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Identification and characterisation of the dominant *Pseudo-nitzschia* species (Bacillariophyceae) along the NE Spanish coast (Catalonia, NW Mediterranean)

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SUMMARY: Potentially toxic species of the genus *Pseudo-nitzschia* were studied along the Spanish NW Mediterranean coast from January 2005 to May 2006. Observation in electron microscopy revealed the presence of *Pseudo-nitzschia brasiliana, P. calliantha, P. delicatissima, P. fraudulenta, P. multistriata* and *P. pungens*. Several strains were isolated from coastal waters and their clonal cultures were compared by combined techniques, including light and electron microscopy and internal transcribed spacer (ITS-1, 5.8S and ITS-2) rDNA sequencing. Cultured isolates were submitted to HPLC analysis of pigments to evaluate the possibility of taxonomic discriminations by means of a simple chemotaxonomic approach. The genus *Pseudo-nitzschia* showed high cell concentrations during most of the year, but the population initiated a sharp decline at all stations in the period between April and May. *P. delicatissima* with *P. calliantha* were found at the northern stations between February and April, and at the southern stations between August and November. *P. brasiliana* and *P. multistriata* were only occasionally detected in the southern region. None of the environmental variables considered was significant to explain the observed spatial and temporal distributions of *Pseudo-nitzschia* species in the area studied. Differences in the growth rate and cell yield of the species indicate that biotic factors may play a role in the observed distributional patterns.

Keywords: diatoms, *Pseudo-nitzschia*, harmful algal blooms, autoecology, ITS morphology, pigments.

RESUMEN: Identificación y caracterización de las especies de *Pseudo-nitzschia* dominantes (Bacillariophyceae) en la costa NE de España (Cataluña, Mediterráneo NO). – Se estudiaron especies potencialmente tóxicas de *Pseudo-nitzschia* en la costa española del Mediterráneo NO de enero 2005 a mayo 2006. Observaciones al microscopio electrónico revelan la presencia de *Pseudo-nitzschia brasiliana, P. calliantha, P. delicatissima, P. fraudulenta, P. multistriata* y *P. pungens*. Se aislaron varias cepas de las aguas costeras y fueron comparadas entre si mediante técnicas combinadas que incluyen microscopia óptica, electrónica, secuenciación del ITS-1, 5.8S e ITS-2 rDNA. Cultivos de las cepas aisladas fueron analizadas con HPLC para estudiar la posibilidad de discriminarlas por medio de un método quemotaxonómico simple. El género *Pseudo-nitzschia* muestra concentraciones celulares altas durante la mayor parte del año, pero la población inicia un declive brusco en todos los puntos de muestreos en el periodo entre abril y mayo. *P. delicatissima* y *P. calliantha* fueron encontradas en las estaciones del norte entre febrero y abril, y en las del sur entre agosto y noviembre. *P. brasiliana* y *P. multistriata* fueron detectadas sólo ocasionalmente en las estaciones del sur. Ninguna de las variables ambientales consideradas explica satisfactoriamente la distribución temporal y espacial de las especies de *Pseudo-nitzschia* en el área de estudio. Diferencias en la tasa de crecimiento y producción celular de las especies indica que los factores bióticos pueden jugar un papel importante en los patrones de distribución observados.

Palabras clave: diatomeas, *Pseudo-nitzschia*, proliferaciones algales nocivas, autoecología, ITS, morfología, pigmentos.

INTRODUCTION

Diatoms comprise one of the species-richest phytoplankton taxonomic groups. They have a worldwide distribution, can be found in both freshwater and marine environments, and are characterised by an extraordinary morphologic diversity (Round *et al.*, 1990; Mann and Droop, 1996; Round, 1996). It is estimated that between 1365 and 1783 different planktonic diatoms occur in marine environments (Sournia *et al.*, 1991). These include members of the genus *Pseudo-nitzschia*, commonly found in phytoplankton populations, with the highest cell concentrations in coastal regions (Lundholm *et al.*, 2002a)

The genus *Pseudo-nitzschia* currently harbours 29 different species of needle-shaped, raphid pennate diatoms forming chains of variable length (Hasle and Syvertsen, 1997; Lundholm *et al*., 2002a; Lundholm *et al.*, 2003; Lundholm *et al.*, 2005). There are 11 or 12 species of *Pseudo-nitzschia* (depending on the inclusion of *P. pseudodelicatissima*) able to produce the neurotoxin domoic acid (DA) which is associated with amnesic shellfish poisoning (ASP) in humans (Bates *et al.*, 1989; Bates and Trainer, 2006) and has also been found to induce mass mortality in sea birds, fish and mammals (Bates, 2000; Mos, 2001; Sierra-Beltran *et al.*, 2005; Schaffer *et al.*, 2006).

For the Mediterranean Sea, ASP events related to *Pseudo-nitzschia* were first described in 2001 (Amzil *et al*., 2001), but along the south coast of France the presence of the toxin DA (attributed to *P. pseudodelicatissima*) was detected as early as 1998. Recent publications at this point describe detection of DA in Greek shellfish (Kaniou-Grigoriadou *et al*., 2005), in *P. multistriata* (Orsini *et al.*, 2002) and in *P. calliantha* (Inès and Asma, 2006).

The diatom genus *Pseudo-nitzschia* (initially referred to as *Nitzschia*) has been known to occur in the NW Mediterranean Sea for many decades (Massuti and Margalef, 1950; Margalef, 1969), including the species *Nitzschia delicatissima*, *N. pungens* and *N. seriata*. *Pseudo-nitzschia delicatissima* (referred as *N. delicatissima*) was observed during the aestival stratification (Margalef, 1969).

In the context of the monitoring programme of the Catalan Water Agency (ACA), performed by the Institute of Marine Sciences in Barcelona (ICM-CSIC), the distribution and abundance of *Pseudo-nitzschia* and other potentially harmful algae are continuously evaluated along the NE coast of Spain (Catalonia). This programme, initiated in 1995, currently includes 16 representative harbours, several beaches and two coastal bays (Vila *et al.*, 2001; Furones *et al.*, 2004). Because only optical microscope analyses were done, taxonomic identifications at species level were not performed. Such discriminations are, however, important for the understanding of bloom formation, because ecological preferences may vary significantly among species, whereas not all of them are actually toxic. Discrimination of species within the genus *Pseudo-nitzschia* currently requires the use of SEM (scanning electron microscopy) and/or TEM (transmission electron microscopy) techniques for morphological characterisation.

In the present study a combination of TEM and SEM was employed to elucidate, for the first time, spatial and temporal distributions of *Pseudo-nitzschia* species along the NE Spanish coast during one and a half years. Morphologic characteristics of the detected species were carefully analysed and compared with data available in the literature. To characterise the different *Pseudo-nitzschia* sp. identified at the sample location and enable comparative studies of specific characteristics in the future, a culture collection was initiated using isolates from the different locations sampled. For each strain, cell yield and growth rate were determined and the samples were submitted to HPLC analysis of pigments. Special attention was given to possible differences in composition and/or relative importance of individual pigment compounds, which would allow some degree of species classification using a simple analytical tool. Moreover, ITS region has been shown to be very informative (Coleman, 2003; Amato *et al.*, 2007) for the identification of the genus *Pseudo-nitzschia*. The ITS-1, 5.8S, and ITS-2 sequence data were used as a means of confirming the identification already performed by light and electron microscopy analyses of morphologic features.

Finally, to identify the potential biotic and abiotic variables controlling the observed spatial and temporal distributions of *Pseudo-nitzschia* species in the study area, statistical analysis was performed.

MATERIALS AND METHODS

Sampling

The stations sampled in the present study were selected on the basis of experiences obtained during 10 years of regular monitoring of the coast (Vila,

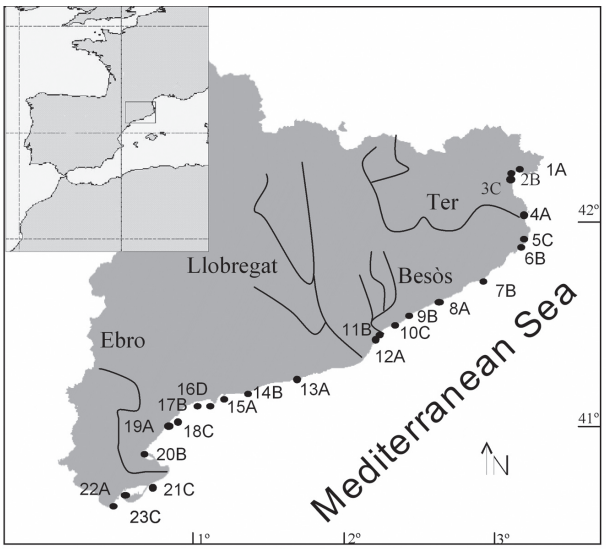

FIG. 1. – Geographic location of Catalonia, Spain, and the specific locations sampled during the years 2005 and 2006. Station's name. 1 Roses; 2 Empuriabrava; 3 Muga; 4 Estartit; 5 Fosca; 6 Palamós; 7 Blanes; 8 Arenys; 9 Premià; 10 Parc del litoral; 11 Olímpic; 12 Barcelona; 13 Vilanova; 14 Torredembarra; 15 Tarragona; 16 La Pineda; 17 Cambrils; 18 Alguer; 19 Ametlla; 20 Ampolla; 21 Eucaliptus; 22 St. Carles; 23 Parc de Garbí. Sample frequency codes: A Weekly from May-Sept., bimonthly rest of the year harbours, B Bimonthly May-Sept., monthly rest of the year harbours, C Bimonthly May-Sept., monthly rest of the year beaches, D Extra beaches.

2001) and included the period from January 2005 to May 2006. These locations (Fig. 1) cover relevant points of interest in the area, and include all those known to be affected by (recurrent) algal blooms. This coastal section is over 500 km long and many locations are densely populated. Chemical conditions along the coast are strongly influenced by inflows of nutrient-enriched freshwater of continental run-off and the rivers, mainly the River Ter (6-20 m^3 s^{-1}) in the north, the Rivers Llobregat (14-30 m^3 s^{-1}) and Besòs (2.6-7.2 m^3 s^{-1}) in the central part, and the large River Ebro (130-630 m^3 s^{-1}) in the south (Idescat, 2006).

Water samples were collected (for details on the sampling frequency see Fig. 1) with a bucket, in which standard hydrographic parameters (temperature and salinity) were directly measured using WTW probe (Model 315). Subsamples (150 ml) for taxonomic identification of the total phytoplankton population using inverted light microscopy (outlined below) were directly fixed with Lugol's iodine solution. Additional subsamples (50 ml) for analyses of nutrients were stored on ice during transport to the laboratory and frozen (-20°C) upon arrival. Nutrient samples were analysed with an autoanalyser as described in Grasshoff *et al.*, (1983). Probable inorganic dissolved nutrient limitations were calculated as in Justic *et al.* (1995). The criteria of probable nutrient limitation is as follows: P limitation (P <0.1 μM; DIN:P >22; Si:P >22), N limitation (DIN <1 μM;DIN:P <10; Si:DIN >1) and Si limitation (Si <2 μM; Si:PO4 <10; Si:DIN <1).

Light microscopy

Subsamples (50 ml) were allowed to settle in counting chambers for 24 h and phytoplankton were subsequently enumerated in an appropriate area depending on the cell number (field or transect) using a Leica-Leitz DM-II inverted bright field microscope with at a 200-400x magnification according to Throndsen, 1995. A total of 618 samples were counted. The limit of detection of the Utermöl method is 20 cells L^{-1}(Utermöhl, 1931). Identification of the species among the genus *Pseudo-nitzschia* in the light microscopy was attempted on the basis of the length and shape of the cells (Trainer and Suddleson, 2005).

Electron microscopy

When observations with the light microscope revealed a *Pseudo-nitzschia* spp. population exceeding 10^4 cells L^{-1}, Lugol-fixed samples were subjected to SEM (63 samples) and TEM (7 samples) procedures. Samples were observed at TEM to distinguish the fine structure of the poroids and confirm identification. Samples with lower concentrations (<10^4 cells L^{-1}) were not studied because little material was left after cleaning of the samples.

For both techniques (SEM and TEM) organic material was removed from the samples with sulphuric acid and potassium permanganate with later addition of oxalic acid as described in Lundholm *et al.* (2002b). For SEM the remaining material was mounted on a polycarbonate filter and this was attached on stubs with colloidal silver and then sputter-coated with gold-palladium. The stubs were screened by a Hitachi S-3500N microscope operating at 5 kV. For analysis with TEM, drops of cleaned material were placed on Formvar-coated copper grids, dried and studied with a Hitachi H800 microscope.

Morphometric characteristics

Cells identified as *Pseudo-nitzschia* were carefully examined for several characteristics under SEM, including the width and length of the valve,

TABLE 1. – Representative morphological characteristics observed for NW Mediterranean *Pseudo-nitzschia* species from field samples compared with literature data. [a] Hasle (1995), Priisholm *et al* (2002), Lundholm *et al* (2003). [b] Kaczmarska *et al* (2005), [c] Orsini *et al* (2002) Number in italic show mean ± SD. All measurements were made with SEM. N= number of observation. *Differences from description

Taxa	Valve shape	Fibulae/ 10 µm	Striae/ 10 µm	Row of poroids	Poroids/ 1 µm	Central nodule	Length (µm)	Width (µm)	N Length and Width	N Fibulae, Striae
P. brasiliana	rectangular	22-27*	23-28*	2	7-10	–	34.1-39.4	2.3-3.3		
		24.6 ± 1.5	*25.2±1.6*		*8.7 ± 0.8*		*36.5 ± 1.6*	*2.7± 0.2*	18	10
a		20-26	20-26		7-10		12-65	1.8-3		
P. calliantha	linear	15-26*	30-40*	1	4-5	+	41.4-123.1*	1.1- 2.4*		
		18.9±2	*35.8 ±1.9*		*4.7±0.5*		*79.3 ± 11.3*	*1.7± 0.2*	1450	32
a		15-21	34-39		4-6		41- 98	1.3-1.8		
P. delicatissima	lanceolate	20-28	36-42	2	9-11	+	32.3-78*	1.01-2.4*		
		23.1±2.2	*37.3±1.9*		*9.9±0.7*		*44. 8 ± 6.7*	*1.6± 0.3*	612	14
a		19-26	35-40		8-12		19-76	1.5-2		
b		20-30	33-42		9-12.5		39-71	1.3-1.7		
P. fraudulenta	lancelolate	19-24	21-24	2	5-6	+	38.9-131.5*	2.9-7.1*		
		21.1±1.5	*22.7±1.2*		*5.5±0.5*		*71.± 20.3*	*5.1± 1.0*	264	10
a		12-24	18-24		4-7		50-119	4-6.5		
b		20-24	21-23		5-6		93-98	5-6		
P. multistriata	lancelolate	22-26	36-42	2-3	9-13	–	35.9-58.5	2.2-4.2*		11
		24.2±1.9	*38.7±2.1*		*11.6±1.3*		*50.1± 4.5*	*3.4± 0.4*	45	
a		22-28	36-46		10-12		34-60	2.3-4		
c		23-32	37-44		11-13		38-50	2.5-4		
P. pungens	Linear-	9-13	9-13	2	2.5-3	–	70.1-155.9	2.2-4.8*		
	lanceolate	*11.2±1.3*	*11.4±1.4*		*3.0±0.2*		*116.9± 24.6*	*3.75± 0.57*	81	10
a		9-15	9-15		2-4		37-142	2-4.5		
b		10-11	10-11		1-3		95-156	3.5-4.2		

the density of striae, fibulae and poroids on valves, the structure of girdle bands and the pattern of perforation in the poroid hymens. In each sample, organisms were identified in a different appropriate area of the filter, from one transect to a whole filter depending on the cell abundance (minimum cell number measured= 30). Length and width of all the whole cells were always measured (Table 1) and the number of measurements of fibulae and striae were variable and depended on the species. The percentages of each species obtained in SEM were applied to the cell counts.

Clonal cultures

From live field samples examined under an inverted microscope (Leica DM-II inverted bright field microscope), cells identified as *Pseudo-nitzschia* spp. were isolated with a glass Pasteur pipette and transferred to a tissue culture flask filled with silicate-containing f/2 or L1 medium (Guillard, 1975; Guillard and Hargraves, 1993). These flasks were subsequently maintained at 19-21±1ºC using a 12:12 h light:dark cycle. Illumination was provided by fluorescence tubes (Gyrolux, Sylvania, Germany), providing a photon irradiance of 100 µmol photons m^{-2} s^{-1}. With these procedures a total of 42 clonal cultures (belonging to five species identified with SEM) were established. In this study we present data on growth rate, cell yield and molecular and pigment characterisation for one representative strain of each species.

Growth rates

The growth rates of the strains in L1 medium prepared in filtered seawater with a salinity of 36 were determined. Batch cultures were grown in 50 mL polycarbonate bottles and maintained in the growing conditions described above. Growth experiments were started with an exponential population of parental strains. Every second day, 1 mL subsamples were fixed in Lugol's iodine and counted in a cell chamber under an optical microscope (Leica-Leitz DM-Il, Leica Microsystems GmbH, Wetzlar, Germany) at a 200-400x magnification, and the number of cells was recorded. Cell abundance was used to calculate exponential growth rates according to the method of Guillard, 1973.

DNA extraction, PCR amplification and sequencing

All samples for sequencing were obtained from 15 ml of pure non-axenic cultures. DNA was extracted with the DNeasy® Plant Kit (Qiagen) following the manufacturer's protocols, with the exception of the elution volume, which was reduced to 25 µl. The ri-

bosomal DNA region, including the ITS-1, 5.8S and ITS-2, was amplified using the primers MicroSSU (5'-GTGAACCTGCG-GAAGGATC-3') and Dino E (5'-CCKSTTCAYTCGCCRTTAC-3') in a 25 µl reaction tube containing 1X polymerase buffer (Invitrogen), 1.5 µM $MgCl_2$, 400 µM dNTP's, 2 µM of each primer, and 1 U Taq DNA polymerase (Invitrogen). All of the amplifications were carried out in an Eppendorf MasterCycler using a program of 40 cycles of 30 sec at 94ºC and 30 sec at 54ºC, followed by 1 min at 72ºC with a pre-cycling incubation of 5 min at 95ºC and a post-cycling incubation of five min at 72ºC. The resulting amplicons of approximately 800 bp were cloned into the vector pCR2.1-TOPO (Invitrogen) for growth in *Escherichia coli* DH5α. Tranformant bacterial clones were screened using PCR and the primers and conditions shown above. One positive transformant clone representative of each isolate was submitted to a private company (Sistemas Genómicos, Valencia, Spain) for bidirectional sequencing. The plasmid clones of the rDNA were obtained in order to maintain a permanent record of these species sequences for future reference and/or other projects. The sequences obtained from these clones are those used in this study. However, direct sequencing of PCR products of some of these strains as well as others obtained during the same time period as this study have not shown any significant indels or intrastrain variation of the IT-S, 5.8S, and ITS-2 rDNA. The sequences were analysed using a BLAST search with no a priori knowledge of the morphometric characteristics or the possible identities of the cells.

Pigment analyses

For pigment analyse algal cells from 50 mL cultures in exponential growth (f/2 and L1) were concentrated on 25 mm GF/F filters by vacuum filtration (-25 Kpa). The filters were extracted in 3 mL 90% acetone and analysed with the HPLC method of Zapata *et al*., (2000). Details of the sample treatment, the chromatographic setup and adaptations of the method employed were previously described by Van Lenning *et al*., (2003). The HPLC was calibrated with authentic pigment standards obtained from DHI, Denmark. Concentrations of accessory pigments detected in individual cultures were normalised to chlorophyll *a* (Chl *a*), the proxy for algal biomass, and their contributions to the total pigment load were calculated. Results obtained for 3 different strains of the same species were averaged and the final results are presented for comparison (Table 2). To follow the variation of the pigment composition in *P. delicatissima* over the growth period a time series was performed. Every day, samples from a culture were taken and subsequently analysed until the stationary phase.

Statistical analysis

To identify potentially variables controlling the temporal and spatial dynamics of *Pseudo-nitzschia* sp on the Catalan coast one matrix was created (temperature, salinity, freshwater content (FWC), chlorophyll *a*, dissolved inorganic nutrients and *Pseudo-nitzschia* species abundance, n=44). The species *P.*

TABLE 2. – Mean relative contributions of Chl *a* (n.c.). degradation products and accessory pigments to the total pigment load (%) determined for a culture collection of five different *Pseudo-nitzschia* species grown in F2 and L1 culture media. Vertical arrangement of accessory pigment is based on descending mean relative importance.

	P. delicatissima			*P. pungens*			*P. calliantha*			*P. multistriata*			*P. fraudulenta*			*Mean
Medium:	F2%	L1%	n.c.	F2%	L1%	n.c.	F2%	L1%	n.c.	F2%	L1%	n.c.	F2%	L1%	n.c.	F2/L1
n=	4	10		2	1		5	1		7	1		5	1		28
Chl *a*	26.8	21.1	-	38.9	19.6	-	46.3	50.2	-	39.5	52.2	-	44.1	54	-	39.3
Chlide *a*	20.5	22.1	1.55	11.6	24.8	0.05	4.7	2.6	0.05	9.2	1.6	0.03	7.5	0.4	0.01	10.5
Chlide *a*-2	n.t.	1.7	0.12	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	1.7
∑ Chl *a*-like:	47.2	45	2.67	50.5	44.4	0.05	51	52.8	0.05	48.7	53.8	0.03	51.5	54.4	0.01	49.9
Fucoxanthin	30.8	30.7	1.83	25.1	30.7	0.6	31.5	30.1	0.6	31.2	27.5	0.53	27.9	26.7	0.49	29.2
Chl c2	7.6	8.5	0.51	15.4	16.5	0.22	10.8	11.1	0.22	9.9	8.5	0.16	8.9	7.9	0.15	10.5
Chl c3	8.5	9.1	n.t.	4.1	3.3	n.t.	1.4	0.9	n.t.	4	6.1	n.t.	2.7	2.3	0.07	4.3
Chl c1	n.t.	n.t.	0.55	n.t.	n.t.	0.02	n.t.	n.t.	0.02	n.t.	n.t.	0.12	4.2	3.5	0.04	3.8
Diadinox.	2.9	2.8	0.18	3	1.5	0.05	2.5	2.5	0.05	3	1.6	0.03	3	1.9	0.03	2.5
cis Fucoxan.	1.7	2.6	0.17	0.7	1.5	0.03	1.8	1.5	0.03	2	1.2	0.02	1.6	2.1	0.04	1.7
β.β-carotene	0.9	0.8	0.05	1	1.3	0.02	0.9	0.8	0.02	0.9	1.1	0.02	0.7	0.8	0.02	0.9
Chl c2-P. gyrans	0.3	0.2	0.01	0.5	0.5	0.01	0.2	0.3	0.01	0.4	0.2	0	0.3	0.2	0	0.3
MgDVP	0.1	0.2	0.01	0.1	0.4	0.003	0.01	0.2	0.003	0.02	0.1	0.001	0.1	0.1	0.002	0.1
∑ Accessory:	52.8	55	3.31	49.8	55.6	0.95	49.1	47.2	0.95	51.4	46.2	0.88	49.5	45.6	0.84	50.2

*Only considering samples in which it was identified; nt: not detected.

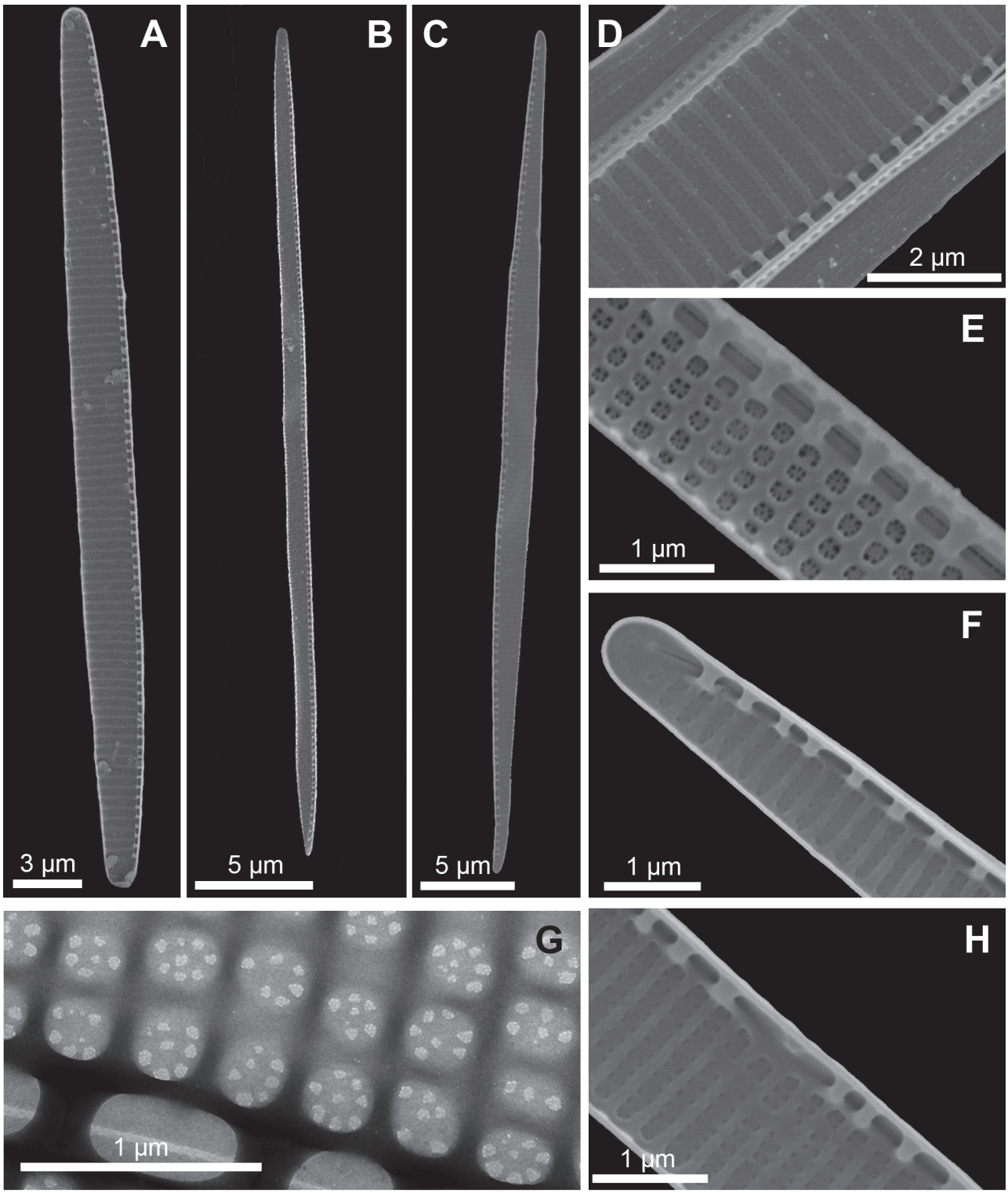

FIG 2. – SEM (scanning electron microscopy) and TEM (transmission electron microscopy) of *Pseudo-nitzschia* species in NW Mediterranean Sea. A and D, *P. brasiliana* (St. 13, 12/09/05); B, E and G, *P. calliantha* (St. 8, 14/03/05); C, F and H, *P. delicatissima* (culture ICMB-102, St. 22).

brasiliana appeared only once in the study period, so the sample was excluded for the statistical analysis. Prior to all analysis abiotic data were transformed v' = log10 (v + 1) and biotic data were transformed v' = (v+1). As no variable showed a normal distribution (Kolmogorov–Smirnov and Shapiro-Wilk) only non-parametric statistical analyses were applied. The following analyses were performed: one-way analy-

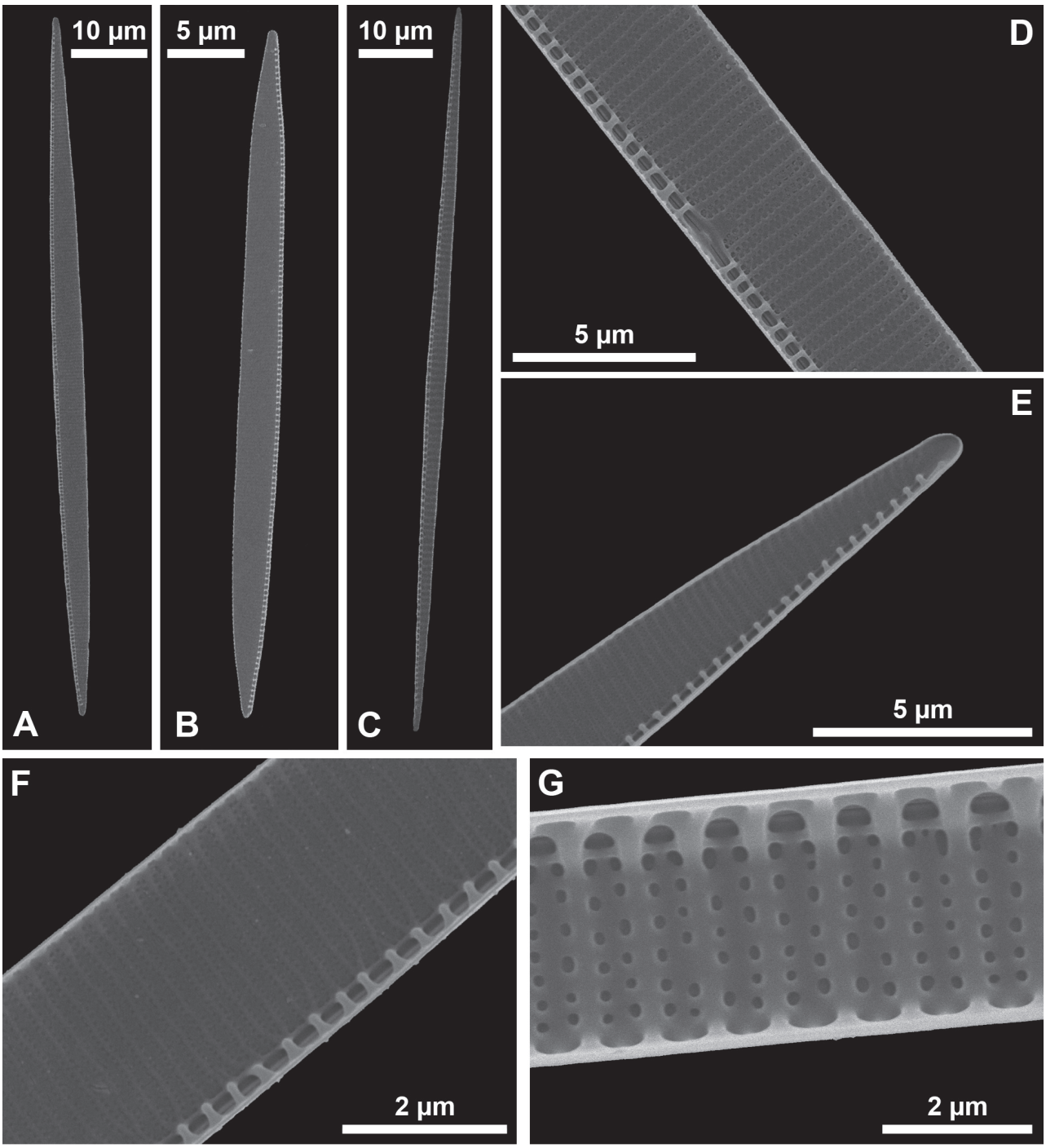

FIG. 3. – SEM (scanning electron microscopy) of *Pseudo-nitzschia* species in NW Mediterranean Sea. A and D, *P. fraudulenta* (St. 22, 4/07/05); B, E and F, *P. multistriata* (St. 15, 28/08/05); C and G, *P. pungens* (St. 11, 27/02/05).

sis of similarities (ANOSIM) with the corresponding pairwise tests if needed, cluster analysis using the group average method and working with Euclidean or Bray Curtis distances with the corresponding similarity profile (SIMPROF) to create objectively-defined groups, multi-dimensional scaling (MDS), BEST (biota and environment matching test), and similarity percentages (SIMPER) in order to determine crucial variables. All these analyses were performed using the Primer 6 statistical package (Clark and Warwick, 2001). Additionally, tables using Spearman rank order correlations among all variables, transformed v' = log (v+1), were created using the STATISTICA 6.1 statistical package (StatSoft, 2003).

RESULTS

Morphological characterisation by light and SEM microscopes and sequencing analyses

During the period 2005-2006 six different *Pseudo-nitzschia* species were identified in the field samples collected. These species include (in alphabetic order): *P. brasiliana* Lundholm, Hasle and G.A. Fryxell (2002), *P. calliantha* Lundholm, Moestrup and Hasle (2003), *P. delicatissima* (Cleve) Heiden (1928), *P. fraudulenta* (Cleve) Hasle (1993), *P. multistriata* (Takano) Takano (1995) and *P. pungens* (Grunow ex Cleve) Hasle (1993).

A summary of the main morphological characteristics for each species is presented in Table 1 and compared with values available in the literature. Images of each species are presented: *P. brasiliana* Lundholm, Hasle and Fryxell (Fig. 2-A and 2-D); *P. calliantha* Lundholm, Moestrup and Hasle (Fig. 2-B, 2-E and 2-G; *P. delicatissima* (Cleve) Heiden (Fig. 2-C, 2-F and 2-H); *P. fraudulenta* (Cleve) Hasle (Fig. 3-A and 3-D); *P. multistriata* (Takano) Takano (Fig. 3-B, 3-E and 3-F); and *P. pungens* (Grunow ex Cleve) Hasle (Fig. 3-C and 3-G).

Considering the cell length, two groups can be distinguished: smaller than 60 μm, which includes *P. brasiliana, P. delicatissima* and *P. multistriata*; and larger than 60 μm, which includes *P. calliantha, P. fraudulenta* and *P. pungens*. Moreover, the width of cells was considered to be an important morphological characteristic (Hasle *et al.*, 1996; Trainer and Suddleson, 2005). Based on this character *Pseudo-nitzschia* species were subdivided into two groups: the "*P. delicatissima* group" with narrow valves (width ≤3 μm) and the "*P. seriata* group*"* with wide valves (width ≥3 μm). The first group includes the three species *P. brasiliana*, *P. calliantha* and *P. delicatissima*. The second group includes *P. fraudulenta* and *P. pungens*. *P. multistriata* has a range of widths that overlaps the 3 μm threshold value (Table 1).

The analyses of the ITS-1, 5.8S and ITS-2 region of the rDNA from our strains were compared with representative strains of GenBank and maximum identity was estimated using a Blast analysis (Table 3).

Growth rates and cell yield

Of the five species, *P. delicatissima* showed the highest growth rates and cell yield recorded in culture (1.61 d^{-1}). The lowest growth rate was obtained from *P. fraudulenta* and the lowest cell yield from *P. pungens* (Table 3).

Pigment composition of cultures

Most pigments identified in the culture collection were common to all five *Pseudo-nitzschia* species considered, regardless of the strain, growth medium or physiological stage. Chl *a* comprised on average 39.3% of the total pigment content (tpc) identified in the cultures. Common accessory compounds were also estimated (Table 2). Trace levels of a Chl c_2-like *Pavlova gyrans*-type pigment (0.3% tpc) and MgDVP pigment (0.1% tpc) should probably be included in the range of common *Pseudo-nitzschia* pigments, but they were not always within detection levels. Chl c_1, the monovinyl analogue of Chl c_2, was only detected in cultures of *P. fraudulenta* (3.8% tpc). The Chl *a* degradation compound Chlorophyllide *a* was detected in all cultures, but was always exceptionally abundant in extracts of *P. delicatissima* and *P. pungens* (20.5–22.1 and 11.6–24.8% tpc in F2 and L1 media, respectively). A second Chlorophyllide *a* form (Chlide *a*-2; 1.7% tpc) with a retention time similar to Chl c_1 was detected in *P. delicatissima*

TABLE 3. – Culture strains of different species from NW Mediterranean. Strain code, GenBank number, growth rate and cell yield of each strain. Reference strains were used from the GenBank: source, accession number and maximum identity estimated using a Blast analysis.

Species	Strain code	Sample location	Genbank number	Growth rate (d^{-1})	Cell yield cell L^{-1}	Genbank source	Accession number	Max ident
P. brasiliana	ICMB175	Olímpic	1042372	-	-	Hasle and Lundholm, 2005	DQ062662	99%
P. calliantha	ICMB119	Rápita	DQ990359	0.67	1.35E+08	Lundholm *et al*., 2003	AY257856	99%
						Lundholm *et al*., 2003	AY257855	99%
P.delicatissima	ICMB102	Rápita	DQ990363	1.61	5.61E+08	Amato *et al.*, 2005	AY764136	99%
						Orsini *et al*., 2004	AY519309	98%
P. fraudulenta	ICMB104	Arenys	DQ990365	0.44	1.70E+08	Lundholm *et al*., 2003	AY257840	98%
						Fehling *et al*.,	AM118040	98%
P. multistriata	ICMB115	Tarragona	DQ990369	0.71	1.34E+08	Lundholm *et al*., 2003	AY257843	98%
P. pungens	ICMB108	Olímpic	DQ990370	0.47	6.03E+07	Hoang, A.L	DQ166533	97%
						Lundholm *et al.*, 2003	AY257846	97%

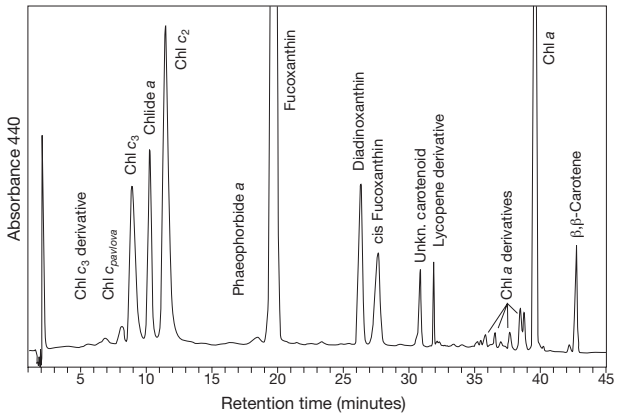

FIG. 4. – Representative absorbance chromatogram obtained for an extract of *Pseudo-nitzschia multistriata* culture, simultaneously containing most pigments and degradation products detected in the culture collection employed.

grown in L1 medium. Synthesis of Chl c_1 comprised the only significant difference in pigment compositions detected, and its possible coelution with Chlide *a*-2 underlined the need for on-line spectral data regarding identification purposes. In aging cultures several additional compounds came within detection levels (Fig. 4), including a Chl c_3 derivative, a phaeophorbide, an unknown carotenoid, a lycopene derivative and a range of Chl *a* derivatives (like allomers). These compounds were not detected during the exponential phase, and none of them was typically associated with a specific species. The carotenoid diatoxanthin is usually considered to be a common compound of the light-harvesting system in diatoms, but during the present study this carotenoid was not positively identified in any extract analysed.

Results obtained with the *P. delicatissima* pigment time-series (Fig. 5) revealed that degradation

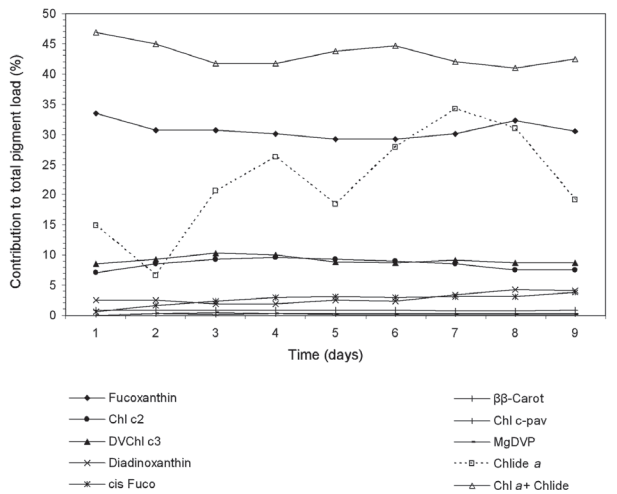

FIG. 5. – Temporal variations in the relative contributions of accessory compounds to the total pigment content observed during growth of *P. delicatissima* in L1 medium.

of Chl *a* occurred at all times, but with an increasing tendency with age of the culture. The sum of the accessory pigments identified in the *Pseudo-nitzschia* culture collection (not including the Chl *a* degradation products) comprised on average 50.2% tpc, with minor variations between species and culture medium (Table 2).The relative importance of Chl *a* + Chlide *a*, however, only showed minor variations between the 9 days of sampling (Fig. 5), with a mean value of 43.3% tpc, similar to the value obtained for the total culture collection analysed (Table 2). In all five species the accessory pigments were strongly dominated by fucoxanthin, and the relative importance of this specific carotenoid oscillated within a narrow range, from 26.7% tpc in *P. fraudulenta* to 30.7% tpc in *P. delicatissima*. The relative importance of fucoxanthin always yielded the lowest values in cultures characterised by the lowest degradation of Chl *a*. Chl c_2, the second most important pigment, showed relatively large variations between species, ranging from 7.9% tpc in *P. fraudulenta* to 16.5% tpc in *P. pungens*, but the values did not change greatly according to the growth media. Large variations were observed even for Chl c_3 (0.89-9.13% tpc), whereas the far less important pigments (<3% tpc) diadinoxanthin, β,β-carotene and Chl $c_{2\text{-}P.\ gyrans}$, showed only minor variations. During the *P. delicatissima* pigment time-series the relative importance of the accessory pigments oscillated within a very narrow window, showing no clear variations according to the culture age.

Environmental variables accompanying high cell densities of *Pseudo-nitzschia* species

During the sampling period, surface water temperature values fell within the range commonly reported for the coastal surface waters of the NW Mediterranean, ranging from 8.2 to 28.9ºC. The periods of cold waters (8 to 16ºC) were January to March 2005 and November 2005 to April 2006, and the warm period was April to October 2005. In general, during the warm period at the south station the temperature was higher than in the northern area (Fig. 6A). Salinity ranged from 18 to 38 in the south, especially at Station 22, where lower salinity was recorded due to the influence of the water discharges of the irrigation channel in this area (Fig. 6B).

The highest DIN concentration was found at Station 8 during the study period with values above 100 μM, whereas at the south station values of approxi-

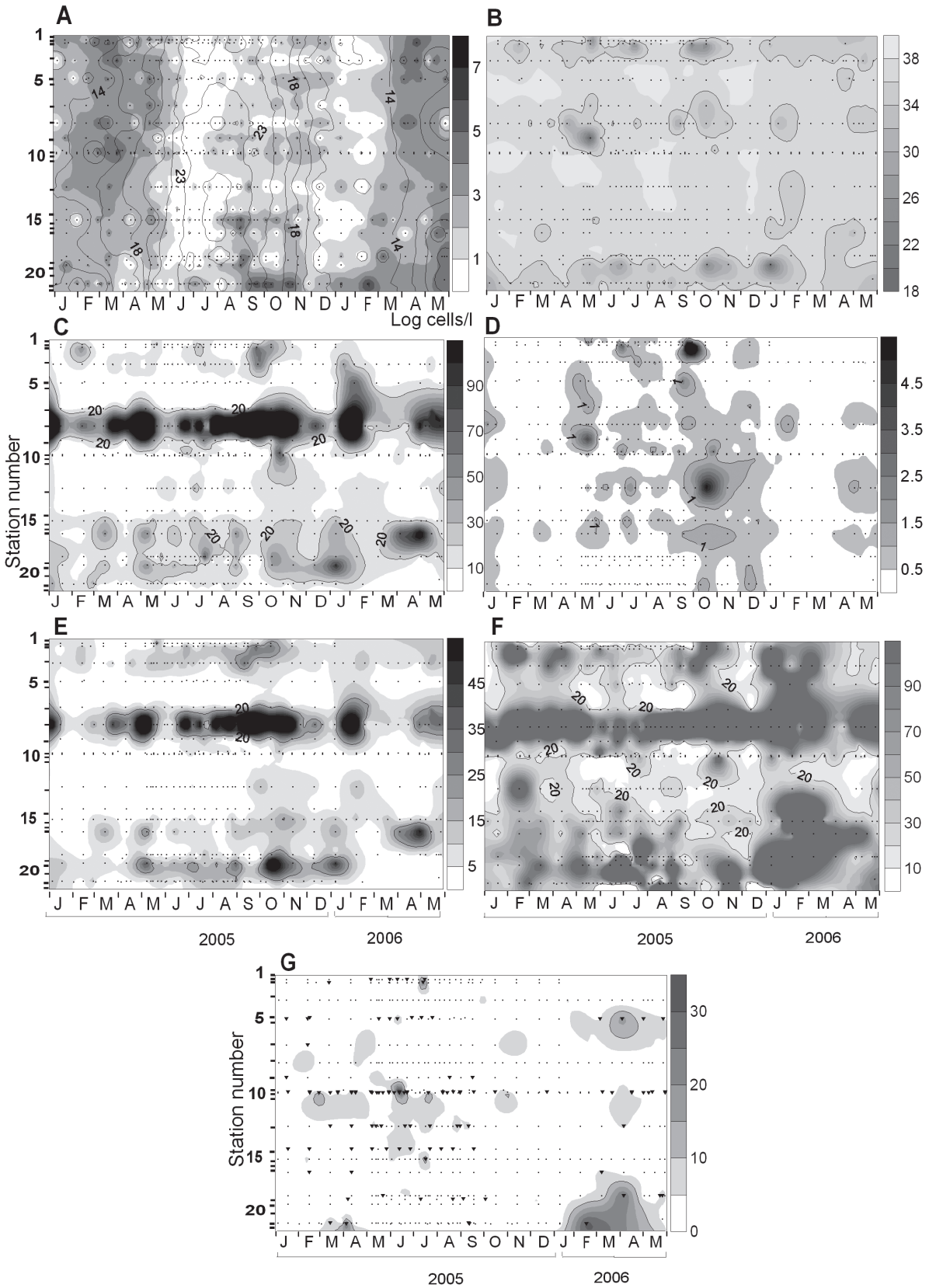

FIG. 6. – Temporal-spatial distribution of; A) *Pseudo-nitzschia* spp and temperature; B) salinity. The dots on the graphs are the sampling stations C) DIN μM; D) PO_4 μM; E) SiO_4 μM; F) ratio DIN/PO_4 (note that there is no limitation at any point); G) ration DIN/SiO_4 (▼ indicated places were there are limitations of SiO_4 according to Justic *et al*, 1995).

mately 20 μM were found (Fig. 6C). PO_4 showed a concentration above 0.5 μM sporadically, but in the period from September to November 2005 there was an increase at all the sample stations (Fig. 6D). Both SiO_4 and DIN showed a high concentration at Station 8 and at the south stations, especially Station 22, where the freshwater supply normally occurs (Fig. 6E). The DIN:P ratio was affected by the high concentration of DIN at Station 8, showing both similar pattern. Moreover, in the south high values were measured (Fig. 6F).The DIN/Si ratio showed high values in the south region from January to April 2006. Probable Si limitation was found mainly at Station 10, and from May to September at station 5, 13 and 14 principaly (Fig. 6G). Probable DIN limitation was found only twice and P limitation three times. During the study period, Chl *a* showed a wide range of values, with a maximum of 91.8 mg L^{-1} in July and a minimum of 0.05 mg L^{-1} in September (data not shown).

Pseudo-nitzschia spp. distribution patterns

Pseudo-nitzschia spp. were present along all the coast, with high cell abundance in winter-early spring (January to May) 2005, and in early 2006. Moreover, in 2005, high cell abundance was observed at the southern stations from late summer to autumn (September to November 2005). Periods of no cell presence were restricted to the warmest months (June and July 2005) on the entire coast and December to January 2006 at the northern stations (Fig. 6A). The distribution patterns of the most abundant species along the coast (the 23 stations sampled were arranged from N to S during the 17 months of study) is presented in Figure 7.

Pseudo-nitzschia brasiliana was only found at Station 13 in September 2005, with a maximum cell abundance of 2.58 x10^5 cells L^{-1} (Fig. 7A).

P. calliantha was mainly found from January to April at the northern stations in both 2005 and 2006. In the south it was present sporadically at Station 20 in January. At Station 22 a persistent bloom of this species was present from August to November 2005 and from January to March 2006 (Fig. 7B). Maximum cell abundance (>10^5 cells L^{-1}) was found at Stations 4, 16, 22 and 23.

P. delicatissima was present during the cold months at the northern stations in 2005. In the south it was present at Station 22, but with lower abundance than *P. calliantha.* In 2006 it was abundant in the south from December to May (Fig. 7C). Maximum cell abundance (>10^5 cell L^{-1}) was found at Stations 4, 16, 18, 19 and 23.

P. fraudulenta was present in 2005 in the north from February to April with relative low densities (>10^3 cell L^{-1}). In 2006, the species appeared sporadically in the same months in the south (Fig. 7D). In this study, the maximum cell abundance of the species was 2.61 x 10^5 cells L^{-1} and it was mainly mixed with *P. calliantha*.

P. multistriata was only found at Stations 15, 16 and 19 from August to September 2005 in bloom concentrations (cell abundance >10^5 cell L^{-1}) (Fig. 7E).

P. pungens was present in the north from February to May in 2005 and sporadically at other stations (Fig. 7F). Its cell abundance was no higher than 10^5 cells L^{-1}.

Statistical analysis

No significant differences between samples from the north and south stations were noted in the ANOSIM tests either with biotic and abiotic variables or between seasons. However, using only data of abundances of *Pseudo-nitzschia* species and performing a cluster with its corresponding SIMPROF test ($p<0.05$), three groups of samples were differentiated as is shown in the MDS test (Fig. 8A): one (a) characterised by a high abundance of *P. delicatissima,* which includes all samples from Stations 01, 03, 17 and 21; one (b) characterised by high abundances of *P. delicatissima* and *P. calliantha*, which includes all samples from Stations 07, 10, 12 and 18; and one (c) characterised by high abundances of *P. calliantha,* which includes all samples from Stations 02 and 05 (Table 4). Means and standard deviation (SD) of abundances of each group are shown in Table 5. In contrast, using only data of abiotic variables and performing both a cluster and a SIMPROF test ($p<0.05$), two groups of samples were identified, as shown in MDS (Fig. 8B): one (a) characterised by a low concentration of NO_3 and SiO_4, which includes all samples from Stations 01, 02, 03, 05, 07, 10, 11, 12, 15, 16, 18, 21 and 22; and one (b) characterised by a high concentration of NO_3 and SiO_4, which includes all samples from Station 17 (Table 4). Means and SD of abundances of each group are shown in Table 5. Abiotic and biotic groups did not match and this result was confirmed by BEST analysis, which demonstrated that no abiotic variable or combina-

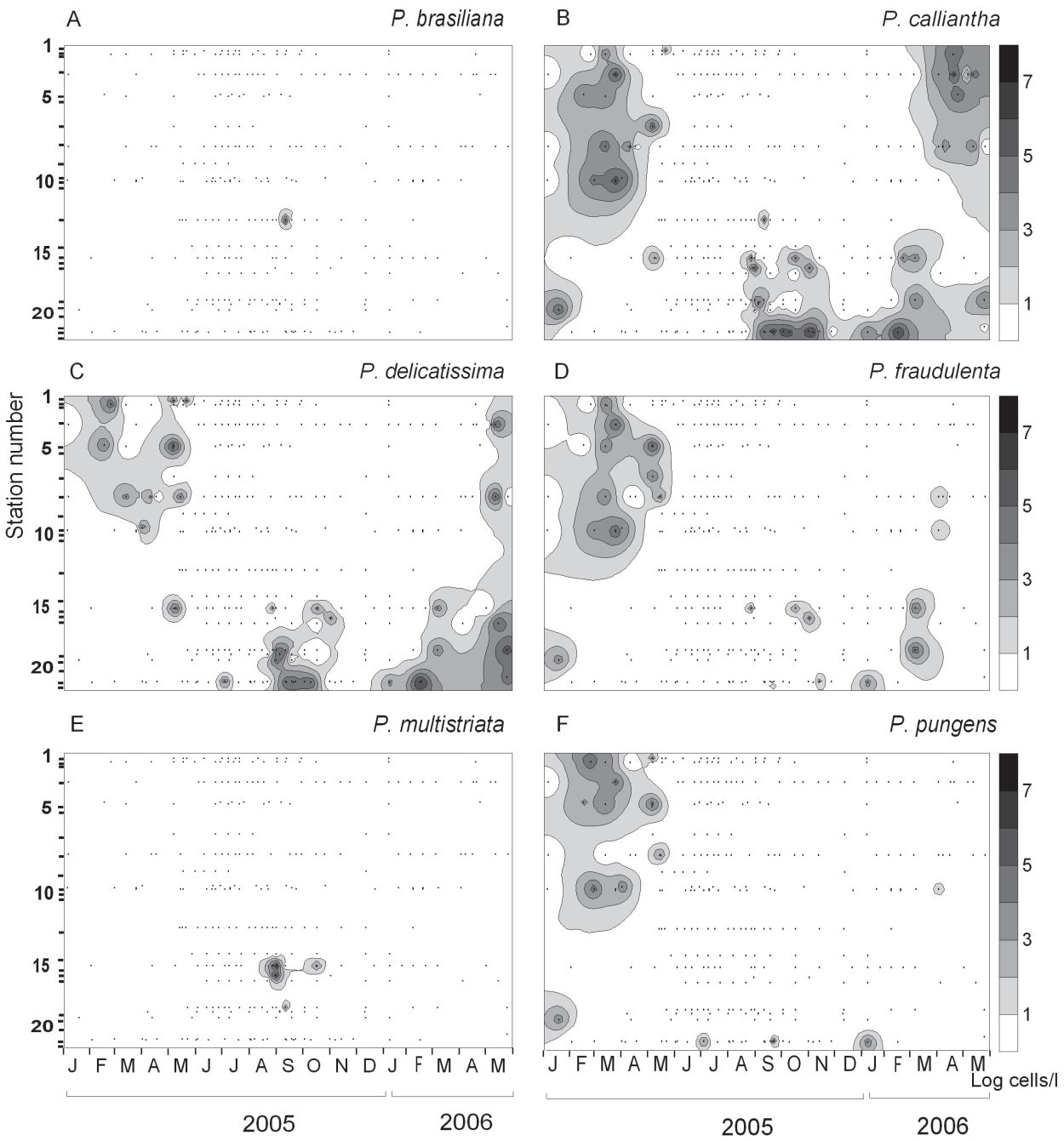

FIG. 7. – Temporal-spatial distribution of the most abundant *Pseudo-nitzschia* species.

tion of abiotic variables could accurately explain the distribution of *Pseudo-nitzschia* species in the area of study. This result was again corroborated by the correlation table (Table 6), which showed a low degree of association between abiotic variables and abundances of *Pseudo-nitzschia* spp. Nevertheless, several species of *Pseudo-nitzschia* showed significant correlations with some abiotic variables: *P. delicatissima* with temperature (positive correlation), salinity (negative correlation), freshwater content (positive correlation) and NO_2 (positive correlation); and *P. fraudulenta* with salinity (positive correlation), freshwater content (negative correlation) and NO_2 (negative correlation). Furthermore, *P. delicatissima* was negatively correlated with *P. fraudulenta*, and in relation to abiotic variables, temperature was negatively correlated with salinity and positively correlated with freshwater content, SiO_4 was positively correlated with NO_3, and PO_4 was positively correlated with NH_4.

TABLE 4. – Summary of critical variables and stations for each abiotic and biotic groups. Station only present in that group in bold.

Abiotic group	St. nr.	Station	Biotic group	St. nr.	Station
a	01	**Roses**	**a**	01	**Roses**
	02	**Empuriabrava**		03	**Muga**
↓ NO_3	03	**Muga**	↓ *P. calliantha*	08	Arenys
↓ SiO_4	04	Estartit	↑ *P. delicatissima*	15	Tarragona
	05	**Fosca**		17	**Cambrils**
	07	**Blanes**		19	L'Ametlla
	08	Arenys		21	**Eucaliptus**
	10	**Parc del Litoral**		22	St. Carles
	11	**Olímpic**			
	12	**Barcelona**	**b**	04	Estartit
	15	**Tarragona**		06	Palamós
	16	**Palamós**	↑ *P. calliantha*	07	**Blanes**
	18	**Alguer**	↑ *P. delicatissima*	08	Arenys
	19	Ametlla		10	**Parc del Litoral**
	20	Ampolla		11	Olímpic
	21	**Eucaliptus**		12	**Barcelona**
	22	**St. Carles**		18	**Alguer**
	23	Parc Garbi		19	Ametlla
				20	Ampolla
b	04	Estartit		22	St. Carles
	08	Arenys		23	Parc Garbí
↑ NO_3	17	**Cambrils**			
↑ SiO_4	19	Ametlla	**c**	02	**Empuriabrava**
	20	Ampolla		04	Estartit
	23	Parc Garbí	↑ *P. calliantha*	05	**Fosca**
			↓ *P. delicatissima*	06	Palamós
				08	Arenys
				11	Olímpic
				15	Tarragona
				19	Ametlla
				20	Ampolla
				23	Parc Garbí

TABLE 5. – *Pseudo-nitzschia* abundance (mean ± standard deviations) of each biotic group and abiotic variables (mean ± standard deviations) of each abiotic group.

Biotic group	N	*P. calliantha*	*P. delicatissima*	*P. fraudulenta*	*P. multistriata*	*P. pungens*
a	8	510±947	30898±17394	474±1341	233±660	8171±17720
b	20	251431±267987	325707±599481	27238±66053	0±0	7610±13804
c	16	13020±11446	1603±1841	5807±9286	1509±5428	3604±7729

Abiotic group	N	Temp	Sal	FWC	Chl-*a*	NO_3	NO_2	NH_4	PO_4	SiO_4
a	36	17.6±5.2	36.8±1.1	42.4±29.6	2.5±1.6	8.6±10.6	0.3±0.3	3.0±2.6	0.4±0.4	5.1±3.7
b	8	19.6±5.3	35.1±1.5	87.2±38.9	5.8±6.3	71.2±63.9	0.4±0.3	2.6±1.3	0.4±0.2	27.9±18.4

TABLE 6. – Spearman correlations between abiotic variables and *Pseudo-nitzschia* spp. abundances. Bold correlations are significant at $p<0.01$. *P.d., P. delicatissima; P.f., P. fraudulenta; P.m., P. multistriata; P.p., P. pungens.*

	P.d.	*P.f.*	*P.m.*	*P.p.*	Temp	Sal	FWC	Chl-*a*	NO_3	NO_2	NH_4	PO_4	SiO_4
P. calliantha	-0.03	0.05	-0.07	-0.14	0.09	0.06	-0.06	0.14	-0.08	0.09	-0.29	-0.06	-0.19
P. delicatissima		**-0.56**	-0.04	-0.18	**0.53**	**-0.42**	**0.42**	-0.04	0.12	**0.41**	-0.01	0.15	0.12
P. fraudulenta			0.04	0.31	-0.38	**0.47**	**-0.47**	-0.04	-0.06	**-0.45**	0.10	0.01	-0.13
P. multistriata				-0.19	0.28	0.29	-0.29	-0.03	0.12	0.23	0.20	0.31	0.19
P. pungens					-0.33	0.20	-0.20	0.05	-0.27	-0.25	-0.06	0.01	-0.13
Temp						**-0.38**	**0.38**	0.09	0.08	0.25	-0.06	0.28	0.29
Sal							-1.00	-0.17	-0.23	-0.35	-0.08	0.08	-0.31
FWC								0.17	0.23	0.35	0.08	-0.08	0.31
Chl-*a*									0.16	0.21	0.23	-0.03	0.04
NO_3										0.36	0.11	0.13	**0.68**
NO_2											-0.09	-0.11	0.32
NH_4												**0.41**	0.12
PO_4													0.32

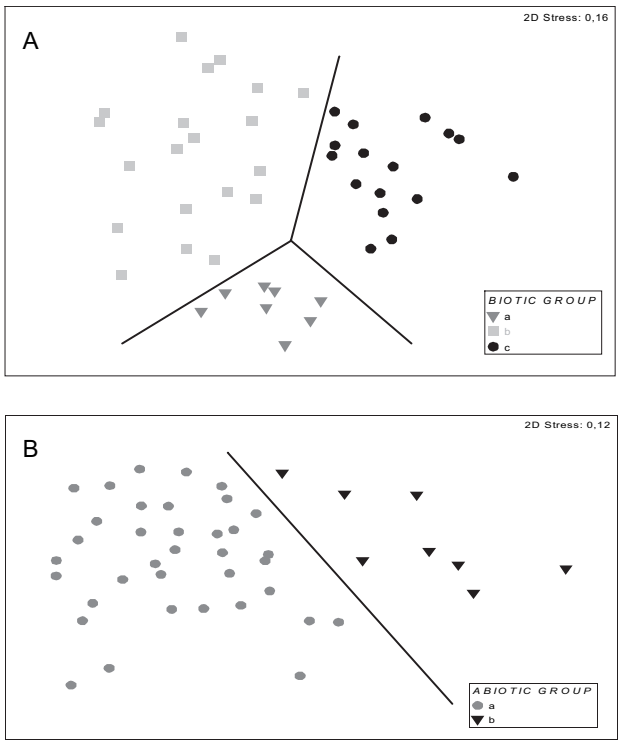

FIG. 8. – Multi-Dimensional Scaling (MDS) of A) *Pseudo-nitzschia* abundances, and B) abiotic data. Statistically different groups are shown.

DISCUSSION

Improvements in taxonomic identification and differentiation of *Pseudo-nitzschia* under the optical microscope have been a continuous challenge for many studies carried out during the last decade. Detailed examination of such organisms requires electron microscopic examination of ultrastructure and analysis of genetic variations. We studied strains of different *Pseudo-nitzschia* species isolated from the Spanish coast (NW Mediterranean) from 2005 to 2006. The strains were characterised using morphological, molecular, and physiological methodologies in order to understand the species present in the field. Moreover, field samples were observed with both optical and electron microscopy to identify the ecological variables under which these species proliferate in high densities.

The morphometric measurements of the *Pseudo-nitzschia* species found on the Catalan coast fit with those reported in previous studies on *P. brasiliana, P. multistriata* and *P. pungens*. However, some data differ for *P. calliantha*, *P. delicatissima* and *P. fraudulenta.* We reported length values of up to 123.1, 78 and 132.5 μm, while other works reported maximum lengths of 98, 71 and 98 μm, respectively. The maximum length values were recorded during periods of high cell abundance. It has been hypothesised that during a bloom, sexual reproduction occurs in order to restore the size of the species (Drebes, 1977; Bates and Davidovich, 2002; Mann, 2002). This suggests that in high cell abundance of *Pseudo-nitzschia* sp cells are larger because sexual reproduction has occurred.

Another important morphological aspect is the width of the cells that are used in defining species. Following the literature, the "*P. seriata* group" included wide species (width ≥3 μm) (Hasle and Syvertsen, 1997). From this study, *P. multistriata* had a width range that overlapped the 3 μm threshold value. Since other morphological characters differentiate *P. multistriata*, we agree with (Orsini *et al*., 2002), who suggest that the width of the cell is an ambiguous character for grouping species.

The ITS-1, 5.8S, and ITS-2 sequence data, via a BLAST search, were used to strengthen the identification already performed by light and electron microscopy analyses of morphologic features of the species cultured. In each case the first species listed in the BLAST results was that identified by morphological analysis. It is worth noting that many such BLAST searches provided results which showed sequences of near complete identity but sometimes with two to four different species names interspersed. This indicates some degree of disagreement between morphology and sequence identity (which may be either due to incorrect morphological identification or sequences which were submitted without any careful examination by electron microscopy) or the possibility of pseudo-species. For this reason we point to the need for the morphometric analysis accompanying the sequencing in order to avoid the erroneous identification of species in GenBank.

In HPLC results obtained for natural field samples or unialgal cultures we usually observe that concentrations of Chl *a* comprise about 50% of the total pigment load (tpc) detected. However, in cultured isolates of *Pseudo-nitzschia* analysed in the present study the relative contribution of Chl *a* was far less. This was attributed to a marked degradation of Chl *a* to Chlide *a*. The sum of Chl a and its degradation product Chlide *a* accounted, on average, for 49.9% tpc. The highest degradation was observed in extracts of *P. pungens* and *P. delicatissima* grown in L1 medium, regardless of the culture age. Since HPLC pigment samples were extracted in acetone, which (unlike methanol) hinders activity of chloro-

phyllase, degradation of Chl *a* must have occurred in the cell itself. Such degradations probably indicate unsuitable culture conditions and might not occur in natural environments.

The carotenoids diadinoxanthin (detected in all samples) and diatoxanthin (not observed in any sample) form part of a 2-component xanthophyll cycle in all chromophyte algal groups, including diatoms. The relative proportions of diadinoxanthin and diatoxanthin can therefore not be employed for chemotaxonomic discriminations between *Pseudo-nitzschia* species. Based on the pigment time-series on *P. delicatissima*, relative contributions of accessory pigments do not vary with culture age, nor do they usually vary significantly as a function of the culture media employed.

None of the environmental variables studied seemed to play an important role in either the spatial or the temporal distribution of *Pseudo-nitzschia* spp. (BEST and MDS analysis). However, even if there is no statistical relationship between abiotic and biotic variables, samples could be objectively grouped depending on: a) the *P. delicatissima* and *P. calliantha* abundances, and b) the NO_3 and SiO_4 concentrations. The distinction between the groups based only on these two species of *Pseudo-nitzschia* spp is obvious, as both species are the most abundant in the Catalan coast. The differences between groups in the NO_3 and SiO_4 concentrations responds to the fact that it is possible to distinguish stations with high freshwater influence—and consequently a high concentration of both inorganic nutrients—from stations with poor inflows of freshwaters and low nutrient concentrations. In addition, all correlations obtained in this study between abiotic variables and abundances of *Pseudo-nitzschia* spp. were low, confirming the lack of relationship between biotic and abiotic variables, which was also demonstrated by BEST analysis. These results are not in agreement with those described by Penna *et al*. (2006), which showed a negative relation between *Pseudo-nitzschia* spp. and phosphate.

Although none of the specific abiotic variables measured explain either spatial or temporal distributions of the species in our coastal area, some general trends can be described and thus compared with previous knowledge of each species. *P. brasiliana* was found in September with a water temperature of 24.9ºC. This agrees with the fact that the species has been observed mainly in warmer waters for e.g. Brazil, the Gulf of Panama, the Gulf of Mexico, the Gulf of California, Vietnam, Indonesia, Thailand and South Korea. The species has recently been described in Mediterranean waters (Quijano-Scheggia *et al.*, 2005).

P. calliantha has been found in Denmark, Norway, the North Atlantic, Scotland, Kiel Bay, Ría de Vigo in Spain, the Black Sea, the Adriatic Sea, northern Canada, the Gulf of Mexico, the Bermudas, Chile, Vietnam and Australia: geographically widespread observations which indicate a fairly cosmopolitan distribution (Lundholm *et al.*, 2003). Recently, a toxic clone was found in Tunisia (Inès and Asma, 2006). In the bay of Banyuls-Sur-Mer, NW Mediterranean Sea, *P. calliantha* was found associated with warmer temperatures and relatively nutrient-rich waters (Quiroga, 2006). On the other hand, Caroppo *et al*. (2005) found that *P. calliantha* was negatively correlated with water temperature and positively correlated with nutrient availability. No significant relationship between temperature or dissolved inorganic nutrients and the abundance of *P. calliantha* was found during the study period.

P. delicatissima is common, occasionally as the predominant diatom species, in the north Atlantic (Hasle and Syvertsen, 1997). In the Gulf of Naples it was found to produce regular blooms in late spring, with less recurrent peaks in late summer (Orsini *et al.,* 2004). Caroppo *et al.* (2005) found that the dynamics of these species appears to be significantly correlated with the environmental features. In the present study we found only a correlation with NO_2 and *P. delicatissima* show high cell abundance under very different abiotic conditions. Moreover, this species showed the highest growth rate and cell yield in lab conditions. Both facts suggest that the species could be described as one of the *r*-selected phytoplankton species, which have optimised their fitness for conditions with ample resources and a high growth rate. In fact, when phytoplankton were ranked after their growth rates, one of the highest values was recorded for diatoms (Stolte and Garcés, 2006).

P. fraudulenta is a cosmopolitan species (Hasle and Syvertsen, 1997) in the Mediterranean Sea that is more abundant during spring but never attains a density higher than 10^5 cells L^{-1} (Zingone *et al.*, 2006). On the Catalan coast, this species appears mixed with other *Pseudo-nitzschia* species such us *P. pungens*. Moreover, it showed the lowest growth rate in the lab, suggesting that it took a long time to reach high cell densities.

P. multistriata appears in spring in the Mediterranean Sea (Zingone *et al*., 2006). However, in

this study the species showed high cell abundance in summer as reported from inlets of southern Japan, where it forms blooms in summer (Hasle and Syvertsen, 1997). The species has been reported as toxic (Orsini *et al.,* 2002) but our cultures showed no toxicity (Franco, pers. comm.).

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