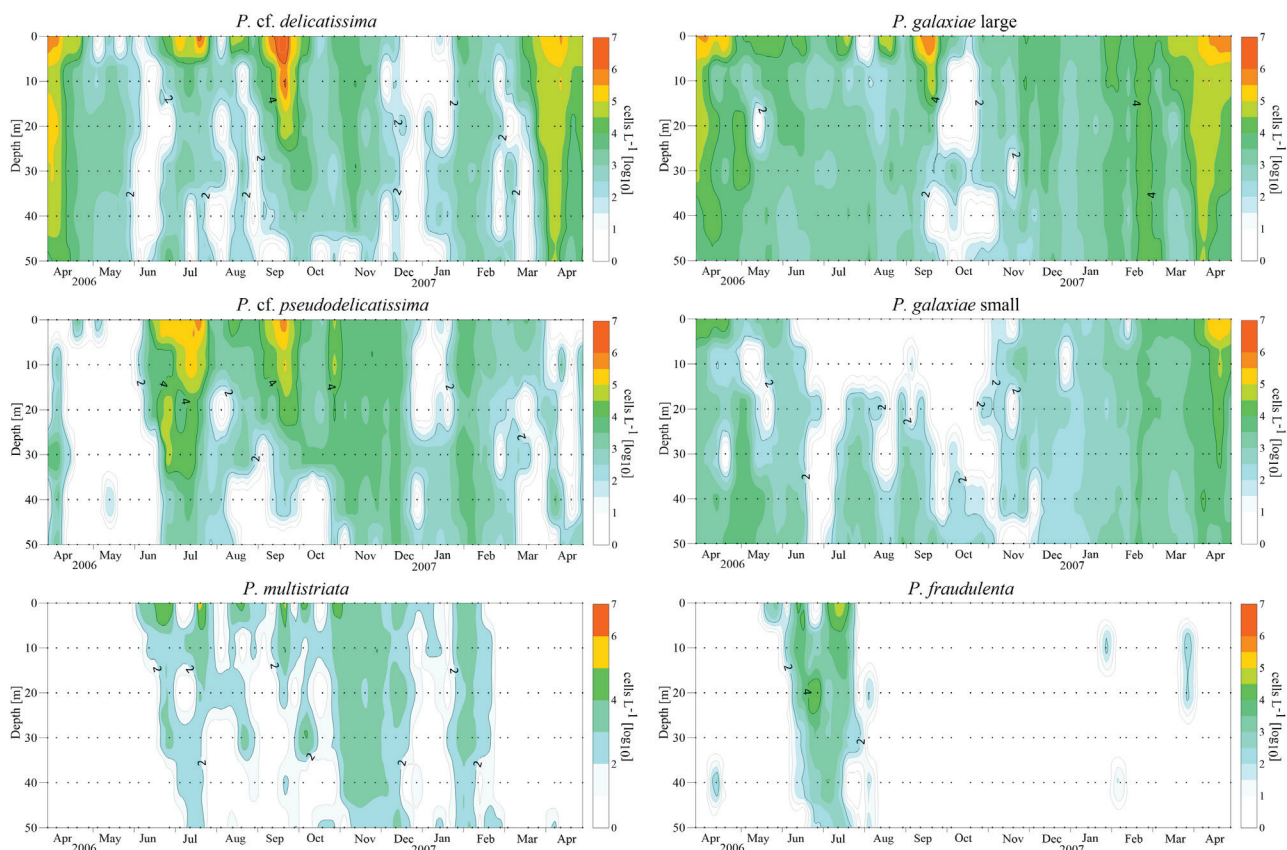
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Fig. 4: Temporal and vertical distribution of cell concentrations of (A) *Pseudo-nitzschia* cf. *delicatissima*, (B) *P. galaxiae* large, (C) *P.* cf. *pseudodelicatissima*, (D) *P. galaxiae* small, (E) *P. multistriata* and (F) *P. fraudulenta* throughout the water column at LTER-MC. Note that cell concentrations of *P.* cf. *delicatissima* occurring from July to December were attributed to *P. allochrona* (see text) (plotted with Surfer® from Golden Software, LLC).

the thermocline with total abundances generally <2 x 10⁴ cells L⁻¹.

In early summer all taxa were detected at our sampling site, with the exception of *P. galaxiae* small, which was not present in the upper portion of the water column (0.5-20 m) until the middle of October. On June 20ᵗʰ, *P.* cf. *pseudodelicatissima* and *P. fraudulenta* appeared in the upper layers above the thermocline (0.5-30 m). The former showed maximum cell concentrations (3.13 x 10⁴ cells L⁻¹) at surface and relatively high values (>4.53 x 10³ cells L⁻¹) also down to 30 m depth. *Pseudo-nitzschia fraudulenta* was present until the base of the thermocline with cell abundances up to 4.74 x 10⁴ cells L⁻¹ on July 18ᵗʰ. *Pseudo-nitzschia multistriata* was detected for the first time on July 4ᵗʰ, and *P.* cf. *delicatissima* re-appeared on July 11ᵗʰ. Based on the study by Percopo *et al.* (2021), the summer/autumn morphotype of *P.* cf. *delicatissima* is to be considered *P. allochrona* and we will use the notation *P.* cf. *delicatissima/allochrona* from here onwards. At this latter sampling date, *P.* cf. *pseudodelicatissima* and *P.* cf. *delicatissima/allochrona* were responsible for a bloom at surface with 4.28 x 10⁵ and 2.73 x 10⁵ cells L⁻¹, respectively. A second, larger bloom of *P.* cf. *delicatissima/allochrona* (~2 x 10⁶ cells L⁻¹) accompanied by *P.* cf. *pseudodelicatissima* (~5 x 10⁵ cells L⁻¹) was recorded two weeks later, on July 25ᵗʰ. *Pseudo-nitzschia* cell concentrations dropped again until the beginning of September. In this period of very low abundance, the two morphotypes

of *P. galaxiae*, *P. pseudodelicatissima* and *P. delicatissima* complexes and, at times *P. multistriata,* were however recorded throughout the water column, including the deeper layers below the thermocline.

Pseudo-nitzschia cf. *delicatissima/allochrona* was the major responsible for a third bloom on September 12ᵗʰ and 20ᵗʰ. On the first date, it reached a concentration of ~1 x 10⁶ cells L⁻¹ at the surface accompanied by *P. galaxiae* large and *P.* cf. *pseudodelicatissima,* which was identified as *P.* cf. *calliantha* based on frustule ultrastructure (Sarno *et al.*, 2010). The following week, when a pronounced thermocline was present, *P.* cf. *delicatissima/allochrona* reached a concentration of 8 x 10⁶ cells L⁻¹ at 0.5 m and about 1 x 10⁶ cells L⁻¹ at 10 m. On this occasion, a massive sexual event involved both *P.* cf. *delicatissima/allochrona* and *P.* cf. *calliantha* (Sarno *et al.*, 2010).

From the beginning of October to the beginning of March 2007, *Pseudo-nitzschia* species concentrations were again low, with minima (<1 x 10⁴ cells L⁻¹) from the middle of December to the middle of January. *Pseudo-nitzschia multistriata* was present throughout the water column, albeit at low concentration, from October till the end of January, together with *P.* cf. *pseudodelicatissima* and *P.* cf. *delicatissima/allochrona*, and the two morphotypes of *P. galaxiae*. *Pseudo-nitzschia* cf. *pseudodelicatissima* almost disappeared from January onwards, when the *Pseudo-nitzschia* assemblage became very sim-

ilar to the one detected in April 2006, i.e., represented by *P. galaxiae* large, *P.* cf. *delicatissima* and lower concentrations of *P. galaxiae* small, which were responsible for the spring bloom in 2007.

No sexual stages of any *Pseudo-nitzschia* species were detected besides those recorded in September 20th 2006 and illustrated by Sarno *et al.* (2010).

Temporal and vertical pattern of Pseudo-nitzschia *abundance as related to environmental factors*

As illustrated above, the highest cell concentrations of *Pseudo-nitzschia* species were recorded in the surface layer; a CCA analysis was performed to summarize the environmental conditions under which blooms of the different species developed (Fig. 5). The two morphotypes of *P. galaxiae* and *P. delicatissima* were related to high DIN and silicate concentrations, low salinity values and lower temperatures. These species bloomed in spring, when the water column was mixed. Their highest cell concentrations were confined to 0.5 m, where a layer with markedly low salinity and relatively high nutrient concentration was present (Fig. 2B, Suppl. Fig. 1), while cell concentration dropped already at 10 m depth. *Pseudo-nitzschia pseudodelicatissima, P.* cf. *delicatissima/allochrona* and *P. multistriata* were instead related to higher temperature and lower nutrient concentrations (DIN, phosphorus and silicates). Blooms of these species were recorded between July and September when the water column was stratified down to 20 m. However, also under these conditions, the highest cell concentrations were always recorded in the surface layer (0.5 m), with no evidence of subsurface maxima. *Pseudo-nitzschia fraudulenta* was detected during a restricted period in summer and it is the only species for which a subsurface maximum was recorded at 20 m (July 4th 2006).

Discussion

Seasonal cycle and cryptic diversity of Pseudo-nitzschia

The seasonal cycle of *Pseudo-nitzschia* species observed at surface during this study generally confirms previous results obtained with light microscopy, with *P.* cf. *delicatissima* and *P. galaxiae* as the most abundant *Pseudo-nitzschia* taxa in the Gulf of Naples (Zingone *et al.*, 2006). *Pseudo-nitzschia* cf. *delicatissima* produces regular blooms in late spring, with less recurrent peaks in late summer (Zingone *et al.*, 2006). The results of molecular studies carried out over the years at LTER-MC have shown that two cryptic species of the *P delicatissima* complex – *P. arenysensis* and *P. delicatissima sensu stricto* - co-occur in spring, while a third cryptic species, *P.* cf. *delicatissima/allochrona,* blooms in summer and autumn (McDonald *et al.*, 2007; Ruggiero *et al.*, 2015; Percopo *et al.*, 2021). The small morphotype of *P. galaxiae* generally blooms in late winter/early spring (Cerino *et al.*, 2005), and our data confirm this pattern, which also matches the results of the clone library investigation, in which ribotype 1, corresponding to the small morphotype, was recorded in winter (Ruggiero *et al.*, 2015). In the present study the medium and large morphotypes of *P. galaxiae* were not discriminated, but the late spring and summer blooms match the seasonal pattern recorded in other years, with the medium morphotype recorded with high abundances in spring and the largest one in summer (Cerino *et al.*, 2005). *Pseudo-nitzschia* cf. *pseudodelicatissima* is irregularly scattered over various seasons, although summer and autumn seem to be preferred by these species (Zingone *et al.*, 2006), as confirmed by the results of our study. The frequent co-occurrence of cryptic species of the *P. pseudodelicatissima* complex in clone library samples did not allow depicting distinct seasonal patterns (Ruggiero *et al.*, 2015). *Pseudo-nitzschia*
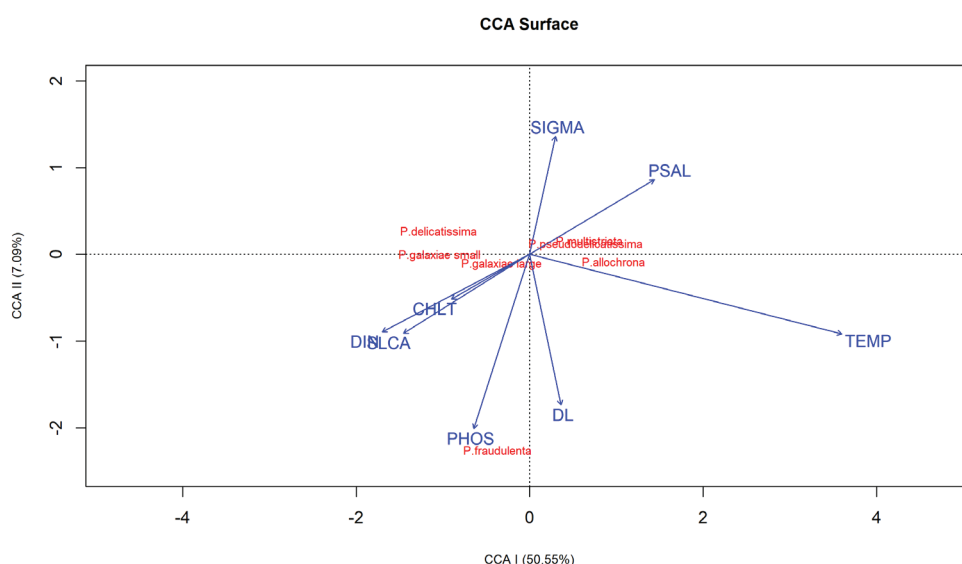
CCA Surface

Fig. 5: Canonical correspondence analysis of abiotic and biotic variables and *Pseudo-nitzschia* species in the surface layer (0.5 m) from April 12th 2006 to April 11th 2007 at LTER-MC. Abiotic variables include: temperature (TEMP), salinity (PSAL), sigma theta (SIGMA), dissolved inorganic nitrogen (DIN), phosphates (PHOS) and silicates (SLCA); biotic variables include chlorophyll *a* (CHLA).

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multistriata, for which cryptic diversity was not found in our molecular studies, was first detected at LTER-MC in 1996; for several years it was mainly recorded in late summer and autumn but, from 2006 onwards, it has broadened its presence towards the summer months (D'Alelio *et al.*, 2010; D. Sarno unpublished data). *Pseudo-nitzschia fraudulenta* cannot be distinguished from *P. subfraudulenta* in LM*;* in the present study the species was mainly recorded in July but ribotypes of the two species were detected, albeit at low abundances, in different seasons (Ruggiero *et al.*, 2015).

Vertical distribution and the reservoir of rare cells

There are few publications presenting detailed information on the abundance of *Pseudo-nitzschia* species along the water column. Some studies occasionally detected aggregation of *P.* cf. *delicatissima* cells in subsurface layers (e.g., Caroppo *et al.*, 2005), others focused on subsurface chlorophyll maxima (e.g., Barnett *et al.*, 2019) or on thin phytoplankton layers located at the level of the pycnocline, where accumulations of *Pseudo-nitzschia* species were detected (Rines *et al.*, 2002; Velo-Suarez *et al.*, 2008).

At LTER-MC the water column stratification begins in April and starts to be eroded in September. Overall, the vertical distribution of *Pseudo-nitzschia* spp. over the year mirrors that of phytoplankton biomass (chl *a*), showing the highest cell concentrations largely confined to the surface layer. This is confirmed by studies carried out on the whole phytoplankton community in summer and autumn when the highest biomass values were recorded in the surface layer (Zingone *et al.*, 1990; 1995). A permanent deep chlorophyll maximum has not been detected at our coastal sampling site (Ribera d'Alcalà *et al.,* 2004) although chlorophyll *a* peaks are occasionally detected at the base of the pycnocline (F. Margiotta, unpublished data). In the present study, subsurface peaks of chlorophyll *a* during the stratification of the water column were present in two cases, namely, May 24th at 40 m and July 4th at 20 m, with no evidence of *Pseudo-nitzschia* cell accumulation. Yet we cannot exclude the presence of thin subsurface layers, whose detection would require specific sampling devices that do not disrupt the physical structure of the water column.

The most interesting result of our study is that *Pseudo-nitzschia* cells were almost always detected down to 50 m depth. The distribution of cells throughout the water column was not limited to periods of deep-water mixing, or to restricted periods after the main bloom events, which could be attributed to mass sinking. Cells with cytoplasmic content, deemed to represent living organisms in the original sample, were always detected below the pycnocline in the summer months and down to 50 m depth also during the winter, when very low abundances of *Pseudo-nitzschia* are recorded at surface. Cryptic diversity within *P. delicatissima* and *P. pseudodelicatissima* complexes does not allow us to depict the seasonal patterns of the individual species based on LM data. Howev-er, we can comment on the distribution of *P. multistriata* and *P. fraudulenta,* which do not show cryptic diversity, and of *P. galaxiae* medium-large and small morphotypes, which correspond to the two dominant ribotypes of this species (McDonald *et al.*, 2007; Ruggiero *et al.*, 2015). In our study*, P. multistriata* was not detected before June in the surface layer, where it bloomed on several occasions between July and October, but persisted along the water column until the beginning of February. A restricted period of relatively high abundance between June and July was recorded in the surface layer for *P. fraudulenta*, but also this species was detected along the water column with very low concentrations on many occasions. The small morphotype of *P. galaxiae*, which bloomed in early spring of both years, was absent above the thermocline (0-20/30 m) from July to October but it was always detected below the thermocline and, in periods of mixing, throughout the water column. The medium-large *P. galaxiae* was instead almost always detected from surface to deeper layers.

In this study we used a counting protocol that allowed us to detect cells with abundances down to 82 cells L^{-1}. This approach is extremely time-consuming and cannot be applied in routine monitoring programs, but revealed the vertical distribution of these diatoms with unprecedented detail. All the data summarized above demonstrate that *Pseudo-nitzschia* species are present in the water column for a much longer period as compared to the restricted temporal windows of the blooms detected at surface and suggest that these diatoms rely on reservoirs of rare cells for the periods in which they are not blooming.

Do species have a temporal window of opportunity for the bloom?

Although long term datasets have shown seasonal patterns for many *Pseudo-nitzschia* species, the environmental or biological mechanisms that induce a bloom are still poorly understood (e.g., Zingone *et al.*, 2003; Fernandes *et al.*, 2014; Bresnan *et al.*, 2015; Smith *et al.*, 2018). The results of our study showed that *P. pseudodelicatissima* complex, *P. multistriata, P. fraudulenta* and *P.* cf. *delicatissima/allochrona* are related to warmer months with longer daylength and a stratified water column, whereas *P. galaxiae* small and *P. delicatissima* complex bloom in cooler months and mixing conditions. At surface, inorganic nutrients were inversely related to salinity, reflecting the impact of land runoff, especially in periods characterized by higher precipitations. This is shown by the low-salinity and nutrient-rich water persisting in the surface layer for several weeks in April-May 2006 and in April 2007. During period of stratification, pulses of lower salinity were restricted to individual sampling dates and were related to the occurrence of thunderstorms in the days immediately preceding them. This was the case for the 11th and 25th July and 20th September 2006 (https://www.ilmeteo.it/portale/archivio-meteo/Napoli/2006/Luglio), when high cell concentrations of *Pseudo-nitzschia* species, as well as chlorophyll *a*, were recorded. Nutri-

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ent concentrations were always low during the summer months, suggesting a rapid growth response of the phytoplankton community to the ephemeral pulses of nutrients due to runoff following heavy rain. While it is possible to depict the proximate conditions at which blooms of the different species occurred over the annual cycle considered in this manuscript, the mechanisms driving their almost regular seasonality supported by other investigations in the study area (Zingone *et al.*, 2006; McDonald *et al.*, 2007; Ruggiero *et al.*, 2015; D'Alelio *et al.*, 2010; Percopo *et al.*, 2021) remain elusive.

Sexual reproduction in Pseudo-nitzschia

Pennate diatoms depend on sexual reproduction to restore maximum cell size (Montresor *et al.*, 2016) but reports of sexual reproduction in the marine environment are extremely limited (e.g., Assmy *et al.*, 2006; Holtermann *et al.*, 2010; Sarno *et al.*, 2010). In the year of this study, a massive sexual event involving two *Pseudo-nitzschia* species (*P.* cf. *delicatissima* and *P.* cf. *calliantha*) occurred on September 20th (Sarno *et al.*, 2010), when gametes were recorded at 0, 10 and 20 m depths along with auxospores of different sizes at times containing the maximum sized initial cell. Sexual stages accounted on average for 7.2% of the whole population for *P.* cf. *delicatissima* and 14.3% for *P.* cf. *calliantha*. It has recently been shown that sinking of *Pseudo-nitzschia* cells in non-turbulent deeper layers of the water column can promote their vertical re-orientation and thus facilitate pairing between opposite mating types (Botte *et al.*, 2013; Font-Muñoz *et al.*, 2019). One of the aims of the present study was to test the presence of sexual stages in deeper layers of the water column, but we could not detect them. This can be due to the fact that sexual reproduction is an ephemeral event that can easily be missed even in weekly monitoring programs. At the LTER-MC site, sampled with a fortnightly interval from 1984 and 1991 and with a weekly one from 1995 up to now, sexual stages of *Pseudo-nitzschia* species have been detected sporadically (D. Sarno, unpublished data) and the only massive event was the one of 2006 (Sarno *et al.*, 2010). It has been shown that *Pseudo-nitzschia* species can accumulate in thin subsurface layers (Rines *et al.*, 2002; McManus *et al.*, 2008; Velo-Suarez *et al.*, 2008), which have thickness in the order of few centimeters up to few meters, may extend horizontally for kilometers and can persist for days (Durham and Stocker, 2012). These structures represent potential sites where sexual reproduction can occur but their sampling require devices that ensure high vertical sampling resolution.

Conclusions

Our study provides evidence for the presence of *Pseudo-nitzschia* species/species complexes throughout the water column for the whole annual cycle. The 'apparent disappearance' of phytoplankton species between subsequent blooms has been related to the formation of resting stages (e.g., Marcus and Boero, 1998); however, only a fraction of planktonic diatoms produce benthic resting stages, which have never been reported for *Pseudo-nitzschia* species (Belmonte and Rubino, 2019). Our study provides evidence for an alternative overwintering strategy, with cells persisting in the water column at low concentration in the period between blooms, which occur in surface waters following species-specific seasonal patterns. Understanding the exogenous and/or endogenous factors that drive the onset of the growth season of the different species within a complex phytoplankton community is still one of the biggest challenges in biological oceanography.

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Supplementary Data

The following supplementary information is available online for the article:

Suppl. Fig. 1: Temporal and vertical distribution of (A) potential density (sigma theta, kg m^{-3}), (B) dissolved inorganic nitrogen (DIN) (mmol m^{-3}), (C) phosphates (mmol m^{-3}) and (D) silicates (mmol m^{-3}) at LTER-MC (plotted with Ocean Data View; Schlitzer, 2018).

Suppl. Fig. S2: Vertical distribution of *Pseudo-nitzschia* species/species complexes (cells L^{-1}) at each sampling date from April 12th 2006 to April 11th 2007 at LTER-MC. To appreciate cell concentrations of the different species in the deeper layers of the water column, where values are markedly lower, some panels are replicated on the right side of the figure with a smaller scale bar. For cruises MC720 (27/06/2006) and MC735 (09/10/2006), marked with a red asterisk, only surface data are available.

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