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Environmental Factors Affecting the Distribution of *Pseudo-nitzschia* in Two Monsoonal Estuaries of Western India and Effects of Salinity on Growth and Domoic Acid Production by *P. pungens*

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Abstract

Species of the diatom genus *Pseudo-nitzschia*, some of which produce the neurotoxin domoic acid (DA), were studied to see how environmental factors affect their distribution in two tropical monsoonal estuaries and how salinity influences the growth and toxicity of *P. pungens*. *Pseudo-nitzschia pungens*, *P. multistriata*, and *P. seriata* were present in both the Mandovi and Zuari estuaries, whereas *P. australis* and *P. pseudodelicatissima* appeared only in the Zuari estuary. Canonical correspondence analysis indicated a significant positive correlation between salinity and the occurrence of *P. seriata* in the Mandovi estuary and of *P. pungens* in the Zuari estuary. A strain of *P. pungens* isolated from the Zuari estuary showed significant variations in specific growth rate between salinities, in support of our finding of a relationship between salinity and its distribution in the field. The lowest growth rate (0.44 day^{-1}) was at a salinity of 5 and it increased to a maximum (1.05–1.19 day^{-1}) at salinities of 15 to 30, declining slightly (0.98 day^{-1}) at a salinity of 35. The isolated strain produced DA but at low levels. DA production rates varied significantly with salinity; they were low and similar at salinities of 5–15 (2.56–3.12 ng ml^{-1} day^{-1}) and increased with increasing salinity, reaching 5.25 ng ml^{-1} day^{-1} at 35. The observed variations in growth rate and DA production by *P. pungens* indicate the need to focus more on salinity as an environmental factor that affects this, and other *Pseudo-nitzschia* species, in these monsoonal waters.

Keywords Domoic acid · Monsoon · Population dynamics · *Pseudo-nitzschia* · Salinity

Introduction

The pennate diatom genus *Pseudo-nitzschia* H. Peragallo is observed in coastal marine environments around the world (Hasle 2002) and includes temperate and tropical species. Knowledge of the geographic distribution of *Pseudo-nitzschia* has increased over past decades at the same time that species have undergone many taxonomic changes, based on frustule morphology and molecular differences (Lelong et al. 2012; Trainer et al. 2012; Teng et al. 2016). Blooms of *Pseudo-nitzschia* spp. are increasing in frequency, especially in areas of nutrient enrichment (Horner et al. 2000; Anderson et al. 2002). Public concern about this genus has increased, as more of its species globally have been shown to produce the neurotoxin domoic acid (DA), the cause of amnesic shellfish poisoning (ASP) in humans and domoic acid poisoning in marine animals (Trainer et al. 2012). To date, 49 species of *Pseudo-nitzschia* have been identified, of which 26 are documented as DA producers (Fernandes et al. 2014; Dao et al. 2015; Teng et al. 2016; Lundholm 2017; https://en.wikipedia.org/wiki/Pseudo-nitzschia).

Numerous studies have reported on the physicochemical variables that affect DA production by *Pseudo-nitzschia* species. Most targeted macro- and micronutrients (e.g., Si, N, P, Fe, and Cu) but others studied temperature, irradiance, and pH (reviewed by Bates 1998; Bates and Trainer 2006; Lelong et al. 2012; Trainer et al. 2012). Several studies have identified salinity as an important factor that affects the growth, seasonality, distribution, and DA production of *Pseudo-nitzschia*

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species (reviewed by Lelong et al. 2012; Lim et al. 2014b; Bargu et al. 2016). Other field studies have examined the correlation between salinity and the distribution of *Pseudo-nitzschia* species (Liefer et al. 2009), or their toxicity (MacIntyre et al. 2011; Schnetzer et al. 2013). However, none have included monsoonal environments. Furthermore, with the exception of Doucette et al. (2008), no other laboratory study has shown how changes in salinity can affect DA production. This is critical because most toxigenic *Pseudo-nitzschia* species occur in coastal waters, including estuarine habitats, where there is a great variation in salinity. A wide salinity tolerance for *Pseudo-nitzschia* species has been demonstrated in both laboratory studies (Brand 1984; Jackson et al. 1992; Villac et al. 2004; Thessen et al. 2005; Markina and Aizdaicher 2016) and in surveys of natural populations (Villac et al. 2004; Thessen et al. 2005). Given the inherently wide salinity fluctuations characterizing many estuaries, as well as the apparently increasing susceptibility of the coastal environment to natural and anthropogenic processes potentially affecting the salinity regime (Turner 2006), knowledge of how salinity influences the growth and toxicity of *Pseudo-nitzschia* species is essential for evaluating the potential impact of toxic blooms on shellfish resources, local economies, and wildlife populations.

Pseudo-nitzschia pungens (Grunow ex Cleve) Hasle is one of the most commonly reported representatives of the genus worldwide (Hasle 2002; Casteleyn et al. 2008; Lim et al. 2014a; Kim et al. 2015). A few strains of *P. pungens* produce DA, but only at low levels compared to the other toxigenic species (Trainer et al. 2012; Lim et al. 2014a). This species has been found at high abundances in the Mandovi and Zuari estuaries, along the west coast of India (Fig. 1) (Patil and Anil 2008; Pednekar et al. 2012). These estuaries are influenced by monsoons, which play a vital role in governing the distribution of salinity and nutrients (Verlencar 1987; Maya et al. 2011). This, in turn, controls the distribution, abundance, and productivity of phytoplankton community (Devassy and Goes 1988; Krishna et al. 2002). During the monsoon season, both estuaries are flushed by freshwater entering upstream, resulting in an extreme drop in salinity (Shetye et al. 2007). Further variations in salinity are then caused by remnants of freshwater introduced by monsoonal runoff and then moved downstream, where it is mixed with saline water at the lower section (Shetye et al. 2007). During the break phase of the monsoon, the estuaries are completely filled with freshwater. The Mandovi estuary experiences a high freshwater influx, with flow rates > 700 m^{-3} s^{-1} at the onset of monsoons (Unnikrishnan et al. 1997; Shetye et al. 1999). The distribution of nutrients in both estuaries is also greatly influenced by land runoff during monsoons, which leads to nutrients being flushed into the estuary, with additions from anthropogenic activities and sediment resuspension (De Sousa 1983; Martin et al. 2008). During the non-monsoon season, freshwater inflow is minimal, and tides are important for maintaining a well-mixed water column (Shetye et al. 1999; Subha Anand et al. 2014).

Our study was undertaken to understand the spatiotemporal occurrence of *Pseudo-nitzschia* species in two salinity-influenced tropical estuaries and to examine the influence of salinity on the growth and DA production by *P. pungens*, the most abundant species.

Methods

Study Site and Sampling

The Mandovi (15.350° N, 73.750° E) and Zuari (15.517° N, 73.817° E) estuaries, together with Cumbarjua Canal, form the major estuarine system of Goa (Fig. 1), both are tropical and monsoonal. The two estuaries were sampled every 2 weeks during both the monsoon (June to September) and non-monsoon (October to May) seasons, starting in June. The Mandovi and Zuari estuaries were sampled during 2007–2008 and 2008–2009, respectively. Duplicate water samples were collected with a Niskin bottle, at the surface and bottom (10 or 11 m), at four stations that covered the entirety of each estuary (Fig. 1): (1) Mouth of the estuary, (2) Lower reach, (3) Middle, and (4) Upper reach. Because the Zuari estuary is wider, the lower, middle, and upper reaches were comprised of two stations each, and the average of each of the two stations is reported. Samples were transported immediately under cold and dark conditions to the National Institute of Oceanography laboratory, 10 min away from Goa, for further processing.

Physicochemical Parameters

Salinity was measured with a salinometer (Atago S/Mill®, Japan) having a salinity range of 0 to ~ 100 and a resolution of 1 salinity unit between 10 and 20 °C. Nitrate, nitrite, phosphate, and silicate were analyzed with a lambda 40 UV/VIS double-beam spectrometer (Perkin Elmer, India Private Limited), using standard procedures (Strickland and Parsons 1972). Water samples were first filtered through Whatman 47-mm diameter grade GF/A glass microfiber filters and stored at − 20 °C for 2 weeks prior to analysis. Surface and bottom water temperatures were obtained with a portable conductivity-temperature-depth (CTD) profiler (Sea-Bird Electronics, Bellevue, Washington, USA). Chlorophyll *a* (Chl *a*) was obtained by filtering 500-ml water samples onto 47-mm glass fiber filters (grade 91 GF/F, Whatman, USA), which were then extracted overnight in 10 ml of 90% acetone in the cold and dark. Sample extracts were then filtered through PTFE filters (0.2 μm, Millipore, USA) to remove GF/F filter debris. Chl *a* was measured in duplicate by high-performance liquid chromatography (Agilent® 1100 series)

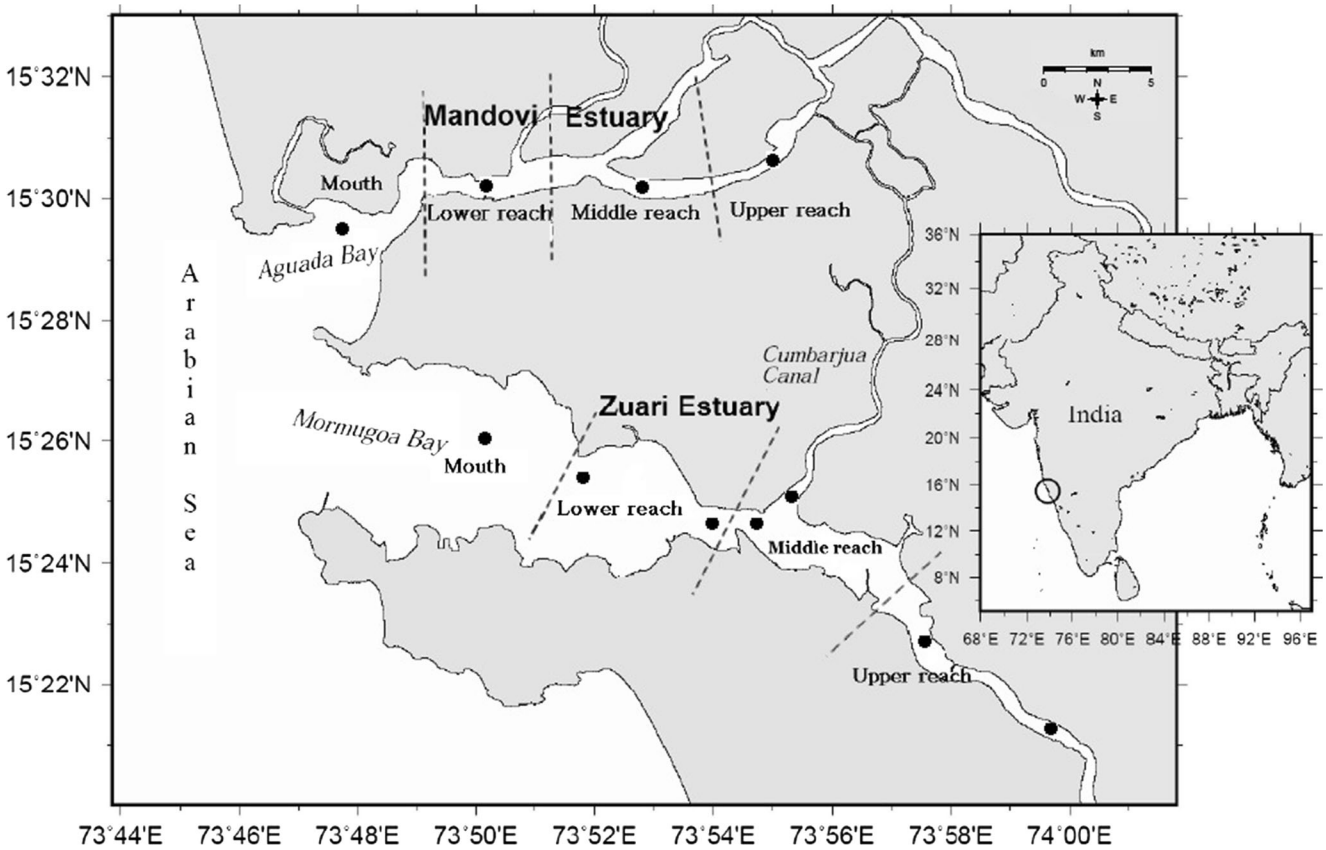

Fig. 1 Map of the Mandovi and Zuari estuaries showing sampling locations in the Mouth, Lower Reach, Middle reach, and Upper reach sections of the estuaries

and separated on a C-18 reverse-phase column using the eluent gradient program of Wright et al. (1991) and as adapted by Parab et al. (2006). Reference pigment standards were obtained from DHIs Water and Environment (Denmark) and Sigma Aldrich Chemicals (USA). Rainfall over the entire west coast of India was obtained from the India Meteorological Department (http://www.imd.gov.in/).

Collection and Identification of *Pseudo-nitzschia* Species

Samples for *Pseudo-nitzschia* and other diatom species were collected in 500-ml opaque plastic bottles, fixed with a few drops of 2% Lugol's iodine solution, and stored under dark and cool conditions for 2 weeks. The settled samples were concentrated to 5–10 ml by carefully siphoning the top layer with a tube covered with a 10-μm Nytex filter on one end. Sample concentrates were then transferred to a 1-ml capacity Sedgewick-Rafter chamber for duplicate cell counts of at least 500 cells, giving a lowest cell count detectable of 1 cell ml^{-1}.

Pseudo-nitzschia spp. cells were cleaned for scanning electron microscopy (SEM) examination using the $KMnO_4$/HCl oxidation method (Miller and Scholin 1998) and then mounted on nylon stubs using carbon conductive tape. The specimens were then coated with ~20 nm of gold in a Hummer 6.2 sputtering unit (Anatech Ltd., Springfield, VA) and viewed by SEM (JEOL JSM-5600) with an EDS attached (JEOL 5800LV). Identifications were based on standard taxonomic keys (Hasle and Syvertsen 1997).

Culture Conditions and Salinity Experiment

A non-axenic culture of *Pseudo-nitzschia pungens* (strain SP-1) was established by isolating a single cell, using a sterile micropipette, from the Middle reach of the Zuari estuary on 19 July 2012. Experiments were carried out during 2012–2013. The culture was maintained and experiments carried out in f/2 medium (Guillard 1975) containing 214 μM Si. The medium was made with filtered seawater collected from the Zuari estuary during the non-monsoon period, when the nutrient concentrations were low (Table 1), and aged for about 1 year prior to use. Cultures were grown at an irradiance of 35 μmol photons m^{-2} s^{-1} (12:12 h light:dark cycle) and a temperature of ~30 °C. This reflects the temperature, i.e., 29–31 °C, and low irradiance level during the non-monsoon period when the sample with the high *P. pungens* cell counts was collected.

Table 1 Average seasonal distribution of salinity, nutrients (μM), temperature (°C; "Temp") and chlorophyll *a* (Chl *a*; mg m^{-3}) in the Mandovi (2007–2008) and Zuari (2008–2009) estuaries during the monsoon and non-monsoon seasons, at the surface (S) and bottom (B). Stations are listed in order, from the Mouth to the Upper reach of each estuary. No bottom samples were collected at the Lower reach of the Mandovi estuary (denoted by a dash). *n* = number of replicate samples collected

Monsoon									Non-monsoon						
Estuary	Depth	Salinity	Nitrate	Nitrite	Silicate	Phosphate	Temp	Chl *a*	Salinity	Nitrate	Nitrite	Silicate	Phosphate	Temp	Chl *a*
Mandovi															
Mouth	S *n* = 24	12.00 ± 7.89	12.03 ± 0.11	1.81 ± 1.01	76.63 ± 39.81	2.34 ± 1.03	28.07 ± 1.07	2.93 ± 1.80	34.04 ± 1.72	1.16 ± 1.16	0.91 ± 1.02	21.69 ± 18.49	0.45 ± 0.48	30.04 ± 0.68	2.84 ± 1.16
	B *n* = 24	22.00 ± 9.88	13.57 ± 4.76	1.77 ± 1.07	81.34 ± 39.52	3.38 ± 1.82	27.18 ± 1.01	1.69 ± 1.02	34.01 ± 1.25	1.24 ± 1.55	0.62 ± 0.61	19.14 ± 14.31	0.77 ± 0.82	27.90 ± 0.71	2.94 ± 1.41
Lower reach	S *n* = 24	17.01 ± 9.37	9.79 ± 9.88	2.30 ± 0.66	22.08 ± 36.81	2.70 ± 1.06	27.95 ± 0.29	2.76 ± 2.24	31.10 ± 0.79	0.86 ± 0.16	0.87 ± 0.06	10.26 ± 2.20	0.30 ± 0.05	31.64 ± 0.32	2.71 ± 1.17
	B	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Middle reach	S *n* = 24	9.03 ± 8.61	10.84 ± 2.16	1.15 ± 1.33	85.35 ± 38.80	1.76 ± 0.98	27.85 ± 1.27	2.61 ± 1.69	31.05 ± 2.91	0.93 ± 1.30	0.80 ± 0.87	25.98 ± 34.67	0.60 ± 0.63	29.55 ± 1.60	4.96 ± 2.53
	B *n* = 24	20.00 ± 7.52	11.21 ± 0.86	1.23 ± 1.29	60.55 ± 23.19	2.39 ± 1.07	27.80 ± 1.26	1.47 ± 0.94	32.00 ± 3.27	0.75 ± 0.97	0.65 ± 0.81	19.79 ± 13.98	0.51 ± 0.83	29.50 ± 1.63	3.97 ± 2.69
Upper reach	S *n* = 24	5.00 ± 6.39	11.02 ± 1.79	1.38 ± 1.21	114.13 ± 15.78	1.72 ± 0.73	27.21 ± 1.36	2.40 ± 2.27	26.00 ± 4.12	1.66 ± 1.21	0.86 ± 0.70	18.42 ± 15.49	0.48 ± 0.34	28.34 ± 1.81	6.56 ± 3.50
	B *n* = 24	10.00 ± 8.29	9.87 ± 1.90	1.24 ± 0.69	103.88 ± 16.01	2.34 ± 1.43	27.20 ± 1.36	1.60 ± 1.41	27.01 ± 2.71	1.49 ± 1.04	0.82 ± 0.71	16.28 ± 10.52	0.49 ± 0.41	28.32 ± 1.81	5.36 ± 2.84
Zuari															
Mouth	S *n* = 24	19.05 ± 9.85	3.32 ± 2.76	1.23 ± 1.18	50.6 ± 9.59	2.81 ± 1.18	28.63 ± 1.33	4.73 ± 2.99	33.02 ± 1.50	1.04 ± 0.47	1.01 ± 1.31	13.2 ± 0.85	0.40 ± 0.09	28.69 ± 0.11	5.55 ± 2.57
	B *n* = 24	24.00 ± 9.23	4.13 ± 2.72	1.79 ± 1.42	51.1 ± 29.23	3.99 ± 2.68	24.89 ± 1.43	3.42 ± 1.16	33.00 ± 1.50	1.14 ± 0.69	3.38 ± 2.75	15.6 ± 10.52	0.52 ± 0.08	28.56 ± 0.11	4.23 ± 2.78
Lower reach	S *n* = 48	16.00 ± 8.81	3.65 ± 2.51	1.20 ± 0.82	43.5 ± 21.01	2.72 ± 1.16	26.86 ± 1.43	6.03 ± 0.57	31.00 ± 1.87	1.82 ± 0.85	1.52 ± 0.81	19.9 ± 4.19	0.43 ± 0.81	29.59 ± 1.32	4.90 ± 0.64
	B *n* = 48	23.00 ± 6.22	4.53 ± 1.94	1.93 ± 0.82	43.9 ± 17.64	3.92 ± 1.70	26.51 ± 1.44	4.18 ± 0.37	32.00 ± 1.35	2.03 ± 0.99	2.05 ± 0.84	18.6 ± 2.66	2.70 ± 1.19	29.00 ± 1.07	5.32 ± 0.67
Middle reach	S *n* = 48	12.00 ± 10.03	5.04 ± 9.93	1.47 ± 1.89	38.3 ± 17.12	2.90 ± 1.18	28.69 ± 2.35	7.59 ± 1.44	29.02 ± 1.72	1.87 ± 1.69	2.05 ± 1.04	25.8 ± 9.88	0.43 ± 0.70	31.12 ± 1.05	6.68 ± 0.28
	B *n* = 48	21.00 ± 10.00	4.50 ± 2.91	1.79 ± 1.35	41.6 ± 18.73	3.84 ± 1.94	26.90 ± 1.34	4.74 ± 0.22	32.00 ± 6.83	2.44 ± 1.72	2.64 ± 1.69	21.0 ± 11.20	0.78 ± 1.25	28.85 ± 0.39	5.31 ± 0.23
Upper reach	S *n* = 48	6.00 ± 8.85	5.34 ± 2.93	0.64 ± 0.63	32.95 ± 20.34	1.49 ± 0.69	27.49 ± 20.34	7.29 ± 0.72	18.00 ± 5.60	2.46 ± 1.59	2.12 ± 1.55	40.25 ± 13.57	0.36 ± 5.60	28.52 ± 12.42	5.94 ± 0.002
	B *n* = 48	8.00 ± 9.99	5.08 ± 3.03	0.81 ± 0.82	34.86 ± 15.69	2.11 ± 1.47	27.19 ± 8.07	5.32 ± 0.43	21.00 ± 5.56	3.27 ± 1.56	2.96 ± 1.44	34.35 ± 8.07	0.46 ± 5.56	28.24 ± 2.18	5.23 ± 0.09

To study the effect of salinity, medium f/2 was prepared at a range of salinities. The aged seawater (salinity of 36) from the Zuari estuary was first filtered through a glass-fiber filter, followed by a 0.22-μm pore size, 47-mm diameter polycarbonate membrane (Whatman Nuclepore). This was then diluted with distilled deionized water to achieve seven lower salinities (5 to 35, in five increments). Nutrients were then added to achieve the concentrations in medium f/2, followed by sterile-filtration through a 0.22-μm pore size, 47-mm diameter polycarbonate membrane. The *P. pungens* cultures were adapted different salinity under the above growth conditions for 12 days prior to carrying out the experiment.

Triplicate experimental flasks were prepared for each of the 12 sampling days. They each contained 100 ml of medium at each of the salinities and were inoculated with 19.5 ml of exponentially growing (day 4) adaptation cultures, to achieve an initial cell concentration of 6000 to 8000 cells ml^{-1}. Growth at the above conditions was monitored daily between 13:00 and 15:00 by aseptically removing 3-ml aliquots from each flask. Cells were preserved with 2% Lugol's iodine, stored for 1 week and counted in triplicate using a 1-ml Sedgewick-Rafter counting chamber. At least 50 fields, or 300 cells, were counted per replicate. Only cells containing chloroplasts were counted. The maximum specific growth rate for the mean of triplicate cultures was determined using cell counts obtained over 3 or more successive days during the exponential growth phase according to Guillard (1975): $\mu\ (day^{-1}) = \ln (N_2/N_1) / (t_2 - t_1)$, where t_2 and t_1 are the sampling times (in days) and N_2 and N_1 are the corresponding cell concentrations at sampling times t_2 and t_1.

Domoic Acid Analysis

To determine domoic acid (DA) production in *P. pungens* cultures, 50-ml aliquots were collected daily from the triplicate cultures flasks, from day 0–12, and frozen at − 20 °C for later DA analysis. Prior to analysis, 30 ml of "whole culture" (cells plus medium; Bates et al. 1998) were sonicated for 1–2 min at 100 W, using a 1-cm diameter probe (Sonics and Materials INC. Danbury, CT, USA) to release DA. Cell debris was removed by filtration through a 0.45-μm pore-size disposable filter (MillexHV, Millipore Corp.). Field samples for DA analysis were collected at each station, using the same method. DA was analyzed by the high-sensitivity 9-fluorenylmethylchloroformate (FMOC) pre-column derivatization method for amino acids followed by reverse-phase HPLC with fluorescence detection; the limit of detection was ~ 16 pg DA ml^{-1} (Pocklington et al. 1990). Results are shown as the total amount of DA (cells plus medium) in the culture expressed either as a concentration (ng DA ml^{-1}) or as the rate of DA production (ng DA ml^{-1} day^{-1}).

Statistical Analyses

Differences in growth and toxin production rates were assessed by a two-way ANOVA, using the univariate test of significance (STATISTICA 6.0, StatSoft, OK, USA). Tukey's post hoc test was performed to examine further variations within salinity. The canonical correspondence analysis (CCA) (ter Braak 1995) was performed to evaluate the temporal variations in the effect of the environmental characteristics of the water column on the phytoplankton communities in the Mandovi and Zuari estuaries, using the MultiVariate Statistical Package (MVSP) program version 3.1 (Kovach 1998).

Results

Salinity

Average salinities were similar in the Zuari and Mandovi estuaries and were lower during the rainy monsoon period than during the non-monsoon period (Table 1). A trend of decreasing salinity was observed between the Mouth and the Upper reaches of both estuaries. During the monsoon period, the lowest salinities were at the Upper reach: 5–10 and 6–8 for the Mandovi and Zuari estuary, respectively. During the non-monsoon period, salinities were greater, and the highest (33–34) were at the Mouth of each estuary.

Nutrients, Temperature, and Rainfall

The average nutrient concentrations were highest during the monsoon season in both estuaries (Table 1). In the Mandovi estuary, nitrate, nitrite, and phosphate concentrations decreased from the Mouth to the Upper reach, while the silicate concentration increased in the opposite direction. Surface and bottom concentrations were similar. All the nutrient concentrations were high during the monsoon season in both the estuaries. In the Mandovi estuary, the highest nitrate concentration (13.6 μM) was observed at the Mouth of the estuary. The Middle and the Upper reach stations showed a similar average nitrate concentration during the monsoon period (10.7 μM), whereas it dropped to 1.21 μM during the non-monsoon period. The highest average phosphate concentration (3.38 μM) was found at the Mouth of the estuary during the monsoon period. Silicate was abundant all year, but the highest average concentration (114.1 μM) was observed during the monsoon season at the Upper reach of the estuary.

In the Zuari estuary, there was no discernible trend in nutrient concentration from the Mouth to the Upper reach. The highest average nitrate concentration (5.34 μM) was at the Upper reach during the monsoon season (Table 1). The highest phosphate (3.99 μM) and silicate (51.1 μM) concentrations were observed at the Mouth of the estuary during the

monsoon period. During the non-monsoon period, nutrient concentrations were lower.

In the Mandovi estuary, temperature varied only slightly during the monsoon period. The highest (28.07 °C) was at the surface of Mouth; the lowest (26.15 °C) was at the bottom of Upper reach (Table 1). During non-monsoon period, the highest temperature (31.64 °C) was at the surface of the Lower reach. In the Zuari estuary, the highest temperature was at the surface of the Middle reach, during both the monsoon (28.69 °C) and non-monsoon (31.12 °C) periods. The rainfall in the Mandovi estuary was 2.52–35.91 cm (average = 19.66 ± 13.35 cm) during 2007–2008. For the Zuari estuary, it was 6.10–27.16 cm (average = 7.04 ± 10.17 cm) during 2008–2009.

Morphology and Identification of *Pseudo-nitzschia* Species

Five species of *Pseudo-nitzschia* were identified: *P. pungens*, *P. multistriata*, *P. seriata*, *P. australis*, and *P. pseudodelicatissima*, based on their morphometrics (Supplementary Table 1). A *Pseudo-nitzschia* species was isolated from the Zuari estuary and brought to culture. Based on morphometrics obtained from light microscopy and SEM images (Supplementary Table 1; Fig. 2), this species is identified as *P. pungens*. Cells are symmetrical and linear to lanceolate in valve view (Fig. 2a, b). Apices are more or less pointed. Cell tips overlap by about one third of the cell length (Fig. 2a). The cell length is 79–91 μm and the valve width is 3.4–4.7 μm. Cells are coarsely silicified and a central interspace is absent.

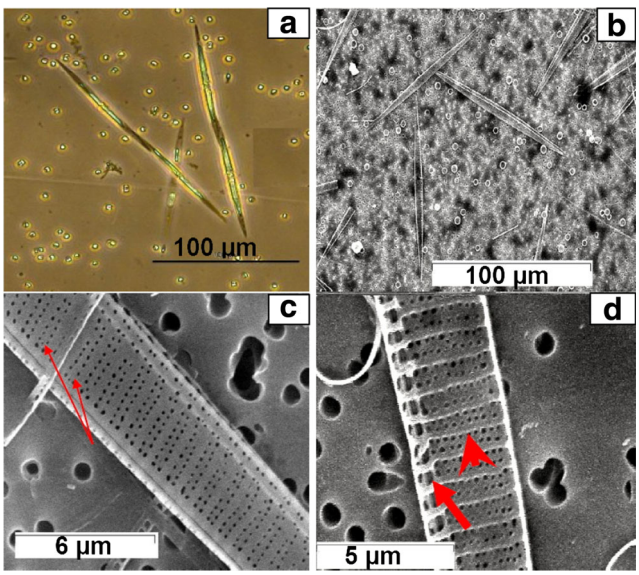

Fig. 2 *Pseudo-nitzschia pungens*. **a** Stepped chains of cells in girdle and valve views, LM; **b** whole valve with pointed ends, SEM; **c** outer valve structure, showing the presence of two to three rows of poroids (arrows), SEM; and **d** inner valve structure with the presence of striae (arrowhead) and fibulae (arrow), SEM

Striae are most often with two rows of poroids or sometimes with a partly formed third row (Fig. 2c, d). The poroid density is 3 in 1 μm. The density of striae and fibulae is both 13–14 in 10 μm (Fig. 2d).

Spatiotemporal Distribution of *Pseudo-nitzschia* Species

In the Mandovi estuary, the percentage of *Pseudo-nitzschia* species relative to the total diatom community ranged from a high value of 21.1% at the Lower reach and 16.2% at the Upper reach, to a low of value < 10% at the Mouth. This low percentage was due to the high abundance of other diatom genera (e.g., *Rhizosolenia*, *Streptotheca*, *Thalassiothrix*, *Skeletonema*, *Pleurosigma*, *Cylindrotheca*, *Chaetoceros*, *Actinocyclus*, *Actinoptychus*), which were dominant at this station.

Three species of *Pseudo-nitzschia* were found in the Mandovi estuary: *P. multistriata*, *P. seriata*, and *P. pungens* (Fig. 3). *Pseudo-nitzschia multistriata* was present mostly during the non-monsoon period, and concentrations generally increased from the Mouth of the estuary (670 cells l^{-1}; Fig. 3a) to the Upper reach (8320 cells l^{-1}; Fig. 3d) during March. At the Lower reach, it was present at low concentrations (190 cells l^{-1}) throughout the study period (Fig. 3b). *Pseudo-nitzschia seriata* was observed during both the monsoon and non-monsoon periods at the Mouth to the Middle reach (Fig. 3a–c) and was absent at the Upper reach during the monsoon period (Fig. 3d). At the Mouth of the estuary, the highest concentration was 1540 cells l^{-1} during non-monsoon period, in March, when the temperature was 28.34 °C. At the Lower reach, the cell concentration was 5670 cells l^{-1}, in May. At the Upper reach, the largest bloom of *P. seriata* (15,320 cells l^{-1}) was in March. *Pseudo-nitzschia pungens* was present only during the non-monsoon period at the Mouth, Middle reach, and Upper reach, although at low concentrations (< 20 cells l^{-1}). At the Lower reach, however, it was present throughout the study period, with the highest cell concentration (4400 cells l^{-1}) during the non-monsoon period, in December (Fig. 3b).

In the Zuari estuary, the highest percentage of *Pseudo-nitzschia* cells relative to total diatoms was observed at the Middle reach (8.8%), followed by the Mouth (7.2%), the Lower reach (3.0%), and the Upper reach (0.1%). The lower two percentages were due to blooms of other diatom genera (e.g., *Rhizosolenia*, *Streptotheca*, *Thalassiothrix*, *Skeletonema*, *Pleurosigma*, *Cylindrotheca*, *Chaetoceros*, *Actinocyclus*, *Actinoptychus*, *Stephanopyxis*, *Gyrosigma*).

Five species of *Pseudo-nitzschia* were found in the Zuari estuary: *P. australis*, *P. multistriata*, *P. seriata*, *P. pseudodelicatissima*, and *P. pungens* (Fig. 4). All of the field samples that contained these toxigenic species were below the limit of detection for DA. *Pseudo-nitzschia australis*

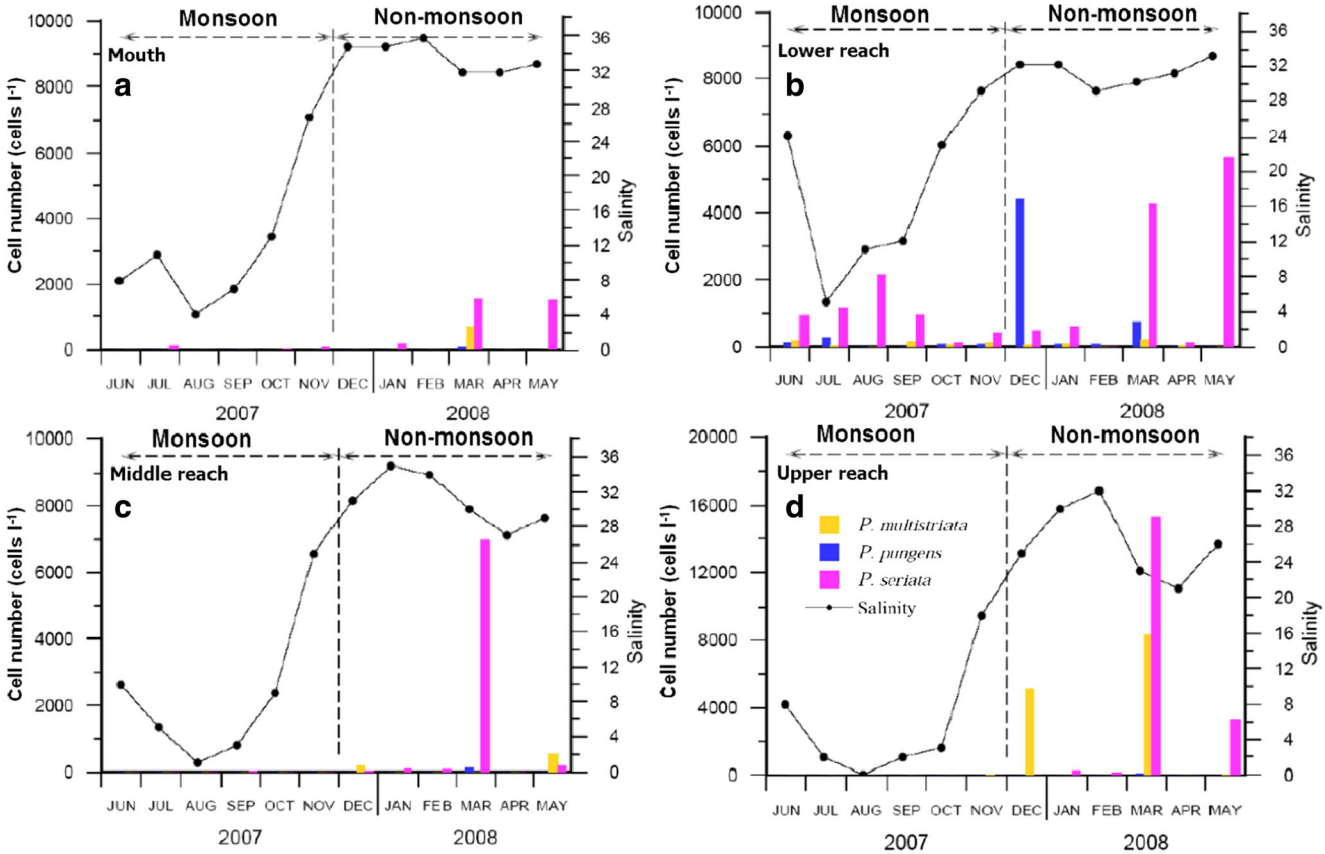

Fig. 3 Spatiotemporal distribution of *Pseudo-nitzschia* species at surface stations in the Mandovi estuary (2007–2008). **a** Mouth, **b** Lower reach, **c** Middle reach, and **d** Upper reach. Vertical dashed line indicates the transition from the monsoon (June to November) to the non-monsoon (December to May) periods

was observed only during the non-monsoon period, from the Mouth of the estuary to the Middle reach. *Pseudo-nitzschia multistriata* was observed at the Mouth only during the monsoon period, but only at very low concentrations (10–30 cells l^{-1}) (Fig. 4a). At the Middle reach, it was detected during both the monsoon and non-monsoon seasons, with the highest cell concentration (2560 cells l^{-1}) in October (Fig. 4c). *Pseudo-nitzschia seriata* was observed at a low concentration (40 cells l^{-1}) at the Middle reach during the non-monsoon season (Fig. 4c). *Pseudo-nitzschia pseudodelicatissima* was detected at the Mouth at a low concentration (< 30 cells l^{-1}) (Fig. 4a). It was present at a high concentration (5760 cells l^{-1}) at the Middle reach during the non-monsoon period (Fig. 4c). *Pseudo-nitzschia pungens* was the most abundant of the *Pseudo-nitzschia* species in the Zuari estuary, in contrast to the Mandovi estuary. It was present only during the non-monsoon period at the Mouth (5052 cells l^{-1}), in February (Fig. 4a), and Lower reach (3188 cells l^{-1}) (Fig. 4b). At the Middle reach, *P. pungens* bloomed to the highest cell concentration (9660 cells l^{-1}) in February, although it was also present at low concentrations during the monsoon season (Fig. 4c). At the Upper reach, the *Pseudo-nitzschia* spp. cell concentration was < 10 cells l^{-1}; hence, the graph is not shown.

Influence of Environmental Factors on the Distribution of *Pseudo-nitzschia* Species

Environmental gradients potentially influenced the abundances of *Pseudo-nitzschia* species, total phytoplankton biomass (Chl *a*), and total phytoplankton concentration (TD) in the Mandovi estuary (Fig. 5a). High TD and Chl *a* concentrations during the monsoon season are related positively to the high concentration of nutrients (nitrate, nitrite, phosphate, and silicate) and to rainfall in our CCA biplots. The abundance of *P. multistriata* (Pm) is positively related to the high concentration of silicate during non-monsoon season at the Upper reach. Elevated salinity and temperature during the non-monsoon period at the Mouth of the estuary are positively related to the abundance of *P. pungens* (Ppu). *Pseudo-nitzschia seriata* (Ps), which is distributed at the center of both axes, also correlated positively with salinity and temperature during the non-monsoon period.

In the Zuari estuary, five *Pseudo-nitzschia* species, along with Chl *a*, TD and seven environmental variables, were selected for CCA analysis (Fig. 5b). Much of the variation in species composition during the monsoon season can be explained by nutrients (nitrate, nitrite, phosphate, and silicate) affected the distribution of *Pseudo-nitzschia* species during

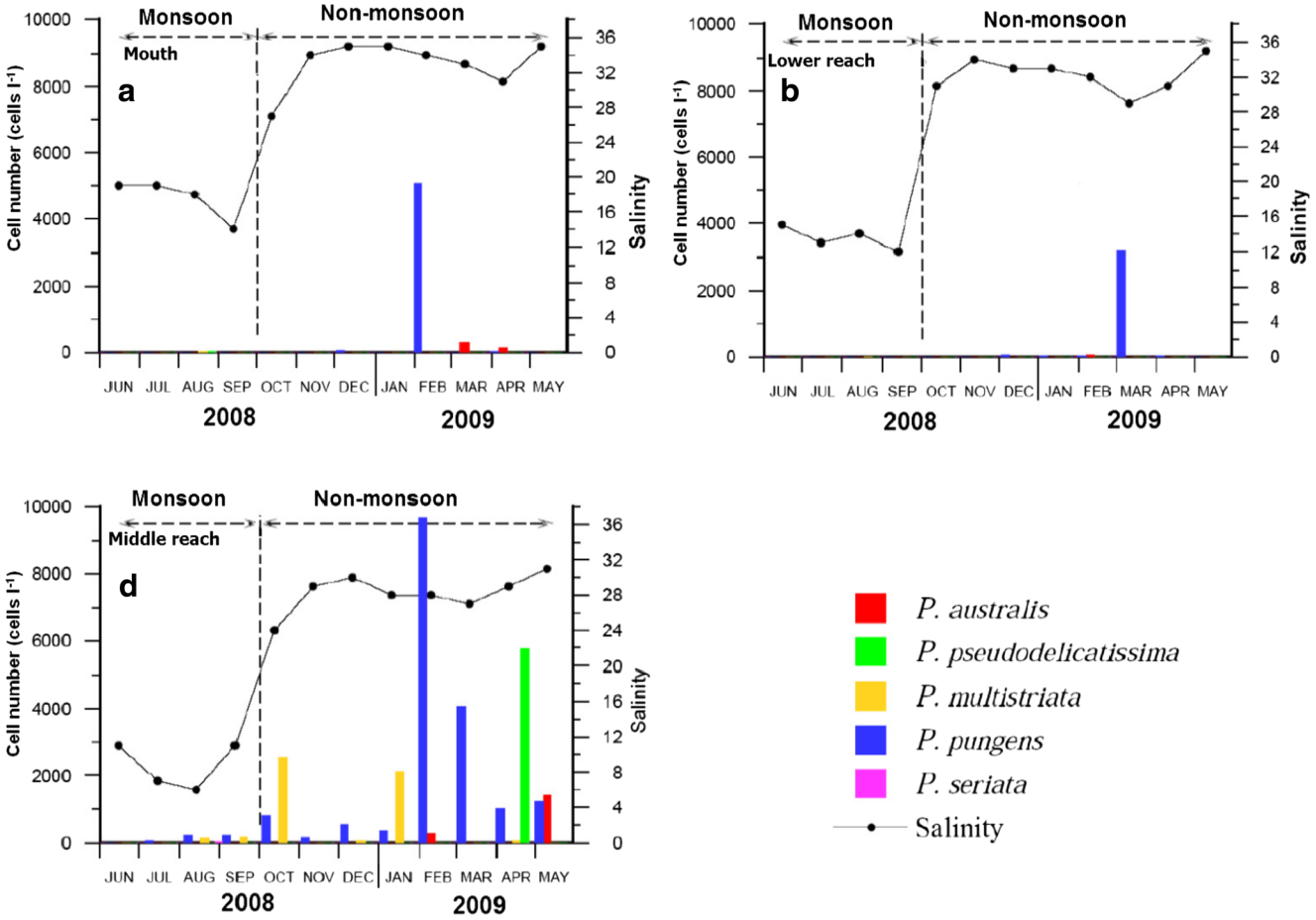

Fig. 4 Spatiotemporal distribution of *Pseudo-nitzschia* species at surface stations in the Zuari estuary (2008–2009). **a** Mouth, **b** Lower reach, and **c** Middle reach. Vertical dashed line indicates the transition from the monsoon (June to September) to the non-monsoon (October to May) periods

monsoon season. *Pseudo-nitzschia multistriata* (Pm) and *P. seriata* (Ps) show positive correlations with nitrate, silicate, phosphate, and rainfall. The high cell abundances of *P. pungens* (Ppu) and *P. australis* (Pa) during the non-monsoon period were governed by salinity and temperature. The distribution of *P. pseudodelicatissima* (Pp), at the center of both axes, shows a positive relation with nitrite and temperature.

Growth Parameters of *P. pungens* in Culture

Batch cultures of *P. pungens* exhibited the classical logistic growth pattern with three phases: lag (days 0–2), exponential (days 3–7), and stationary (days 8–12) (Fig. 6a). Salinity affected the maximum cell concentration attained during the stationary phase. The highest cell concentration at stationary phase (9.6×10^5 cells ml^{-1}) was observed at a salinity of 25 and the lowest (1.3×10^5 cells ml^{-1}) at 5. Salinity also affected growth, although *P. pungens* grew at all the salinities, from 5 to 35. The specific growth rate was lowest (0.44 day^{-1}) at a salinity of 5 and increased to a maximum (1.05 to 1.19 day^{-1}) at salinities of 15–30; it then decreased slightly (to 0.98 day^{-1}) at 35 (Fig. 6b). The ANOVA test showed a significant variation in specific growth rate with respect to salinity. Tukey's post hoc test showed a significant increase in specific growth rate up to a salinity of 30 ($p < 0.001$) and a significant decrease of 35 ($p < 0.001$) (Table 2).

Domoic Acid Production

DA was analyzed by the FMOC pre-column derivatization method for amino acids followed by reversed-phase HPLC with fluorescence detection (Pocklington et al. 1990). Additional tests were required to provide evidence that the suspected peak on the chromatogram was indeed DA (Supplementary Fig. S1). This was a necessary step, as most strains of *P. pungens* have proven to have non-detectable DA (Trainer et al. 2012).

Our spiking tests confirmed that the suspected peak in the chromatograms was DA and thus that our strain of *P. pungens* produced DA (Fig. S1). The overall trend in DA concentration in the "whole culture" (i.e., cells plus medium, expressed per ml) during the course of batch culture growth was similar at each salinity, with peaks on days 10 to 11 during the stationary phase (Fig. 7). Low levels of DA were found during the exponential phase.

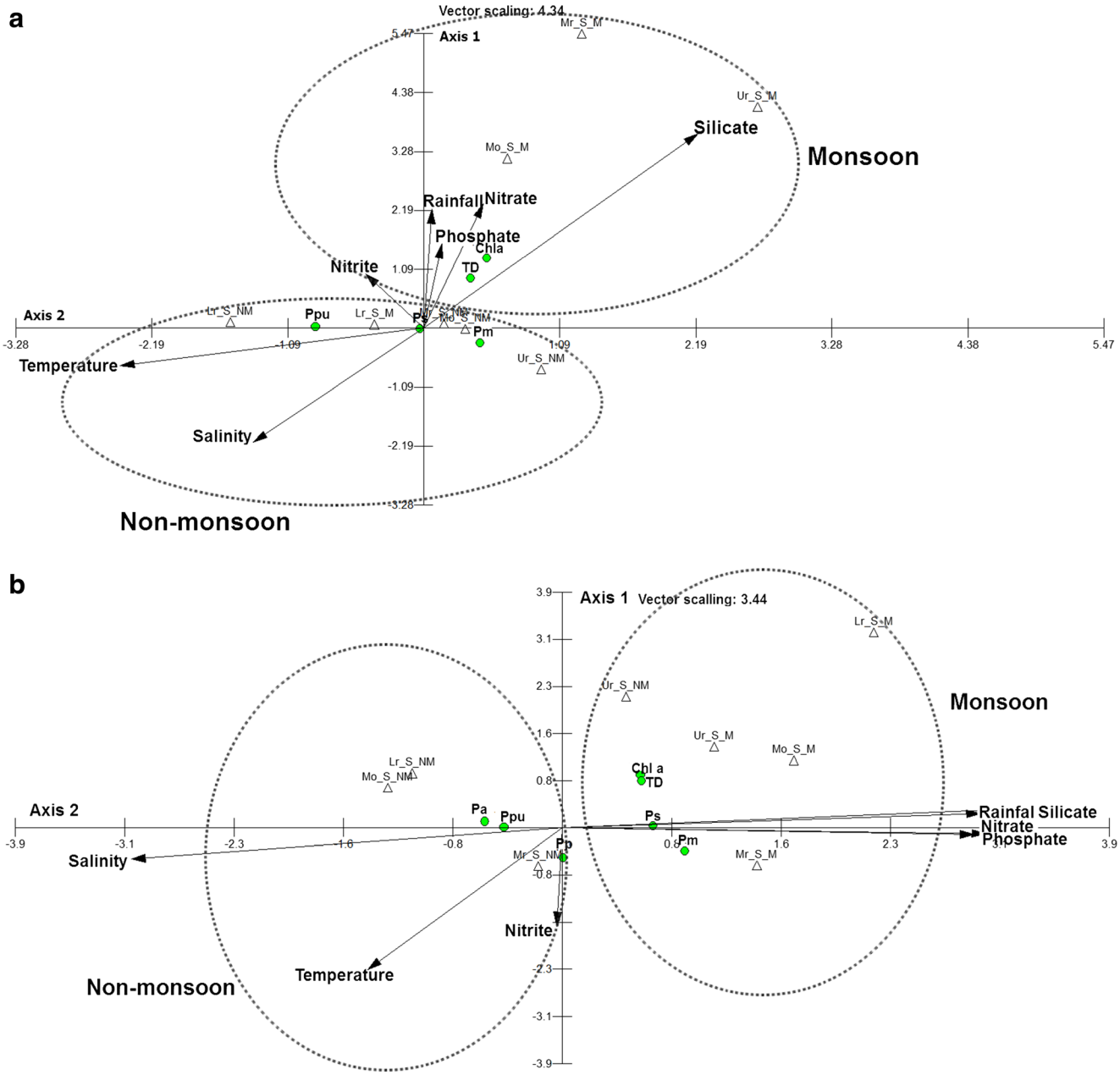

Fig. 5 Canonical correspondence analysis (CCA) joint biplots of *Pseudo-nitzschia* species and stations for analysis in the **a** Mandovi estuary and **b** Zuari estuary. Significant environmental vectors (nitrate, nitrite, phosphate, silicate, temperature, salinity, rainfall) are denoted by arrow lines. *Pseudo-nitzschia* species (circles) are abbreviated as Pm *P. multistriata*, Ps *P. seriata*, and Ppu *P. pungens*. Station names (triangles) are abbreviated as Mo Mouth, Lr Lower reach, Mr Middle reach, and Ur Upper reach. Additional abbreviations for station names: S surface, M monsoon, and NM non-monsoon. Chl *a* chlorophyll *a*, TD total phytoplankton concentration

Salinity affected DA production. The lowest stationary-phase DA concentration (18.7 ng ml^{-1}) was at a salinity of 5 and it gradually increased (to 29.7 ± 0.4 ng ml^{-1}) at salinities of 25 to 35 (Supplementary Fig. S2). DA production rates were lowest and similar at salinities 5 to 15 (2.56 to 3.12 ng ml^{-1} day^{-1}) and thereafter gradually increased with increasing salinity, reaching 5.25 ng ml^{-1} day^{-1} at a salinity of 35 (Fig. 7). There was a significant variation in DA production between salinities ($p < 0.001$; two-way ANOVA) and a significant increase in DA production at salinities from 5 to 35 ($p < 0.001$; Tukey's post hoc test) (Table 2).

Discussion

Here, we show that salinity and nutrients contribute to explaining the distribution of *Pseudo-nitzschia* species during the monsoon and non-monsoon seasons in the Mandovi and

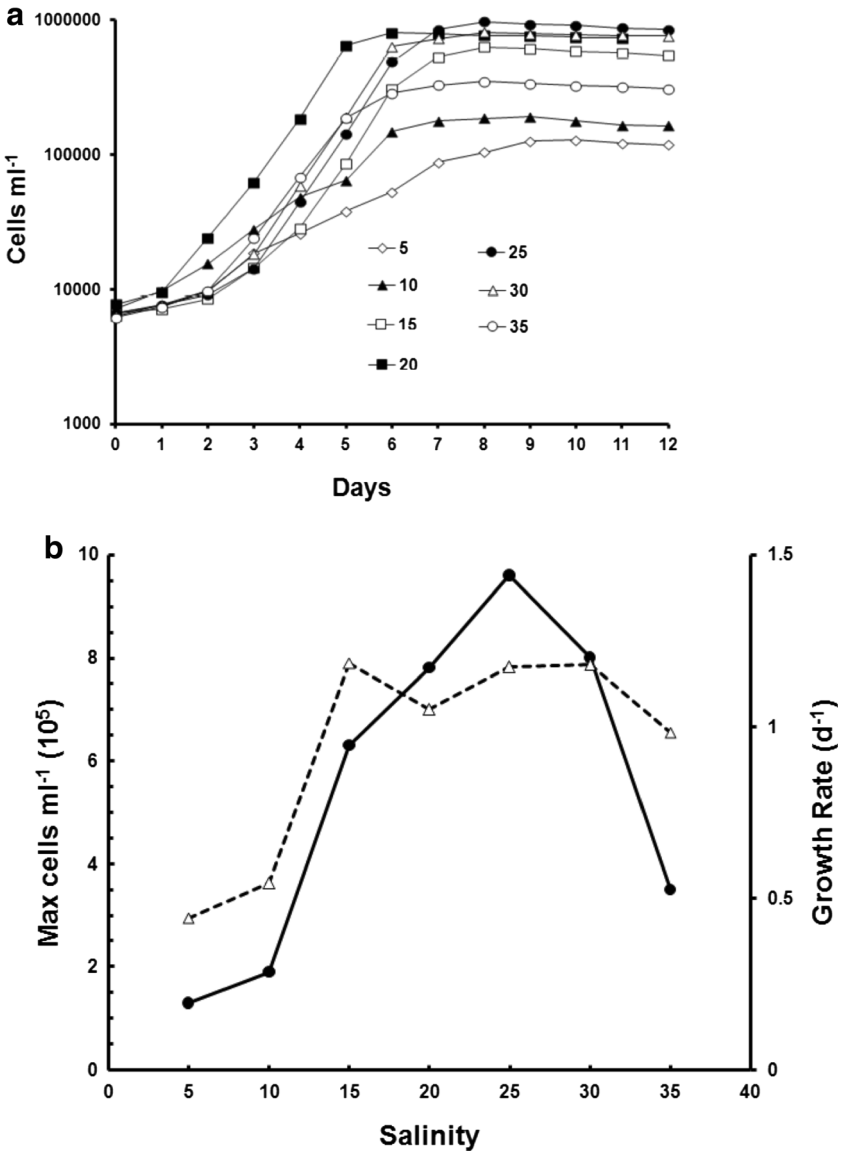

Fig. 6 Effect of salinity on the growth of *Pseudo-nitzschia pungens* isolated from the Zuari estuary. **a** Growth curves, **b** maximum cell concentration at stationary phase (●), and maximum growth rate (▲)

Zuari estuaries, along the west coast of India. It should be noted, however, that any comparisons between the Mandovi and Zuari estuaries are indirect, because they were sampled in successive years. Furthermore, we show that the growth and toxicity of an isolate of *P. pungens*, one of the most abundant *Pseudo-nitzschia* species in the Zuari estuary, were influenced by salinity.

Pseudo-nitzschia species were ~20% of the total diatom species in the Mandovi and Zuari estuaries. Elsewhere, when toxigenic species are abundant and therefore DA is reported in the plankton or molluscan shellfish species, the percentage of *Pseudo-nitzschia* species relative to total diatoms is higher. In Scottish waters, it was 14–77% (Fehling et al. 2006). In Spanish Mediterranean waters, 80–91% of the diatoms were *Pseudo-nitzschia* species (Quijano-Scheggia et al. 2008). On the west coast of the USA, the percentage of *Pseudo-nitzschia* species was low (2.5%) in April 2015 (Du et al. 2016). However, by May, it increased to 23% and then to 90% of total diatoms, indicating a mono-specific bloom of toxic *P. australis*. This led to closures of the harvest of razor clams and Dungeness crabs along the west coast of the USA. In our study, the percentage of *Pseudo-nitzschia* spp. was greater in the Mandovi estuary (21.1%) than in the Zuari estuary (8.8%). This could be related to the higher average amount of rainfall during 2007 in the Mandovi estuary (35.91 ± 13.94 cm), compared to the Zuari estuary (7.04 ± 10.17 cm) in 2008. Higher rainfall leads to a greater addition of nutrients and higher variations in salinity into the Mandovi estuary, which could favor *Pseudo-nitzschia* species.

We report five species of *Pseudo-nitzschia*, although only two of them (*P. australis* and *P. pseudodelicatissima*) were found exclusively in the Zuari estuary. In spite of the fact that each of these species is toxigenic (Trainer et al. 2012), DA was below the detection limit in all field samples, either because

Table 2 Two-way ANOVA result showing the variation in specific growth rate [μ (day^{-1})] and rate of domoic acid (DA) production [DA (ng ml^{-1} day^{-1})] at seven salinities.

Salinity	Rate	F	*p*
5	μ (day^{-1})	0.45	< 0.001
	DA (ng ml^{-1} day^{-1})	3.13	< 0.01
10	μ (day^{-1})	0.56	< 0.001
	DA (ng ml^{-1} day^{-1})	2.49	< 0.001
15	μ (day^{-1})	1.17	< 0.001
	DA (ng ml^{-1} day^{-1})	2.53	< 0.001
20	μ (day^{-1})	1.07	< 0.001
	DA (ng ml^{-1} day^{-1})	3.79	< 0.001
25	μ (day^{-1})	1.18	< 0.001
	DA (ng ml^{-1} day^{-1})	3.45	< 0.001
30	μ (day^{-1})	1.18	< 0.001
	DA (ng ml^{-1} day^{-1})	4.90	< 0.001
35	μ (day^{-1})	0.97	< 0.001
	DA (ng ml^{-1} day^{-1})	5.18	< 0.001

F statistic, *p* probability

their concentrations were too low or the strains (other than *P. pungens*) are non-toxic in those estuaries. It should also be noted that the biomass of *Pseudo-nitzschia* species may be low, in spite of the high nutrient concentrations, because of light limitation due to cloud cover and high water column turbidity (c.f Maya et al. 2011). As shown by the CCA analysis, the spatiotemporal distribution of *Pseudo-nitzschia* species in these estuaries is related to environmental parameters such as nutrients, temperature, salinity, and rainfall. Indeed, nutrients can play an important role in the distribution of *Pseudo-nitzschia* species. Other studies have reported a correlation between *Pseudo-nitzschia* abundances and nutrients (Trainer et al. 2002; Kaczmarska et al. 2005). At the same time, a high Si concentration can increase the cell biomass, whereas Si limitation boosts DA production (Pan et al. 1996; Fehling et al. 2004). Two distinct seasons were observed in the CCA analysis, i.e., a monsoon and non-monsoon season, pertaining to each axis, wherein each species of *Pseudo-nitzschia* was distributed. In the present work, the distribution of *P. multistriata* showed a strong positive correlation with silicate in both estuaries. Its abundance was greater at the end of the monsoon season in October, when a high concentration of silicate was observed at the Upper reach in the Mandovi estuary, and at the Middle reach in the Zuari estuary.

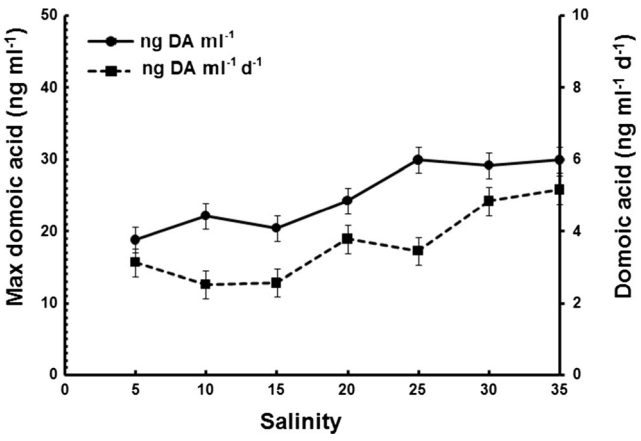

Fig. 7 Effect of salinity on domoic acid (DA) production by *Pseudo-nitzschia pungens* growing in culture at different salinities, expressed as a concentration (ng ml^{-1}; ●) and as a rate (ng ml^{-1} day^{-1}; ■). Error bars indicate ± SD

The abundance of *P. seriata* was higher in waters of the Mandovi estuary during the non-monsoon period. This is the second report of *P. seriata* in this part of the world, after Mochemadkar et al. (2013). Patil and Anil (2008) reported a *Pseudo-nitzschia* sp. from the "Seriata group" in the Zuari estuary (3767 cells l^{-1} at the surface and 30,987 cells l^{-1} at the bottom). Its presence was nevertheless unexpected, because it is usually associated with cold waters of higher latitudes in the Northern and Southern Hemispheres (Couture et al. 2001; Hasle 2002; Fehling et al. 2004; Almandoz et al. 2007; Lelong et al. 2012). In our study, the highest concentration (15,320 cells l^{-1}) at the Upper reach was associated with a high salinity (~25) and temperature (~28 °C). The CCA analysis gave a positive relation between *P. seriata* and salinity and temperature, supporting this finding. Its presence was also associated with a higher availability of nutrients due to high rainfall during the monsoon period. There was no evidence of water discoloration, nor of DA, at this high cell concentration. Nevertheless, it is comparable to that found at Natashquan, Quebec (northern Gulf of St. Lawrence, Canada), where a similar *P. seriata* concentration (14,000 cells l^{-1}) was associated with the presence of low levels of DA (0.89 μg DA g^{-1}) in soft-shell clams (*Mya arenaria*), in August 1999 (Couture et al. 2001). In the Baie des Chaleurs (Quebec, Southern Gulf of St. Lawrence), the *P. seriata* concentrations reached 62,000 cells l^{-1} in 2002, and the DA level in blue mussels (*Mytilus edulis*) was 89 μg DA g^{-1} (Bates et al. unpublished data), which is over the 20 μg DA g^{-1} regulatory limit and resulted in widespread harvesting closures. In Scottish waters, *P. seriata* was most abundant at temperatures of 10–15 °C (Fehling et al. 2004). Given its presence in warm waters of western India, more regions of the world require study to determine if the distribution of this species is more widespread.

Pseudo-nitzschia pungens is ubiquitous, found in temperate and tropical coastal waters (Hasle 2002; Casteleyn et al. 2008; Anderson et al. 2010; Lim et al. 2014a, 2015; Kim et al. 2015). Molecular and morphological studies have delineated three clades of *P. pungens* around the world (Casteleyn et al. 2008; Lim et al. 2014a; Kim et al. 2015). Clade I (*P. pungens* var. *pungens*) is distributed mainly in temperate waters, clade II (both *P. pungens* var. pungens and var. *cingulata*) is from western Pacific waters, and clade III (*P. pungens* var. *pungens*

and var. *aveirensis*) is found in tropical and warm temperate waters. Our morphological data do not allow definitive assignment of the *P. pungens* from the Goa estuaries to a particular clade. However, clade II can be ruled out based on its more restricted distribution.

The present study represents the successful isolation and culturing of *P. pungens* and also shows how growth and DA production of a *P. pungens* strain from the Zuari estuary respond to salinity. Results of the laboratory study support the field observations. Salinities of 15 to 30 were best suited for the growth of our *P. pungens* SP-1 strain (highest specific growth rate: 1.19 day^{-1}); higher (35) and lower (5–10) salinities resulted in a growth rate reduction. The maximum cell concentration (9.6×10^5 cells ml^{-1}) was at a salinity of 25. At the Middle reach of the Zuari estuary, a bloom concentration of *P. pungens* (9680 cells l^{-1}) was observed where the salinity was 20–32, and the temperature was 28–32 °C. Hence, the laboratory and field observations are congruent and help to explain the dominance of *P. pungens*, in relation to all the other species of *Pseudo-nitzschia*, during the non-monsoon period, when the salinity is higher and temperature is warmer. In other parts of the world, blooms of *P. pungens* can occur during the late summer, accompanied by warm temperatures, high salinity, and low nutrients (Almandoz et al. 2007; Lelong et al. 2012). *Pseudo-nitzschia pungens* is euryhaline, growing at salinities of < 6 to > 50, the widest range of all *Pseudo-nitzschia* species (Lelong et al. 2012). By way of comparison, cultures of *P. pungens* from Guanabara Bay (Brazil) grew well in a salinity range of 20–40 (Villac et al. 2004). This species also grew in the colder, higher salinity waters of Chesapeake Bay (Thessen et al. 2005; Thessen and Stoecker 2008). However, other strains of *P. pungens* from the Sea of Japan (Russia) died when grown at salinities of 4 and 8 (Markina and Aizdaicher 2016).

Our strain of *P. pungens* produces DA, based on the identical retention times of the peak of the DA standard and the presumed DA peak on the HPLC chromatogram. At the same time, a spike of a known concentration of a standard DA solution had the same retention time as the presumed DA peak and also increased the DA concentration by the expected amount (Supplementary Fig. S1). This is thus the first study to demonstrate that a *P. pungens* strain from India is toxigenic and the first to show how salinity affects the DA production of this strain. *Pseudo-nitzschia pungens* was considered as non-toxic until 1994 (Bates et al. 1998). It was then shown that some strains were capable of producing low levels of DA. The presence of DA in greenshell mussels (*Perna canaliculus*) in Marlborough Sounds (New Zealand) was attributed to *P. pungens*, one strain of which produced low levels of DA (Rhodes et al. 1996). Another strain in Twin Harbor (Washington State, USA) also produced low levels of DA (40 ng ml^{-1}) (Trainer et al. 1998). Baugh et al. (2006) reported even lower DA levels (0.03 ng ml^{-1}) in an isolate of *P. pungens* from offshore Washington State. Calu et al. (2009) also reported on a toxigenic strain of *P. pungens*, from the Bay of Crozon (France).

Most studies show that toxin production by *Pseudo-nitzschia* species takes place during limitation by P or Si (reviewed by Bates 1998; Bates and Trainer 2006; Trainer et al. 2012), or during Fe limitation or Cu toxicity (Maldonado et al. 2002). Our study adds to the evidence that salinity is an additional controlling factor. DA production increased as the salinity increased from 15 to 35, with the highest rate (5.25 ng ml^{-1} day^{-1}) at 35. On the other hand, specific growth rates remained high (up to 1.18 day^{-1}) at salinities of 15, 25, and 30, declining only slightly at the highest salinity, 35. This pattern is similar to that found by Thessen et al. (2005) and Doucette et al. (2008). For example, *P. multiseries* had the highest DA production as well as growth rates at salinities of 30 to 40 (Doucette et al. 2008). Pan et al. (1998) suggested that when growth is limited by P or Si, energy becomes available for DA production. In our study, *P. pungens* was able to maintain both high growth and DA production at a salinity of 15. This is in contrast to *P. multiseries* (Doucette et al. 2008), perhaps because nutrients were still available at the low cell concentrations achieved in our cultures. Previous studies have shown that an increase in salinity boosts the production of the amino acid taurine (Jackson et al. 1992) and the sugar alcohol sorbitol (Stewart et al. 1997), which may regulate the osmotic pressure in the case of *P. multiseries*. Further work is required to establish if DA is indeed an osmoregulatory compound.

Our laboratory cultures of *P. pungens* showed the highest growth at salinities of 15 to 25, the same range at which field populations of this species were also the most common relative to the other *Pseudo-nitzschia* species. This is also the salinity range at which our strain of *P. pungens* had the highest DA production rates. Although *P. pungens* was the dominant *Pseudo-nitzschia* species in the Zuari and Mandovi estuaries, it was only one of the potentially toxigenic members of the *Pseudo-nitzschia* genus present; strains of *P. multistriata*, *P. seriata*, *P. australis*, and *P. pseudodelicatissima* from our waters should also be isolated into culture and tested for their ability to produce DA.

Both the Zuari and Mandovi estuaries, along the west coast of India, serve as important economic zones for fisheries and tourism. The sites chosen for our study (especially the Upper and Middle reaches) have active fisheries (prawns, oysters, and freshwater fishes) during both the monsoon and non-monsoon seasons. Although the DA levels produced by our strain of *P. pungens* studied were low, other *Pseudo-nitzschia* species that may produce higher toxin levels were also found at these active fisheries zones. Our study thus raises the prospect of potential human health and economic consequences of *Pseudo-nitzschia* species from these tropical estuaries. It also broadens our knowledge of the distribution of these species in

monsoonal waters. In light of the importance of tides and freshwater runoff influencing the salinity and nutrient patterns during the monsoon period, additional field and laboratory studies are required to refine the relationship between salinity and other physicochemical factors in the development and toxicity of *Pseudo-nitzschia* blooms.

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