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Bloom dynamics of the genus *Pseudo-nitzschia* (Bacillariophyceae) in two coastal bays (NW Mediterranean Sea)

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SUMMARY: The spatial and temporal variations in the composition of *Pseudo-nitzschia* during bloom events from August 2005 to February 2006 were characterised in two bays of the NW Mediterranean Sea (Alfacs and Fangar Bay) by means of scanning electron microscopy (SEM). The study provides detailed records of the *Pseudo-nitzschia* community at the species level and describes its relationship with both the surrounding environmental conditions and biotic factors such as the accompanying phytoplankton community. The size distributions of several species of *Pseudo-nitzschia* were monitored during the bloom events. These measurements may serve as indicators of the physiological status of the cells. The species observed in the two bays were *Pseudo-nitzschia calliantha, P. delicatissima, P. fraudulenta, P. multistriata*, and *P. pungens.* In Alfacs Bay, a mixed species bloom of *P. calliantha* and *P. delicatissima* began in late August 2005 and lasted 11 weeks. In Fangar Bay, the *Pseudo-nitzschia* bloom was limited to the period from early August to late September 2005 and comprised *P. calliantha* and *P. delicatissima*. Commonly, the proliferation of *Pseudo-nitzschia* was mono-specific or was accompanied by other diatoms. Two objectively defined groups were identified by the statistical analysis in Alfacs bay; the first was made up only of winter samples and the second of summer and autumn samples. The first group was defined by a high concentration of NO_3^- and low concentrations of NH_4^+, conditions associated with a high abundance of *P. delicatissima* and a low abundance of *P. calliantha*. The second group expressed the opposite characteristics. A succession of different blooming species of *Pseudo-nitzschia* lasting months in Alfacs Bay is described.

Keywords: *Pseudo-nitzschia*, bloom dynamics, phytoplankton community.

RESUMEN: Dinámica de las proliferaciones del género *Pseudo-nitzschia* en dos bahías costeras (NO del Mar Mediterráneo). – Las variaciones en la composición de una proliferación de *Pseudo-nitzschia* se caracterizaron de agosto 2005 a febrero 2006 en dos bahías del Mediterráneo noroeste (bahía de Alfacs y Fangar) por medio de microscopia electrónica de barrido (SEM). Este estudio provee descripciones detalladas de la comunidad de *Pseudo-nitzschia* hasta nivel de especie y describe las relaciones con las condiciones ambientales y factores bióticos como por ejemplo la comunidad fitoplanctónica acompañante. La distribución de tamaño de algunas especies de *Pseudo-nitzschia* fue estudiada durante el evento de proliferación y se propone como posible indicador del estado fisiológico de las células. Las especies observadas en las dos bahías fueron *Pseudo-nitzschia calliantha, P. delicatissima, P. fraudulenta, P. multistriata* y *P. pungens.* En la bahía de Alfacs una proliferación mixta de *P. calliantha* y *P. delicatissima* comenzó en agosto 2005 y duró 11 semanas. En la bahía de Fangar la proliferación de *Pseudo-nitzschia* estuvo limitada al periodo entre principios de agosto y fines de septiembre. Normalmente las proliferaciones de *Pseudo-nitzschia* fueron monoespecíficas o acompañadas de otras diatomeas. Mediante el análisis estadístico de los datos en la bahía de Alfacs, se definieron dos grupos de muestras: las de invierno y las de verano-otoño. El primer grupo se definió por la alta concentración NO_3^- y baja concentración de NH_4^+, condiciones que acompañaron una gran abundancia de *P. delicatissima* y baja abundancia de *P. calliantha*. El segundo grupo presentó las características opuestas. Se describe la sucesión de especies de *Pseudo-nitzschia* que proliferaron durante meses en la bahía de Alfacs.

Palabras clave: *Pseudo-nitzschia*, dinámica de proliferaciones, comunidad fitoplanctónica.

INTRODUCTION

In recent decades, there have been numerous reports of toxic phytoplankton blooms occurring worldwide. Increased research efforts have certainly contributed to the detection of new, harmful species in some areas. In addition, the growth of shellfish farming as an industry has led to increased monitoring of harmful phytoplankton and marine toxins.

Several species of the marine diatom genus *Pseudo-nitzschia* can be problematic due to their toxin-producing abilities. At least eleven such species are known to release domoic acid (DA), which causes amnesic shellfish poisoning (ASP) (Bates *et al.*, 1998; Bates, 2000; Moestrup *et al.*, 2004).

Alfacs and Fangar Bays (Ebro River Delta, NW Mediterranean Sea) are the most important aquaculture sites (fish and shellfish farming) along the NE coast of Spain (Catalonia). In these bays, a monitoring programme aimed at detecting toxic phytoplankton species and related toxins present in shellfish harvesting areas has been in place since 1989. This effort is part of national and international programmes whose goals are to prevent food intoxication and to improve the management of shellfish-producing areas. Accordingly, the potential presence of the paralytic shellfish poisoning (PSP), diarrheic shellfish poisoning (DSP), and ASP toxins has been monitored as mandated by current legislation. Although no episodes of ASP on the Catalan coast have been reported, monitoring for ASP toxicity remains an important task due to the frequent occurrence of *Pseudo-nitzschia* spp. blooms in this area. Additionally, ASP events along the Mediterranean coast of France (Ifremer, 2003) and southern Mediterranean were recently reported (Fernández *et al.*, 2004).

In routine monitoring programmes, diatoms of the genus *Pseudo-nitzschia* are not identified further because optical microscopy is not able to reliably distinguish these organisms at the species level. By contrast, electron microscopy and molecular methods are valuable tools for the accurate identification of several harmful algae (Hasle, 1995; Orsini *et al.*, 2002; Priisholm *et al.*, 2002; Lundholm *et al.*, 2003; Lundholm *et al.*, 2006); indeed, specific probes have already been developed for the identification of *Pseudo-nitzschia* species (Miller and Scholin, 1998; Miller and Scholin, 2000; Cho *et al.*, 2002). However, preliminary studies of several local *Pseudo-nitzschia* species from Alfacs Bay did not result in the development of species-specific probes (Elandaloussi *et al.*, 2006) due to the unexpected presence of cryptic diversity within these ''species,'' which had been assumed to be cosmopolitan and ubiquitous (Parsons *et al.*, 1999; Hasle, 2002; Orsini *et al.*, 2004).

The occurrence of blooms comprising *Pseudo-nitzschia* species have been related to high nutrient concentrations in several areas (Parsons *et al.*, 2002; Trainer and Hickey, 2003; Spatharis *et al.*, 2007). Both natural eutrophication in upwelling regions anthropogenic inputs of nutrients are known to favour the growth of these species. It is therefore important to identify more precisely the conditions that pro-

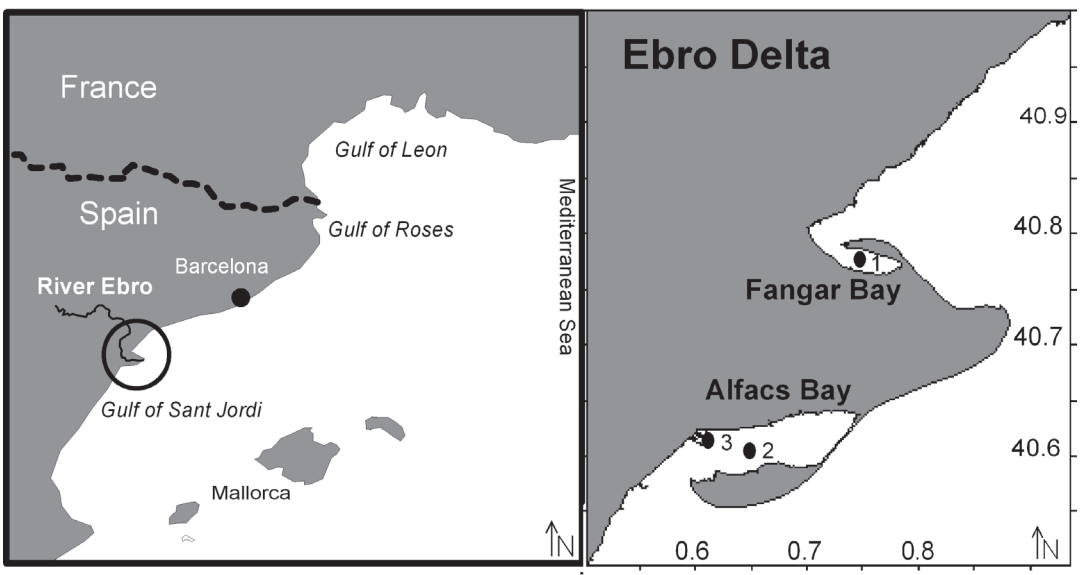

FIG. 1. – Study area showing the location of sampling stations in Alfacs and Fangar Bays, NW Mediterranean Sea.

mote the development of *Pseudo-nitzschia* blooms in order to minimise the possible toxic effects on the local shellfish industry.

Diatoms reproduce asexually, cycling through a miniaturisation process in which there is a progressive decrease in cell size through successive divisions. The organisms recover their original size during sexual reproduction, which consists of meiosis, gamete fusion, and the formation of auxospores, during which the cells recover their maximum length (Round *et al.*, 1990; Chepurnov *et al.*, 2004). Inside the densely populated bloom, the probability that sexually compatible organisms of the same species will meet is increased; therefore the probability of detecting cells of increased size is likewise higher. Consequently, the proportion of larger organisms could be greater in a bloom than in non-blooming natural populations. In order to analyse the variations in cell size that occur during different phases of a bloom, and thus determine the life-cycle status of a diatom species, the size-frequency distribution of diatom cells can be measured over short time intervals within the growing season.

Nonetheless, field data that allow the interpretation of *Pseudo-nitzschia* bloom dynamics at the species level are lacking. Therefore, the aims of the present study were: (i) to determine the diversity among *Pseudo-nitzschia* species in two bays of the Ebro Delta, (ii) to assess the influence of abiotic and biotic factors on this diversity, (iii) to explore whether cell size serves as an indicator of life-cycle status in *Pseudo-nitzschia* sp., and (iv) to describe the phytoplankton species accompanying blooms of *Pseudo-nitzschia*.

MATERIALS AND METHODS

Study area

Alfacs and Fangar Bays are two semi-enclosed coastal areas within the Ebro River Delta (NW Mediterranean Sea 40º40'N, 0º40'E) (Fig. 1). The two bays are separated from the Mediterranean Sea by a wide sandy barrier.

Alfacs Bay is a semi-confined mass of sea water, with a surface area of 49 km^2 and an average depth of 3.13 m. The mouth of the bay is 2 km wide. The edge of the bay is surrounded by a shallow shelf (18 km^2) that falls in a gentle slope from 0 to 1.5 m, with an average depth of 0.64 m. Further out, the shelf slopes more steeply, descending to a muddy central basin, which has a maximum depth of 6.5 m and an area of 31 km^2. The bay receives freshwater inputs of about 275×10^6 m^3 $year^{-1}$ from its northern edge between April and October but much less during the rest of the year, a seasonal pattern caused by the water demands imposed by rice cultivation in the region. The input of water transports considerable quantities of inorganic nutrients and organic matter into the bay (Camp and Delgado, 1987; Prat *et al.*, 1988), which favours the development of dense phytoplankton populations (Delgado, 1987). Alfacs Bay is a stratified type B estuary, according to the criteria of Pritchard (1955). The salinity of the water in the bay is influenced by precipitation as well as by freshwater discharge from delta irrigation channels. The bay is characterised by a salinity-dominated stratification, with a superficial layer (0 to 2-3 m deep) of low salinity (30-35) and an outward movement, and a deep salty layer (salinity: 36-38) with an inward movement. Complete mixing of the two layers is rare and occurs only in response to very strong wind events. Water renovation of the bay varies and is related to the freshwater inputs; the mean residence time of water in the bay is approximately 10 days (Camp and Delgado, 1987). Thus, volume exchange in the bay, as determined by a box model describing estuarine circulation and assuming steady state, is 3×10^6 m^3 day^{-1} in the closed-channel period and 8×10^6 m^3 day^{-1} in the open-channel period (Camp, 1994). Weather conditions in the Mediterranean are such that, almost every year, the water temperature in Alfacs Bay in early summer is about 30–32ºC. This accounts for the oxygen depletion that occurs in the deepest waters of the innermost areas of this shallow bay.

Fangar Bay is smaller than Alfacs Bay; it is located in the northern part of the Ebro Delta, is approximately 12 km^2 wide and is very shallow, with a maximum depth of approximately 4 m. The mouth of the bay is oriented in a northwest direction, such that Fangar Bay is exposed to strong northwesterly winds originating from the Ebro Valley. The edge of the bay is delimited by a shallow shelf, which slopes gently from 0 to 1.5 m and then more pronouncedly until the central basin, with a maximum depth of 4 m, is reached. The bay receives freshwater inputs of 185×10^6 m^3 $year^{-1}$ from April to October and 55×10^6 m^3 $year^{-1}$ during the rest of the year. Like Alfacs Bay, Fangar Bay is a stratified type B estuary, according to the criteria of Pritchard (1955). The mean

residence time of water in the bay is approximately 1-2 days (Camp and Delgado, 1987).

Sampling

Water samples were collected weekly at a fixed station situated approximately at the centre of each bay (Station 1 for Fangar and Station 2 for Alfacs). Water samples were obtained from the surface and from the bottom of the water column. For Alfacs Bay, sampling was conducted from September 2005 to February 2006; for Fangar Bay, sampling was conducted during the *Pseudo-nitzschia* bloom, which lasted from August to October 2005. Alfacs Bay was chosen as the priority study site, while sampling at Fangar Bay depended on the presence of *Pseudo-nitzschia* blooms. Additionally, for Alfacs Bay, monthly phytoplankton samples were taken at Station 3 between January 2005 and February 2006, located within Sant Carles harbour, inside the bay, in order to quantitatively describe the composition of the phytoplankton community. During the *Pseudo-nitzschia* bloom period, Station 2 was also sampled for the same objective. A WTW probe was used to measure hydrographic parameters (temperature and salinity) directly at the surface (0.5 m) and at the bottom (6 m). For taxonomic identification of the total phytoplankton population, subsamples (150 ml) were directly fixed with 1% formalin. Additional subsamples (50 ml) were prepared for nutrient analysis using an autoanalyser, as described in Grasshoff *et al*., 1983. Potential nutrient limitations were estimated following the criteria of Justic (1995): P limitation (P<0.1 μM; dissolved inorganic nitrogen (DIN): P>22; Si:P>22); N limitation (DIN<1 μM; DIN: P<10; Si: DIN>1); and Si limitation (Si<2 μM; Si:P<10; Si: DIN<1).

Subsamples (150 ml) for the quantification of total chlorophyll *a* (Chl *a*) were transported to the laboratory at 4ºC in the dark. In vivo fluorescence was measured with a Turner Designs AU fluorometer (Holm-Hansen *et al.*, 1965).

Light microscopy

Water samples aliquots (50 ml) for the phytoplankton identification were allowed to settle in counting chambers for 24 h, after which algae were enumerated according to Throndsen (1995), using a Leica DM-IL inverted microscope at a 200-400× magnification, depending on species abundance. In each sample, the entire phytoplankton community was quantified. The phytoplankton community was identified to the species or genus level according to Tomas (1997) and Moestrup *et al.* (2004). When identification was not possible, the different taxa were grouped as centric diatoms, pennate diatoms, or small and large dinoflagellates. Moreover, ciliates, tintinnids, and rotifers were grouped as microzooplankton.

Electron microscopy

Samples in which the concentrations of *Pseudo-nitzschia* spp. cells were $>10^4$ cells L^{-1} were examined by scanning electron microscopy (SEM) in order to identify the diatoms to the species level. Organic material was removed from the samples as described in Lundholm *et al*. (2002). The remaining material was mounted on a polycarbonate filter that was attached to stubs with colloidal silver and then sputter-coated with gold-palladium. The stubs were screened using a Hitachi S-3500N microscope operated at 5 kV. Lower abundance of cells could not be studied in this way due to the limitations posed by the fixation methods.

Morphometric characteristics

In order to follow the size distribution during the bloom, cells identified by SEM as *Pseudo-nitzschia* were examined and the following information was obtained: width and length of the valve, density of striae, fibulae and poroids, structure of the girdle bands, and perforation patterns of the poroid hymen. In each sample, 30–50 cells were identified; the exact number depended on the species abundance and the sample composition. The results were expressed as a percentage, extrapolated to the whole sample. In each species, four classes of cell sizes were defined; for example, the size classes of *P. delicatissima* cells were <35, 35–45, 45–55, and >55 μm. Since *P. calliantha* was the most abundant species over an 8-month period, it was used for further statistical analyses. Correlations between cell size and *P. calliantha* abundance were tested using the STATISTICA 6.1 statistical package. For the other species, the abundance period was too short to allow statistical testing of the data.

Temporal and spatial statistical analyses

Since data on *Pseudo-nitzschia* blooms in Fangar Bay were limited to a 2-month period, only data

from the diatoms sampled in Alfacs Bay were used for statistical analyses.

A matrix (n=28 for each variable) was created to identify potentially important variables controlling the temporal and spatial dynamics of *Pseudo-nitzschia* spp. Each sample was categorised with respect to four factors: season using whole-month criteria (summer: 8 August 2005 to 26 September 2005), autumn (3 October 2005 to 27 December 2005) or winter (03 January 2006 to 20 February 2006); surface-depth; time (with 1 corresponding to the first sample, collected on 8 August 2005, and 197 the latest, corresponding to the sample collected on 20 February 2006); and cluster group, a factor based on CLUSTER analysis and Euclidean distances with the corresponding similarity profile (SIMPROF) and divided into three categories (*a*, *b*, and *c*). Afterwards, all raw data or columns in which the values were equal to zero were deleted and two sub-matrices were created: one consisting of abiotic variables (temperature, salinity, chlorophyll *a*, and DIN) and the other of biotic variables (*Pseudo-nitzschia* species abundance). Prior to all analyses, the abiotic data were transformed according to $v' = \log10 (v + 1)$ and the biotic data according to $v' = (v+1)$. Since not all variables were distributed normally (as determined by Kolmogorov–Smirnov and Shapiro-Wilk tests), only non-parametric statistical analyses were applied. The following analyses were performed: i) one-way analysis of similarities (ANOSIM), with corresponding pairwise tests if needed; ii) cluster analysis using the group average method and SIMPROF to create objectively defined groups; and iii) multi-dimensional scaling (MDS), in which the Euclidean distance and

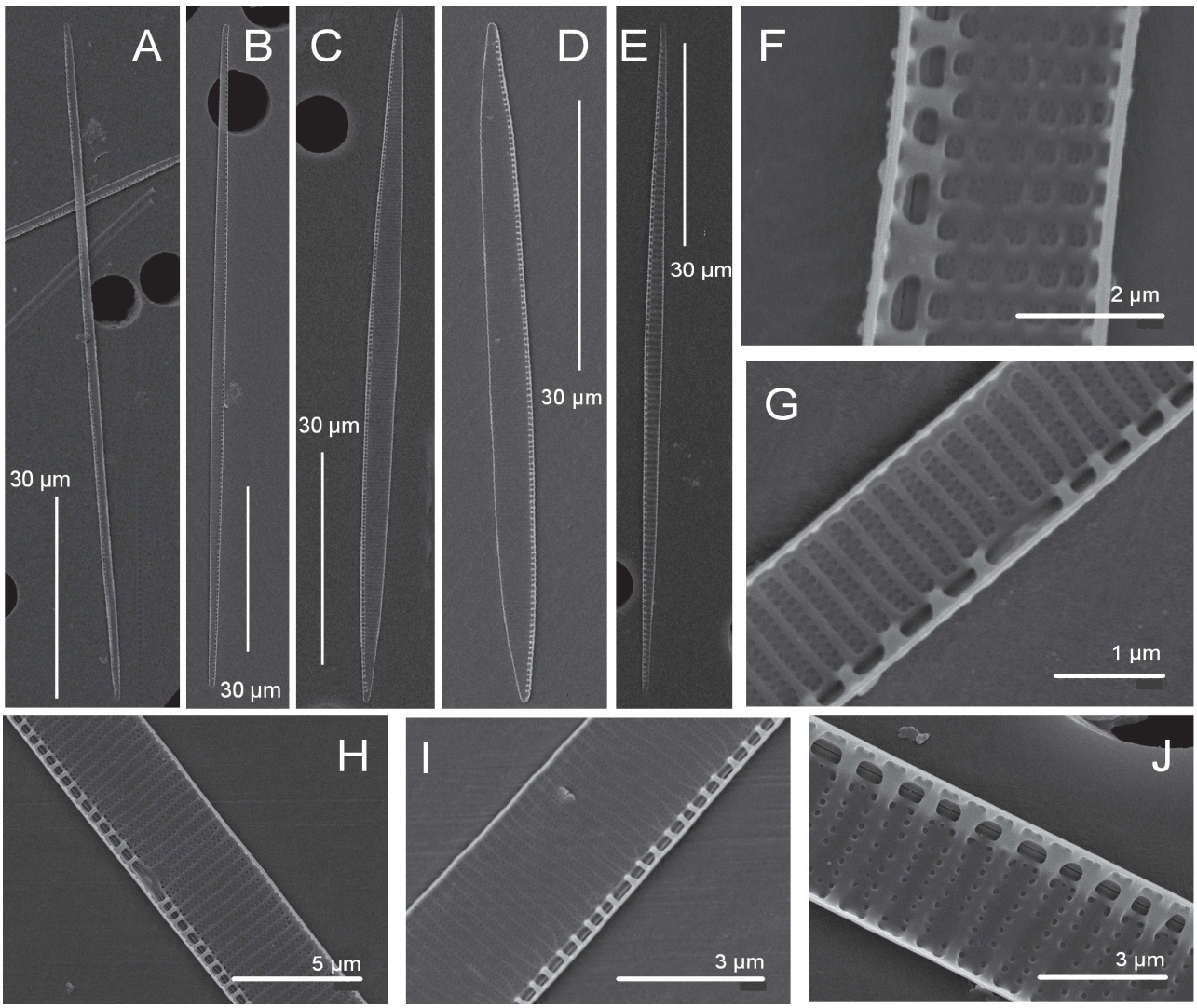

FIG. 2. – Scanning electron microscopy images of *Pseudo-nitzschia* species collected from the Ebro Delta. A, F, *P. calliantha*, B, G, *P. delicatissima*, C, H, *P. fraudulenta*, D, I, *P. multistriata*, E, J, *P. pungens*.

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). Numbers in italic show mean ± SD. All measures were done with SEM. N= number of observation.

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. calliantha	linear	15-21	30-37	1	4-5	+	41.4-122.7	1.2- 2.2		
		18.6±1.3	*35.3 ±1.4*		*4.7±0.5*		*80.5± 10.8*	*1.7± 0.2*	1190	28
a		15-21	34-39		4-6		41- 98	1.3-1.8		
P. delicatissima	lanceolate	20-28	35-40	2	8-11	+	32.3-71.8	1.01-2.4		
		22.7±1.7	*36.6±1.7*		*9.3±0.8*		*44.9 ± 6.7*	*1.6± 0.3*	392	19
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	+	38.9-126.6	2.9-5.6		
		21.3±1.8	*22.5±1.2*		*5.8±0.5*		*60.8.± 15.8*	*4.21±0.5*	130	6
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	2.7-4.1		
		24.7±2.1	*38.7±2.1*		*11±1.4*		*48.3± 8.7*	*3.3± 0.6*	6	6
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-lanceolate	10-13	9-13	2	2.5-3	–	73.8-146.7	2.6-4.5		
		11±1.2	*11.4±1.5*		*2.9±0.2*		*112± 17.6*	*3.4± 0.5*	35	5
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		

similarity percentages (SIMPER) were employed to determine crucial variables. All analyses were carried out using the statistical software Primer 6 (Clark and Warwick, 2001). Additionally, Spearman rank-order correlations were created using the STATISTICA 6.1 statistical package.

RESULTS

Species identified in the Ebro Delta bays

The morphospecies identified from August 2005 to February 2006 in Alfacs and Fangar Bays were: *Pseudo-nitzschia calliantha, P. delicatissima, P. fraudulenta, P. multistriata*, and *P. pungens* (Fig. 2, Table 1). The morphometric characteristics corresponded to those previously reported (Hasle, 1995; Priisholm *et al.*, 2002; Lundholm *et al*., 2003) and were in agreement with a study on *Pseudo-nitzschia* composition carried out recently in the same geographic area (Quijano-Scheggia *et al.*, 2008).

Environmental conditions in the bays of the Ebro Delta

Temperatures in Alfacs Bay (Station 2) varied widely during the study period, reaching a maximum of 26.9ºC in September 2005 and a minimum of 8.1ºC in February 2006 (Table 2). Salinity ranged from 30.8 to 38. The lowest salinity values were recorded at the surface in October and November 2005. High values at the bottom were measured in January and February 2006 (Fig. 3). DIN concentrations were higher in Alfacs Bay during the win-

TABLE 2. – Maximum, minimum and mean values for chemical and biological parameters measured at the surface and the bottom of each bay. The measurement period in each bay corresponded to the bloom period (cell abundance of *Pseudo-nitzschia* $>10^5$ cells L^{-1}). which extended from August 29 2005 to February 20 2006 in Alfacs Bays and from August 8 to October 2005 in Fangar Bay. Probable inorganic dissolved nutrient limitations (DIN, P, and Si) are indicated (n= case number of limitation). N is the total number of the measures.

	Max	Min	Means ± SD	n	N
Alfacs					
Temperature ºC	26.9	8.2	18.18 ± 6.1		41
Salinity	38.0	30.8	34.9 ± 1.6		41
chlorophyll *a* µg L^{-1}	10.7	1.0	4 ± 2.1		43
NO_3^- µM	38.78	0.69	8.91± 8.9		34
NO_2^- µM	1.07	0.05	0.52 ± 0.28		34
NH_4^+ µM	30.44	0.02	5.88 ± 7.56		34
PO_4^{3-} µM	1.37	0.08	0.44 ± 0.28		34
SiO_2 µM	13.53	0.25	5.31± 3.7		34
DIN limitation				2	34
P limitation				19	34
Si limitation				0	34
Fangar					
Temperature ºC	27.8	19.8	24.2 ± 2.5		17
Salinity	36.6	28.0	34.0 ± 2.3		14
chlorophyll *a* µg L^{-1}	11.7	1.0	4.8 ± 2.8		17
NO_3^- µM	4.37	0.15	1.67 ± 0.93		14
NO_2^-- µM	0.93	0.07	0.35 ± 0.28		14
NH_4^+ µM	30.85	1.33	12.08 ± 10.16		14
PO_4^{3-} µM	1.35	0.02	0.53 ± 0.32		14
SiO_2 µM	37.73	3.58	12.45 ± 8.47		14
DIN limitation				2	14
P limitation				5	14
Si limitation				0	14

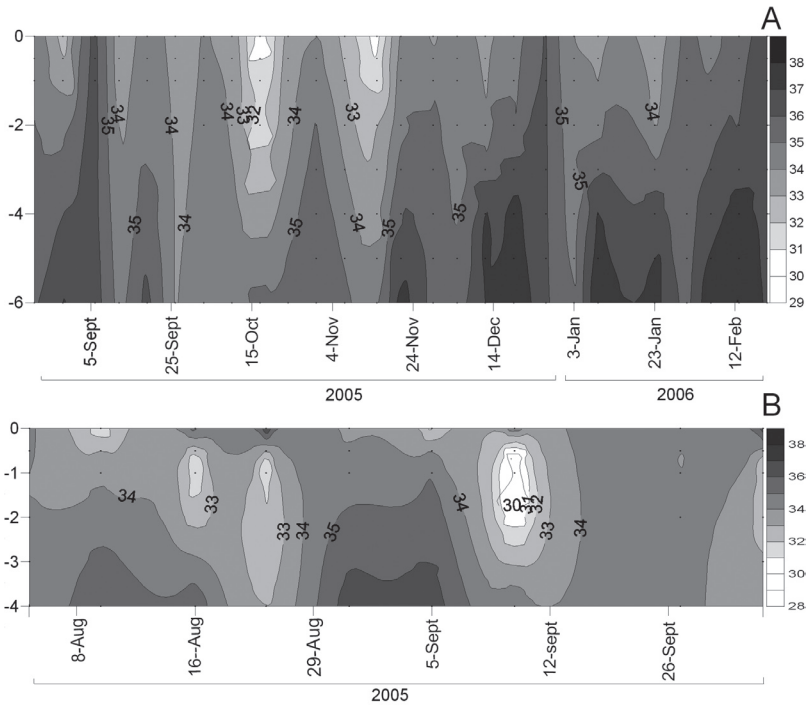

FIG. 3. – Temporal-spatial distribution of salinity in Alfacs (A) and Fangar (B) Bays. Depth in metres.

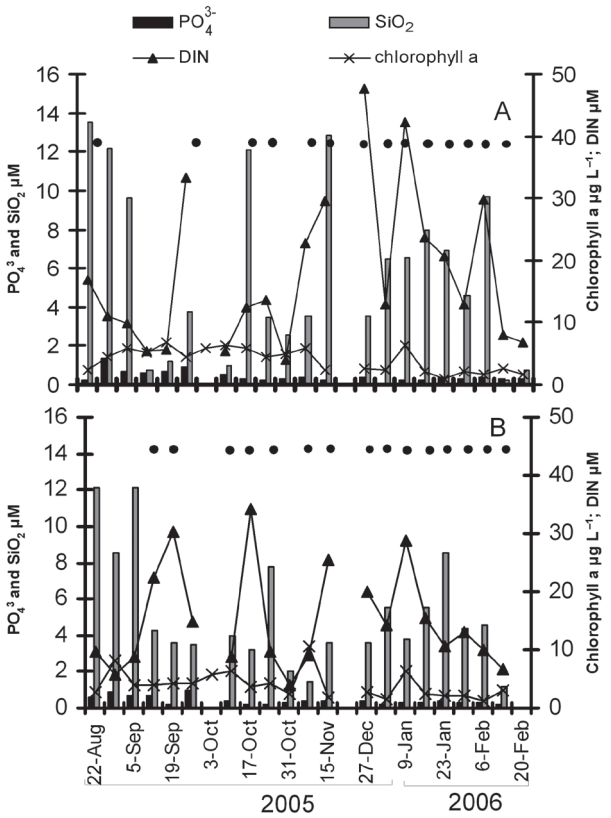

FIG. 4. – Weekly biological and chemical parameters in Alfacs Bay at the surface (A) and bottom (B) as measured at a central station. Chlorophyll *a* concentration (µg L^{-1}) and dissolved inorganic nitrogen (DIN, µM) are shown on one axis. Dissolved inorganic P and Si (µM) on the other axis. Probable inorganic P limitations are indicated in the graph as •.

ter period and mainly at the surface station. Based on our estimates, there were very few cases of DIN limitation during the summer months. However, in winter, the PO_4^{3-} concentration was <1.4 µM and was thus the limiting nutrient (Fig. 4A, B and Table 2); the inorganic N/P ratio ranged from 6.8 to 200.8. For silicates, the values were extremely variable, with high levels (>10 µM) measured in summer and autumn (Fig. 4A, B). From December to February, the silicate concentration was quite stable and no limitations in this nutrient were observed (Fig. 4, Table 2).

The temperature at Fangar Bay (Station 1) varied less than the temperature at Alfacs Bay during the study period. Salinity measurements indicated stratification between 29/8 and 5/9 just after and just before the occurrence of low salinity conditions (Fig. 3). During this study, DIN concentrations at Station 1 were higher at the bottom than at the surface and few cases of DIN limitation were observed. At the bottom of the measurement station, limitations in PO_4^{3-} occurred relatively frequently (50% of the data), but only once at the surface station. Silicate concentrations were similar at the surface and at the bottom, reaching a maximum on 12 September. No limitations in the amounts of silicate were observed (Fig. 5, Table 2).

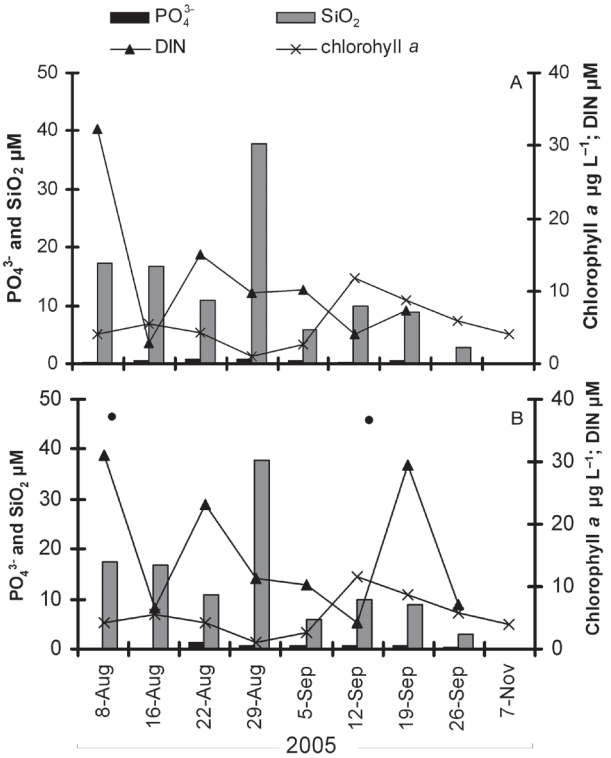

FIG. 5. – Weekly biological and chemical parameters in Fangar Bay at the surface (A) and bottom (B) as measured at a central station. Chlorophyll *a* concentration (µg L^{-1}) and dissolved inorganic nitrogen (DIN, µM) are shown on one axis. Dissolved inorganic P and Si (µM) on the other. Probable inorganic P limitations is indicated in the graph as •.

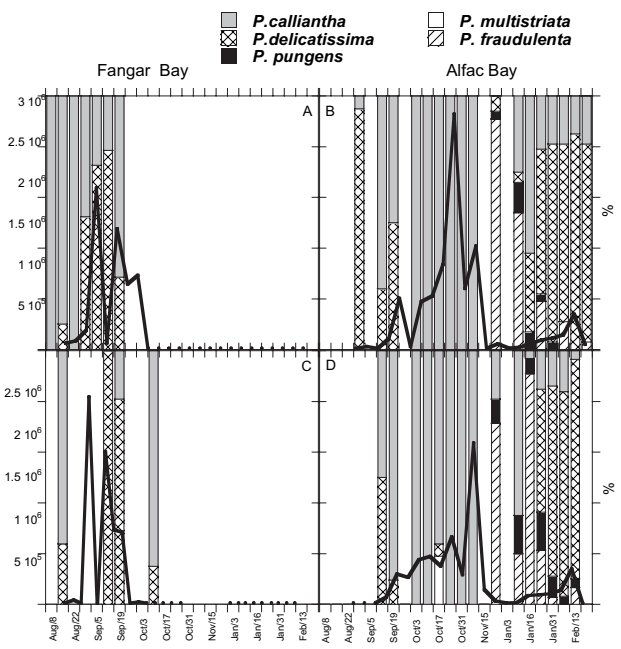

FIG. 6. – Weekly *Pseudo-nitzschia* cell abundance (cells L^{-1}) and percentage of each *Pseudo-nitzschia* species (%) in Fangar Bay at the surface A) and bottom C), and in Alfacs Bay at the surface B) and bottom D).

Bloom composition of *Pseudo-nitzschia* spp.

During the 7 months of the study in 2005–2006, diatoms of the genus *Pseudo-nitzschia* bloomed (exceeding cell concentrations of 10^5 cells L^{-1}) for approximately 4 months in Alfacs Bay, whereas the extent of the *Pseudo-nitzschia* spp. bloom in Fangar Bay was shorter (3 months).

In Fangar Bay, the proliferation was characterised by the presence of *P. calliantha*, with low densities (<10^5 cells L^{-1}) recorded on August 8 at the surface (Fig. 6A, C). A mixed bloom of *P. delicatissima* and *P. calliantha* with an abundance as high as 2×10^6 cells L^{-1} was detected during late August/early September at the surface of Station 1. By contrast, *P. delicatissima* (<10^6 cells L^{-1}) was dominant at the bottom of the station in early September. The bloom finished at the end of September 2005.

In Alfacs Bay, the bloom began late in August and consisted of low concentrations (<10^5 cells L^{-1}) of a mixture of the species *P. calliantha* and *P. delicatissima*, without any difference in the vertical profile. Beginning in October and continuing for the next 6 weeks, high cell densities (>10^6 cells L^{-1}) were recorded. The proliferation at the surface was limited to *P. calliantha*, whereas at the bottom *P. calliantha*, *P. pungens*, and *P. multistriata* were recorded. In December 2006, *P. fraudulenta* became the main species albeit at low concentrations. From January 9 until February 13, a mixed bloom with low abundances (<10^5 cells L^{-1}) was present in Alfacs Bay. The species composition was *P. delicatissima*, *P. calliantha, P. pungens*, and *P. fraudulenta* in different percentages. *P. delicatissima* was dominant from January 23 until the end of the bloom (Fig. 6 B, D).

Size distribution of *Pseudo-nitzschia* spp. in Alfacs Bay

P. calliantha predominated during the October 2005 bloom at Alfacs Bay. During the period of maximum cell abundance, there was a significant percentage of large cells (>80 µm) of this species (r=0.71, significance level of 0.02). A considerable proportion of large *P. calliantha* cells were also recorded on November 7; the cell abundance at this date was 1.56×10^6 cells L^{-1} (Fig. 7A). The highest cell abundance of *P. delicatissima* recorded in Alfacs Bay during the study period occurred be-

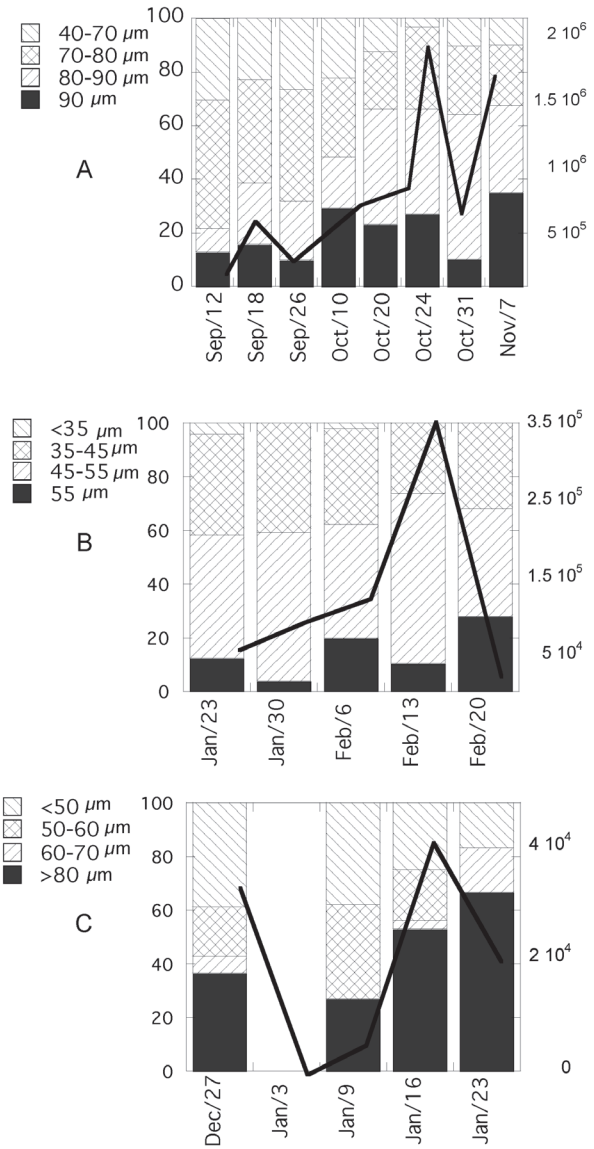

FIG. 7. – Cell abundance (cell L^{-1}) and percentage of size distribution during the blooms of *Pseudo-nitzschia* spp in Alfacs Bay. A) *P. calliantha* (n=394), B) *P. delicatissima* (*n*=202), and C) *P. fraudulenta* (*n*=124)

tween January and February 2006. In general, an increase in cell size paralleled the increase in cell abundance. The maximum percentage of *P. delicatissima* cells >45 μm occurred at a cell abundance of 3.5×10^5 cells L^{-1} (Fig. 7B). *P. fraudulenta* was abundant from December 2005 to January 2006; the maximum cell abundance for this species was 4×10^4 cells L^{-1}. The largest proportion of cells >60μm (83%) occurred on January 23 (Fig. 7C). In Fangar Bay, the study period was too short to investigate the cell-size distribution with statistical confidence.

Composition of the phytoplankton community

In Alfacs Bay (Station 3), from January to March 2005, the phytoplankton community was dominated by nanoflagellates and coccolithophorids (Fig. 8). Nanoflagellates were also dominant in May, August, and December 2005 and in January 2006. Blooms of diatoms were recorded mainly in April, when *Chaetoceros* spp. and *Leptocylindrus danicus* predominated while in June only *Chaetoceros* spp. were observed. *Pseudo-nitzschia* appeared in July; the cell abundance was initially low but increased during the following months. In October and November, *Pseudo-nitzschia* was highly abundant, representing >80% of the diatom community and >74% of the group made up of diatoms, dinoflagellates and nanoflagellates. The species composition was dominated by *P. calliantha* (95%). At the end of the year, *Pseudo-nitzschia* declined in abundance, but in February 2006, it represented 91% of the diatom community, with *P. delicatissima* as the most abundant species (98%).

At Alfacs Bay Station 2, the phytoplankton community showed three marked periods in terms of species abundance: Period 1, of *Pseudo-nitzschia* dominance, Period 2, of dinoflagellate dominance, and Period 3, of dominance by diatom species other than *Pseudo-nitzschia*. During the *Pseudo-nitzschia*

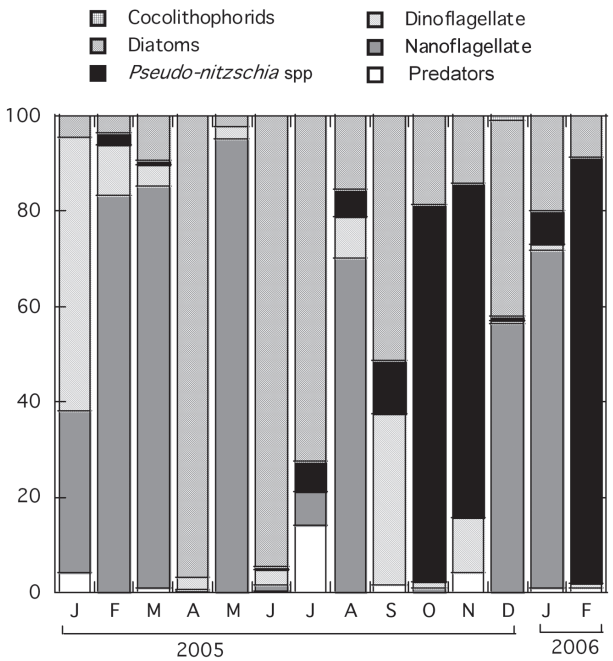

FIG. 8. – Relative abundance of the principal phytoplankton group in Alfacs Bay (Station 3) The category "predator" includes rotifers, tintinids, ciliates, nauplius, copepods, and larvae of bivalves

bloom period (>10^5 cells L^{-1}, Period 1), from 19 September 2005 to 7 November 2005 and from 23 January 2006 to 13 February 2006, this genus accounted for >98% of the community (data not shown). During those periods in which the abundance of *Pseudo-nitzschia* was <10^5 cells L^{-1} (Period 2, from 21 November 2005 to 23 January 2006), dinoflagellates, specifically *Karlodinium* spp., *Karenia* sp., and *Prorocentrum triestinum*, predominated on only one occasion, whereas they were far less represented during the diatom bloom. Phytoplankton composition at the bottom of the station were similar to those at the surface. During Period 3, other diatoms, i.e. *Lioloma pacificum*, *Thalasssionema nitzschioides*, *Chaetoceros* spp. and *Pleurosigma* sp., dominated the community composition (>95% of the community).

At the surface of Station 1 (Fangar Bay), during the *Pseudo-nitzschia* bloom from 8 August 2005 to 26 September 2005, diatoms were also dominant (data not shown). *Pseudo-nitzschia* spp. were accompanied by *Pseudosolenia calcar-avis*, *Rhizosolenia setigera*, and *Pleurosigma* sp. Dinoflagellates represented >50% of the community only once (August 22, 2005); the most abundant species was *Gonyaulax polygramma*. At the bottom of Station 3, dinoflagellates, represented by *Gonyaulax polygramma* and *Prorocentrum triestinum*, were dominant on three occasions when the abundance of *Pseudo-nitzschia* was low (<10^4 cells L^{-1}).

Statistical analysis

The ANOSIM analysis revealed no significant differences between samples taken at the surface or at depth, whereas the differences among seasons were significant (p<0.01). The pairwise test demonstrated that samples obtained in winter were significantly different from those obtained in summer and autumn (p<0.05); the summer samples were not significantly different from those collected in autumn. Therefore, the analyses led to the identification of two groups of samples (Fig. 9). The cluster analysis generated a dendrogram in which the SIMPROF analysis (p<0.05) created three objectively defined groups (*a*, *b*, and *c*); this result was confirmed by ANOSIM analysis (significant differences among the three groups p<0.01). The pairwise test (p<0.05) showed that group *c* differed significantly from groups *a* and *b*, and that group *a* was not significantly different from group *b*. Groups *a* and *b* represented species present in winter and group *c* those present in summer and autumn (Fig. 9, Table 3). This statistical concordance between cluster and group confirmed that categorisation of samples on a temporality basis, performed prior to the statistical analysis, was an accurate approach to evaluate the samples. However, there were three samples that did not match these classifications; these were transitional, i.e. obtained between seasons (samples 142, 190, 197).

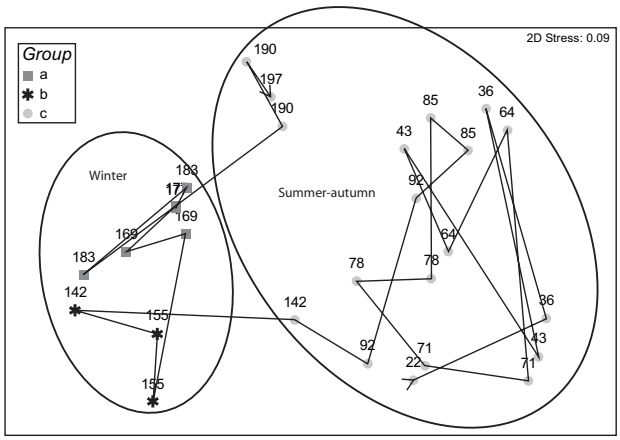

FIG. 9. – MDS (2-d minimum stress: 0.09) result of abiotic data. The black arrow shows the time sequence of sampling. Cluster and season groups are also shown

TABLE 3. – Mean values (N = 28) for abiotic and biotic parameters of Alfacs Bay with respect to cluster group (*a*, *b* and *c*) (see text) and season (summer, autumn and winter). Temperature (Temp), salinity (Sal), chlorophyll *a* concentration (Chl-*a*, µg L^{-1}), dissolved inorganic nutrients (µM), *Pseudo-nitzschia* abundance per species (cells L^{-1}), and total *Pseudo-nitzschia* abundance (TPSN). Variables with a significant role defining these groups are indicated in bold. *P.cal., P.calliantha*; *P.del., P. delicatissima*; *P.pun., P. pungens*; *P.mul., P. multistriata*; *P.fra., P. fraudulenta.*

	Temp	Sal	Chl-*a*	NO_3^-	NO_2^-	NH_4^+	PO_4^{3-}	SiO_2	*P.cal.*	*P.del.*	*P.pun.*	*P.mul.*	*P.fra.*	TPSN
a	10.28	35.73	1.65	**14.70**	0.63	**0.91**	0.34	6.44	**0.91**	**3.55**	0.28	0.00	0.24	4.99
b	16.97	34.93	5.03	**37.22**	0.65	**1.64**	0.28	4.63	**1.57**	**0.05**	0.51	0.00	2.14	4.27
c	19.46	34.71	4.69	**4.21**	0.29	**8.17**	0.42	3.62	**4.10**	**1.31**	0.03	0.06	0.19	5.69
Summer	24.40	34.18	4.90	**2.43**	0.48	**12.05**	0.71	4.40	**3.45**	**1.65**	0.00	0.00	0.00	5.10
Autumn	18.61	34.54	4.99	**7.95**	0.24	**7.79**	0.33	4.02	**4.97**	**0.02**	0.07	0.09	0.54	5.70
Winter	12.45	35.75	2.71	**15.66**	0.54	**1.07**	0.30	4.65	**1.02**	**3.43**	0.27	0.00	0.46	5.17

Multidimensional scaling (MDS), with a minimum 3-d stress value of 0.03 and minimum 2-d stress value of 0.09, confirmed the representation of the samples and was coincident with the cluster result. The test also showed a marked temporality, observable by the factor "season, cluster groups" and by the factor "time" (Fig. 9). The abiotic variables that differentiated the two groups (winter, which included groups *a* and *b*, and summer-autumn, which included group *c*) were NO_3^- and NH_4^+ (SIMPER analysis with minimum contribution of 30%). Specifically, the NO_3^- concentration was higher and the NH_4^+ concentration lower in winter while the opposite was true in summer and autumn (Tables 3 and 4). Moreover, these two groups were also characterised by two biotic variables (SIMPER analysis with minimum contribution of 30%) with respect to the factor season as well as at the cluster level: *P. delicatissima* was more abundant and *P. calliantha* less abundant among the winter samples and among cluster groups *a* and *b*, while within summer and autumn samples and cluster group *c* the opposite held regarding the abundances of these two species. Finally, the correlations shown in Table 5 confirmed the following results: concentrations of the inorganic nutrient NO_3^-, which were higher during the winter, were negatively correlated with those of NH_4^+ and with temperature. The concentrations of NH_4^+, which were higher during summer and autumn, were positively correlated with temperature. In addition, *P. delicatissima* abundance, which was greater during the winter, was negatively correlated with NH_4^+, and *P. calliantha* abundance (variables related to the summer and autumn seasons). By contrast, *P. calliantha* was positively correlated with NH_4^+. Other significant correlations between *Pseudo-nitzschia* species and other species or abiotic variables were: *P. pungens* correlated negatively with temperature, NH_4^+, and *P. calliantha* and positively with NO_3^- and NO_2^-; and *P. fraudulenta* correlated negatively with temperature and *P. calliantha* and positively with NO_3^- and *P. pungens*. These correlations established a higher-temperature affinity group consisting only of *P. calliantha*, and a lower-temperature affinity group consisting of *P. delicatissima*, *P. pungens*, and *P. fraudulenta* (Tables 4 and 5). Moreover, chlorophyll *a* was significantly and positively correlated with temperature and significantly and negatively correlated with salinity.

TABLE 4. – Summary of statistical analyses performed on data collected from Alfacs Bay. Two groups were defined distinguishing winter (cluster groups *a* and *b*) from summer and autumn (cluster group *c*) (see text). These groups were characterised by high (+) or low (-) concentrations of NO_3^- and NH_4^+ and by high (+) or low (-) cell abundances of *Pseudo-nitzschia delicatissima* and *P. calliantha*. Other variables did not play a significant role.

	Winter (a + b)	Summer-Autumn (c)
NO_3^-	+	-
NH_4^+	-	+
P. delicatissima	+	-
P. calliantha	-	+

DISCUSSION

Our understanding of the population ecology of most *Pseudo-nitzschia* species is still limited by difficulties in taxonomic identification. Firstly, an im-

TABLE 5. – Spearman correlation coefficients for transformed abiotic and biotic variables: temperature (Temp), salinity (Sal), chlorophyll *a* concentration (Chl-*a*, μg L^{-1}), dissolved inorganic nutrients (μM), *Pseudo-nitzschia* abundance per species (cells L^{-1}) and total *Pseudo-nitzschia* abundance (TPSN). Significant ($p<0.01$) correlations are indicated in bold. *P.cal., P.calliantha*; *P.del., P. delicatissima*; *P.pun., P. pungens*; *P.mul., P. multistriata*; *P.fra., P. fraudulenta.*

	Temp	Sal	Chl-*a*	NO_3^-	NO_2^-	NH_4^+	PO_4^{3-}	SiO_2	*P.cal.*	*P.del.*	*P.pun.*	*P.mul.*	*P.fra.*
Sal	-0.32												
Chl-a	**0.57**	**-0.51**											
NO_3-	**-0.73**	0.22	-0.41										
NO_2-	-0.22	-0.02	-0.33	**0.57**									
NH_4^+	**0.58**	-0.38	**0.50**	**-0.49**	-0.33								
PO_4^{3-}	0.09	-0.38	0.13	-0.22	0.15	0.14							
SiO_2	-0.12	-0.19	-0.21	0.38	**0.55**	-0.01	-0.01						
P. calliantha	0.46	**-0.48**	**0.64**	-0.47	**-0.51**	**0.60**	-0.04	-0.17					
P. delicatissima	-0.33	0.28	**-0.63**	0.25	0.38	**-0.52**	-0.04	0.13	**-0.78**				
P. pungens	**-0.50**	0.38	-0.29	**0.72**	**0.56**	**-0.56**	-0.23	0.31	**-0.57**	0.18			
P. multistriata	0.08	0.13	-0.04	-0.04	-0.13	0.32	-0.24	-0.11	0.06	0.01	-0.14		
P. fraudulenta	**-0.49**	0.40	-0.23	**0.69**	0.43	-0.37	-0.16	0.13	**-0.54**	0.06	**0.75**	-0.13	
TPSN	0.14	-0.14	0.34	-0.47	**-0.70**	0.24	-0.17	-0.37	**0.61**	-0.28	**-0.58**	0.08	**-0.62**

portant fraction of the *Pseudo-nitzschia* species in field samples cannot be identified and counted by routine methods (optical microscopy). Moreover, one species name may actually represent a number of cryptic species, such as has been described for *P. delicatissima* (Amato *et al.*, 2007). Therefore, very little is known about the relationship among particular species and the environmental conditions that favour their growth, although general tendencies have been proposed (Parsons *et al.*, 1999; Trainer *et al.*, 2002; Kaczmarska *et al.*, 2007).

The present study is the first to provide detailed, species-level records of *Pseudo-nitzschia* communities present in two bays of the Ebro Delta and to assess the relationship of these diatom communities with environmental conditions and biotic factors. In our study, the physical and chemical conditions of the waters in which *Pseudo-nitzschia* was detected in high abundance included large variations in salinity, temperature and nutrient composition. Possible limitation in DIN did not seem to play an important role in the spatial and temporal distribution of *Pseudo-nitzschia* proliferation in the bays.

Statistical analysis of the abiotic data collected during the study period showed that measurements made at the surface did not significantly differ from those made at the bottom of the three stations. This result was expected given the low depth of the water column in Alfacs Bay. Nevertheless, unambiguous and marked differences with respect to temporality of the samples were detected following statistic analyses, which identified two objectively defined groups: one made up of winter samples and the other of summer and autumn samples. The inorganic dissolved nutrients NO_3^- and NH_4^+ were the principal variables that explained this distinction, i.e. high NO_3^- and low NH_4^+ concentrations during winter and the opposite during summer and autumn. These two sample groups could also be distinguished on the basis of the biotic data, with *P. calliantha* more abundant in summer and autumn and *P. delicatissima* more abundant in winter. By contrast, *P. delicatissima* was dominant in Fangar Bay in summer. Inorganic dissolved nitrogen loading in coastal waters has been postulated to promote an increase in the abundance of *Pseudo-nitzschia* spp. (Hasle *et al.*, 1996; Parsons *et al.*, 2002). Cusak *et al.* (2004) and Kaczmarska *et al*. (2007) found that the presence of *P. delicatissima* was correlated with environments rich in nitrates, while environments rich in phosphate favour the growth of *P. pungens*. In the case of Alfacs Bay, significant correlations of the main *Pseudo-nitzschia* species (*P. delicatissima* and *P. calliantha*) depended on the particular N form, mainly NH_4^+ and NO_3^-. Regarding other environmental variables, *P. pungens* and *P. fraudulenta* were negatively correlated with temperature, in agreement with the findings of Hasle *et al*. (1996). These authors recorded high *P. pungens* cell densities in northern European waters during the autumn.

Statistical analyses with abiotic factors were not performed in Fangar Bay since insufficient data were collected. Nonetheless, the dynamics of the *Pseudo-nitzschia* bloom can be described. The bloom comprised species different from those making up the blooms in Alfacs Bay (*P. calliantha* and *P. delicatissima*) and was of shorter duration. Another important difference between the bays was that *P. delicatissima* was present in high abundance in Alfacs Bay in winter, but in Fangar Bay in summer. These features were probably due to fact that the bay is smaller and is thus influenced to a greater extent by exchange with water from the open sea.

In natural environments, auxospores are very rarely observed. For this reason, the occurrence of sexual reproduction is often detected by monitoring variations in cell size (Mann, 1988; Mann, 2002). Nonetheless, information on the diatom life cycle is mainly limited to benthic species (Mann, 1988, 1994; Mann *et al.*, 1999; Mann *et al.*, 2003; Chepurnov and Mann, 2004; Mann and Chepurnov, 2005). The main difficulty in the case of planktonic species is the overlap between distinct cohorts in the field (Mann, 1988; Cerino *et al.*, 2005). For *Pseudo-nitzschia galaxiae*, Cerino *et al*. (2005) reported two different morphotypes and hypothesised that sexual reproduction occurs at extremely low rates and only at a specific time of the year, or occurs in cells ranging widely in size. Moreover, it may be the case that until cells attain a cardinal point with respect to size (size threshold), sexual reproduction is not triggered. Although, some authors reported long-time cell reduction in *Pseudo-nitzschia* (order of years)(Davidovich and Bates, 1998), our observations suggested that this genus is affected by a quick size reduction of about 40% over a 3-month period (e.g. cultures of *P. calliantha,* data not shown). Similarly, an abrupt reduction in the size of *Pseudo-nitzschia* species has been reported (Chepurnov *et al.*, 2005). Based on the fact that the cell sizes of *P. calliantha*, *P. fraudulenta*, and *P. delicatissima* increased during the bloom period in Alfacs Bay, we hypothesise that sexual repro-

duction must occur during such events. Although we did not observe auxospores in field samples, which would have confirmed sexual reproduction in *Pseudo-nitzschia*, large cells of the above-mentioned species were found in large proportions when the cell densities reached the maximum values during the course of the bloom. Since only sexual reproduction restores the vegetative cell size of *Pseudo-nitzschia* species (Drebes, 1977; Mann, 2002; Chepurnov *et al*., 2005), our data suggest that sexual reproduction of these species occurred.

The composition of the phytoplankton community in Alfacs Bays in January 2005 was dominated by nanoflagellates and dinoflagellates, both of which evolve more efficiently under conditions of low nutrients and low irradiance. The dominant components of phytoplankton exposed to high-nutrient conditions were centric diatoms in April, June, and July and pennate diatoms in September–November 2005 (among them *Pseudo-nitzschia*). During the *Pseudo-nitzschia* proliferations, in which cell abundance exceeded 10^6 cell L^{-1}, the genus represented the major fraction. The environmental window that allows *Pseudo-nitzschia* species to proliferate and dominate the phytoplankton community in Alfacs bay is quite broad, in view of the fact that i) nutrients do not seems to limit their growth and ii) different species take advantage of different biotic conditions. These features allowed a succession of different blooming species of *Pseudo-nitzschia* lasting months in Alfacs Bay.

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