

Species occurrence of the potentially toxigenic diatom genus *Pseudo-nitzschia* and the associated neurotoxin domoic acid in the Argentine Sea



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ABSTRACT

The marine diatom genus *Pseudo-nitzschia*, the major known producer of the neurotoxin domoic acid (DA) responsible for the amnesic shellfish poisoning (ASP) syndrome in humans and marine mammals, is globally distributed. The genus presents high species richness in the Argentine Sea and DA has been frequently detected in the last few years in plankton and shellfish samples, but the species identity of the producers remains unclear. In the present work, the distribution and abundance of *Pseudo-nitzschia* species and DA were determined from samples collected on two oceanographic cruises carried out through the Argentine Sea (~39–47°S) during summer and spring 2013. Phytoplankton composition was analysed by light and electron microscopy while DA was determined by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The genus *Pseudo-nitzschia* was recorded in 71 and 86% of samples collected in summer and spring, respectively, whereas DA was detected in only 42 and 21% of samples, respectively. Microscopic analyses revealed at least five potentially toxic species (*P. australis*, *P. brasiliiana*, *P. fraudulenta*, *P. pungens*, *P. turgidula*), plus putatively non-toxicogenic *P. dolorosa*, *P. lineola*, *P. turgiduloides* and unidentified specimens of the *P. pseudodelicatissima* complex. The species *P. australis* showed the highest correlation with DA occurrence ($r = 0.55$; $p < 0.05$), suggesting its importance as a major DA producer in the Argentine Sea. In the northern area and during summer, DA was associated with the presence of *P. brasiliiana*, a species recorded for the first time in the Argentine Sea. By contrast, high concentrations of *P. fraudulenta*, *P. pungens* and *P. turgidula* did not correspond with DA occurrence. This study represents the first successful attempt to link toxigenicity with *Pseudo-nitzschia* diversity and cell abundance in field plankton populations in the south-western Atlantic.

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1. Introduction

The marine diatom genus *Pseudo-nitzschia* H. Peragallo is distributed worldwide and often represents an important, even dominant component of phytoplankton assemblages (Lelong et al., 2012). The genus now comprises 48 recognized species (Teng et al., 2014, 2015, 2016; Percopo et al., 2016), most of them described within the last two decades. Among these species, at least 23 have been mentioned as potential producers of domoic acid (DA) (Teng

et al., 2015, 2016), a secondary amine neurotoxin. This neurotoxin can be accumulated throughout the marine food web, causing amnesic shellfish poisoning (ASP), a neurological syndrome with even lethal effects on sea birds, marine mammals and humans in extreme cases (Fire and Van Dolah, 2012).

The existence of toxigenic and non-toxicogenic strains of the same species has been recurrently mentioned for several *Pseudo-nitzschia* species (e.g. Rhodes et al., 1996; Villac et al., 1993; Thessen et al., 2009; Sahraoui et al., 2011). Environmental factors, such as ambient nutrient concentrations, temperature, salinity, irradiance, photoperiod and pH, have been known to influence and under some circumstances to induce DA production in laboratory culture experiments (Lelong et al., 2012; and references therein). In addition, it has been recently observed that DA toxin content

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increased when cells of *Pseudo-nitzschia* species were exposed to copepod grazing (Tammilehto et al., 2015; Harðardóttir et al., 2015). In the field, however, is difficult to provide strong evidence for the association of environmental conditions with DA production by *Pseudo-nitzschia* species (Marchetti et al., 2004; Schnetzer et al., 2007; Guannel et al., 2015).

Toxicogenic *Pseudo-nitzschia* species are typically found in coastal waters, whereas open-ocean strains are usually found to be non-toxicogenic or producers of only very low DA cell concentrations (Marchetti et al., 2008; Trick et al., 2010). Nevertheless, it is unclear if this tendency reflects genetic distinctiveness (“ecotypes”) or is largely a function of ambient environmental influences on toxin production. From a global perspective, most toxic DA events have been associated with the occurrence of *Pseudo-nitzschia australis* Frenguelli, which together with *P. multiseriata* (Hasle) Hasle and *P. seriata* (Cleve) Peragallo present the highest per cell DA concentrations measured in laboratory cultures (Trainer et al., 2012; and references therein). In any case, there is a great inter- and intraspecific variability in DA cell quotas measured among *Pseudo-nitzschia* strains isolated into culture from different regions of the world (Trainer et al., 2012). In addition, highly variable cell quotas are typically found among natural *Pseudo-nitzschia* populations (Schnetzer et al., 2007; Thessen and Stoecker, 2008), but some of this apparent variability may be attributable to DA estimates based upon total cell densities of *Pseudo-nitzschia*, which usually includes several co-occurring species.

Several previous studies have focused on the occurrence of *Pseudo-nitzschia* species in coastal and shelf waters from the Argentine Sea. These taxonomic surveys, conducted exclusively by means of light and electron microscopy analyses, have revealed a high species richness at the morphological level (Negri and Inza, 1998; Ferrario et al., 1999, 2002; Sastre et al., 2001; Almandoz et al., 2007; Sunesen et al., 2009). Similarly, DA has been frequently detected in phytoplankton samples from different zones of the Argentine Sea in the last few years (Negri et al., 2004; Sastre et al., 2007; Cadaillón, 2012; Krock et al., 2015). Indeed, *Pseudo-nitzschia* blooms have been suggested as potentially responsible for calf mortalities of southern right whales along the Valdés Península

(D’Agostino et al., 2015; Wilson et al., 2015). During these episodes, DA production was mainly associated with elevated cell densities of *P. australis* (Negri et al., 2004) or *P. fraudulenta* (Cleve) Hasle (Sastre et al., 2007), but the co-occurrence of several other *Pseudo-nitzschia* species (Cadaillón, 2012; Krock et al., 2015), makes it difficult to unequivocally identify the responsible taxa. Likewise, analyses of DA toxin production of local strains of *P. pungens* (Grunow ex Cleve) Hasle (Sar et al., 2006) and *P. multiseriata* (Montoya et al., 2008) revealed either negative or positive results, respectively, while DA production for other species found in the Argentic Sea has not been yet evaluated. All this indicate that DA production by *Pseudo-nitzschia* species in Argentine waters and the south-western Atlantic remains poorly understood.

In the present work, the distribution and abundance of *Pseudo-nitzschia* species and DA was analysed from two sampling cruises carried out through the Argentine Sea (~39–47°S), covering coastal, shelf and shelf-break waters during summer and spring 2013. The main goal was to reveal the identity of major DA producers in the field, by associating abundance and distribution of *Pseudo-nitzschia* species and DA.

2. Material and methods

2.1. Plankton and toxin sampling

Water samples were collected during two oceanographic expeditions in the Argentine Sea (Fig. 1). The first expedition was conducted in late austral summer on the R/V *Bernardo Houssay* from 11 to 22 March 2013, with 24 sampling stations located between ~39 and 43°S. This cruise was divided into two legs K1 and K2, which comprised 8 and 16 sampling stations, respectively. The second expedition was carried out in austral spring aboard the R/V *Puerto Deseado* from 26 October to 9 November 2013, with 43 sampling stations located between ~39 and 47°S. At each station, temperature and salinity were measured by CTD (conductivity-temperature-depth) profiler, except from leg K2 of Expedition 2, for which only temperature data were obtained with a multiparameter probe.

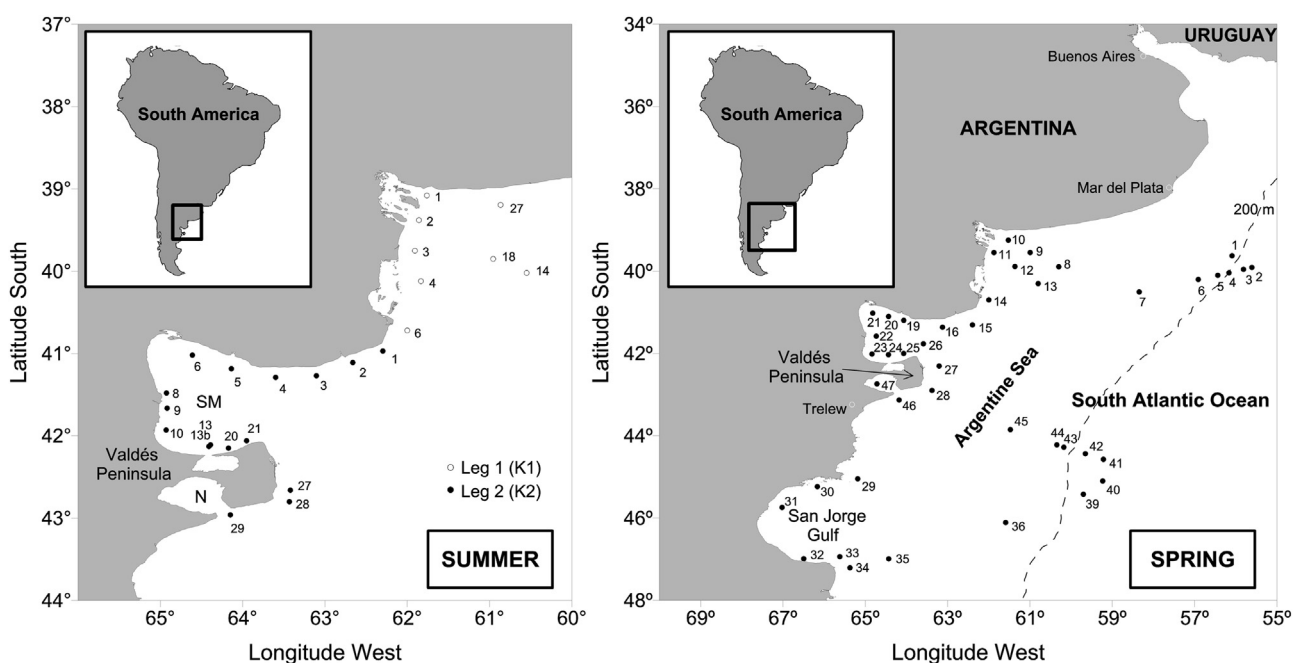


Fig. 1. Maps of the study area showing the location of sampling stations in the Argentine Sea during summer and spring cruises. SM=San Matías Gulf. N=Nuevo Gulf.

Niskin bottle samples were taken from 3 and 10 m depth and mixed at equal volume for determination of total plankton community composition and quantitative analyses. In addition, vertical net tows through the upper 20 m of the water column were conducted with a 20 μm -mesh Nitex net for both taxonomic and phycotoxin analysis. Each net haul concentrate was diluted up to 1 L with 0.2 μm -filtered seawater. An aliquot of 100 mL was fixed with Lugol's iodine solution for species identification and enumeration. The rest was sequentially filtered through Nitex mesh of 200, 50 and 20 μm in PVC cylinders by gravity filtration and split into aliquots for toxin extraction. The particulate material retained on each mesh was re-suspended in 40 mL of filtered seawater and transferred into 50 mL centrifuge tubes. Pellets obtained by centrifugation were stored at -20°C for later toxin analysis. Toxin results from fractions 20–50 μm and 50–200 μm were combined in a single value (20–200 μm) throughout the manuscript and figures. In order to exclude the potential DA accumulation in large zooplankton (e.g., copepods), aliquots corresponding to the higher size fraction ($>200 \mu\text{m}$) were not considered in this study.

2.2. Phytoplankton taxonomic and quantitative analysis

Net and Niskin bottle samples were analysed by a combination of light and electron microscopy methods to determine *Pseudo-nitzschia* species identity and cell abundance. The abundance (in cells L^{-1}) of the genus *Pseudo-nitzschia* in samples collected by Niskin bottles was determined with a phase-contrast Leica DMIL LED inverted microscope according to the procedures described by Utermöhl (1958). Subsamples of 50 mL were settled for 24 h in a composite sedimentation chamber.

An aliquot of each net sample was washed with distilled water several times for desalting and then treated to remove organic material, following Prygiel and Coste (2000). Raw (untreated) and cleaned cell specimens were then mounted on permanent slides with Naphrax mounting medium according to Ferrario et al. (1995). Cleaned material from 16 samples, selected by the presence of potentially toxic *Pseudo-nitzschia* species after light microscopic examination of slides, was also mounted onto glass stubs or filtered onto 0.2 μm polyamide or 2 μm polycarbonate filters and sputter-coated with Au-Pd for scanning electron microscopy (SEM) analysis to confirm species identity. The SEM observations were carried out with a Jeol JSM-6360 LV and a FEI Quanta FEG 200 electron microscope.

Permanent slides were analysed under a phase-contrast Leica DM2500 microscope. In order to estimate the relative cell abundance of *Pseudo-nitzschia* species within the genus, all specimens present on the whole slide (average of 104 *Pseudo-nitzschia* specimens per slide) were enumerated at 1000 \times . In samples with high *Pseudo-nitzschia* species cell densities, specimens were enumerated by examination of linear transects until at least 100 specimens were recorded.

Cell abundance of *Pseudo-nitzschia* species in net samples was determined by counting aliquots of 1 mL in Sedgewick-Rafter chambers with a phase-contrast Leica DMIL LED inverted microscope. These data served for semi-quantitative comparison of cell densities and toxin concentrations. Given the difficulty to identify most *Pseudo-nitzschia* species to species level in raw samples by light microscopy, only total *Pseudo-nitzschia* cell abundance was estimated at this stage. The detection limit was 1000 cells per net tow. Total *Pseudo-nitzschia* density, expressed per net tow (cells NT^{-1}), was multiplied by the relative abundances estimated by the enumeration of permanent slides to obtain the abundance of different species.

2.3. Toxin analysis

Cell pellets from the plankton net tow size fractions were suspended in 500 μL methanol, and subsequently homogenized with 0.9 g of lysing matrix D by reciprocal shaking at maximum speed (6.5 m s^{-1}) for 45 s in a Bio101 FastPrep instrument (Thermo Savant, Illkirch, France). After homogenization, samples were centrifuged at 16,100 $\times g$ at 4°C for 15 min. The supernatant was transferred to a spin-filter (0.45 μm pore-size, Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at 800 $\times g$, followed by transfer to autosampler vials. Analysis of domoic acid was performed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), as described in Krock et al. (2008). Toxin concentrations are expressed as nanograms per net tow (ng NT^{-1}). Detection limits were 4.9 and 6.6 ng DA NT^{-1} for the summer and spring expeditions, respectively.

2.4. Data analysis

A non-parametric Spearman's correlation analysis was employed to determine correlations between *Pseudo-nitzschia* species abundance, DA concentration and environmental factors (temperature and salinity). Unidentified specimens corresponding to the *Pseudo-nitzschia pseudodelicatissima* species complex were considered as a single group for the correlation analysis.

3. Results

3.1. *Pseudo-nitzschia* diversity

Morphological analyses revealed the presence of *P. australis*, *P. brasiliiana* Lundholm, Hasle and Fryxell, *P. dolorosa* Lundholm and Moestrup, *P. fraudulentata*, *P. lineola* (Cleve) Hasle, *P. pungens*, *P. turgidula* (Hustedt) Hasle and *P. turgiduloides* (Hasle) Hasle. Among these species, *P. australis*, *P. brasiliiana*, *P. fraudulentata*, *P. pungens* and *P. turgidula* (Fig. 2) are usually considered as potentially domoic acid producers worldwide. Unidentified specimens corresponding to the *Pseudo-nitzschia pseudodelicatissima* species complex were also observed. The findings of *P. brasiliiana* and *P. dolorosa* represent new records for the Argentine Sea, and thus a brief morphological description is provided here.

Valves of *P. brasiliiana* were linear or linear-lanceolate, 16.7–42 μm long and 2.0–2.5 μm wide, with broadly rounded ends (Fig. 2G). Striae were present in the same number as fibulae (26 in 10 μm). There was no larger interspace in the central part of cell frustule.

Valves of *P. dolorosa* were lanceolate, 40–51.5 μm long and 2.5–2.8 μm wide, with round ends and presence of central interspace (Fig. 3A and B). Striae (33–36 in 10 μm) presented two rows of poroids (7–9 in 1 μm). Fibulae were less dense than striae (19–21 in 10 μm).

In addition, specimens with long and slender valves, 55–84 μm long and 1.5–2.5 μm wide, were also detected. The presence of fibulae and the central interspace could usually be observed by phase contrast light microscopy. Under SEM, these specimens showed striae with only one row of poroids (Fig. 3C–E). The number of striae was 34–40 in 10 μm and fibulae 16–22, delimiting a large central interspace. Poroids were disposed in numbers of 5–6 in 1 μm . The mantle was structured as the valve face, with height of two or three poroid widths. Bands and girdle details could not be observed. Given that neither transmission electron observations nor molecular analysis were carried out during this study, these specimens could only be assigned as part of the *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle species complex *sensu* Lundholm et al. (2003).

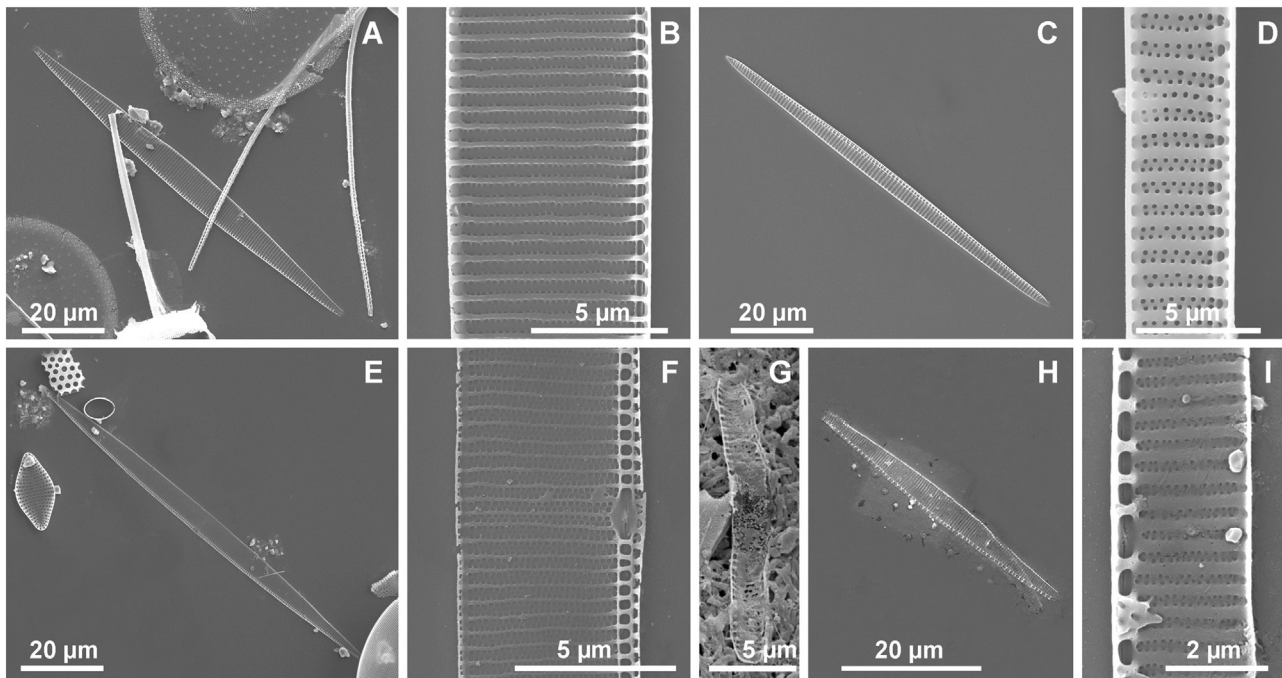


Fig. 2. Scanning electron (SEM) micrographs of potentially toxic *Pseudo-nitzschia* species observed from the Argentine Sea. (A and B) *P. australis*; (C and D) *P. pungens*; (E and F) *P. fraudulenta*; (G) *P. brasiliana*; (H and I) *P. turgidula*.

3.2. *Pseudo-nitzschia* species composition of natural plankton populations

3.2.1. Summer expedition

The genus *Pseudo-nitzschia* was observed in 70.8% of the Niskin bottle samples, with densities ranging between 80 and 13,840 cells L^{-1} (mean: 1.9×10^3 cells L^{-1} ; $n = 17$) and accounting for 0.2–51.6% of total diatom cell densities, with an average contribution of 8.1% (Fig. 4). The highest *Pseudo-nitzschia* cell densities and highest

contribution to diatom cell densities were observed around Valdés Peninsula (St 13–29 K2). In net samples, *Pseudo-nitzschia* cells were also observed at 70.8% of sampling stations, with densities ranging between 3×10^3 and 10.7×10^6 cells NT^{-1} (Fig. 5A). In fact, the density of *Pseudo-nitzschia* species in Niskin bottle and net samples showed a significant positive correlation ($r_s = 0.72$; $p < 0.05$).

Four *Pseudo-nitzschia* species were observed during this expedition: *P. brasiliana*, *P. australis*, *P. fraudulenta*, *P. pungens* and unidentified specimens of the *P. pseudodelicatissima* complex

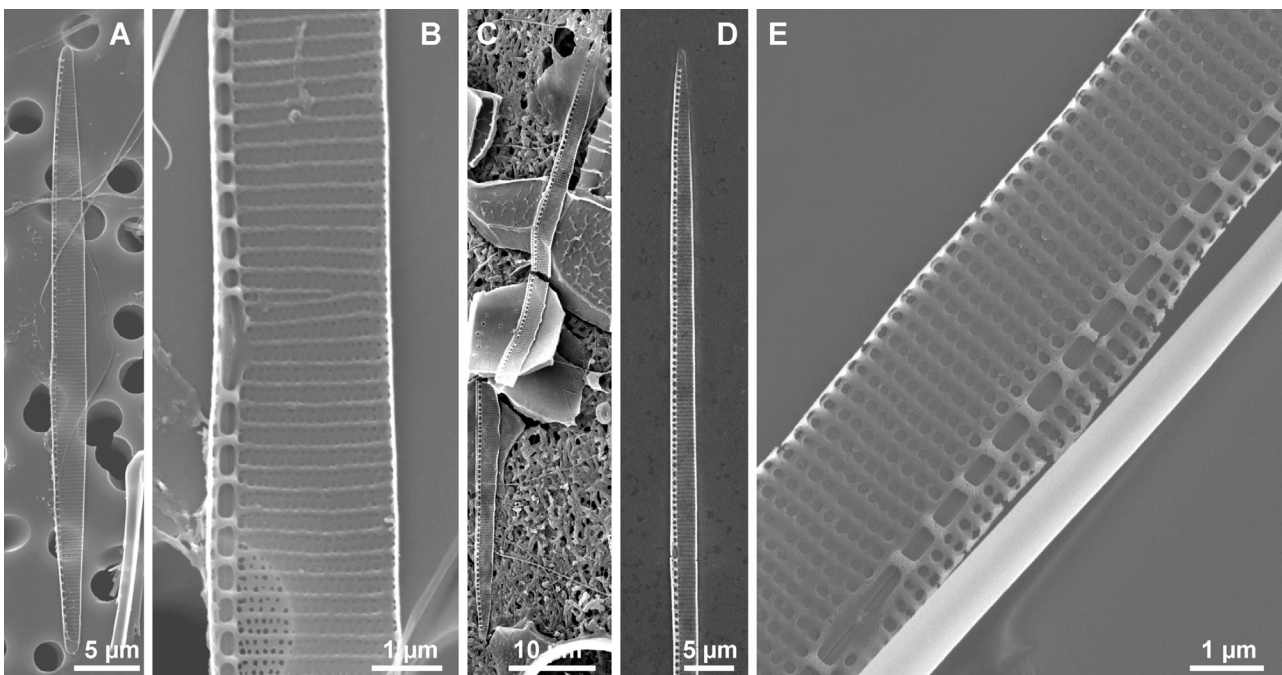


Fig. 3. Scanning electron (SEM) micrographs of other *Pseudo-nitzschia* species observed from the Argentine Sea. (A and B) *P. dolorosa*; (C–E) Specimens corresponding to the *Pseudo-nitzschia pseudodelicatissima* complex but not identified to species.

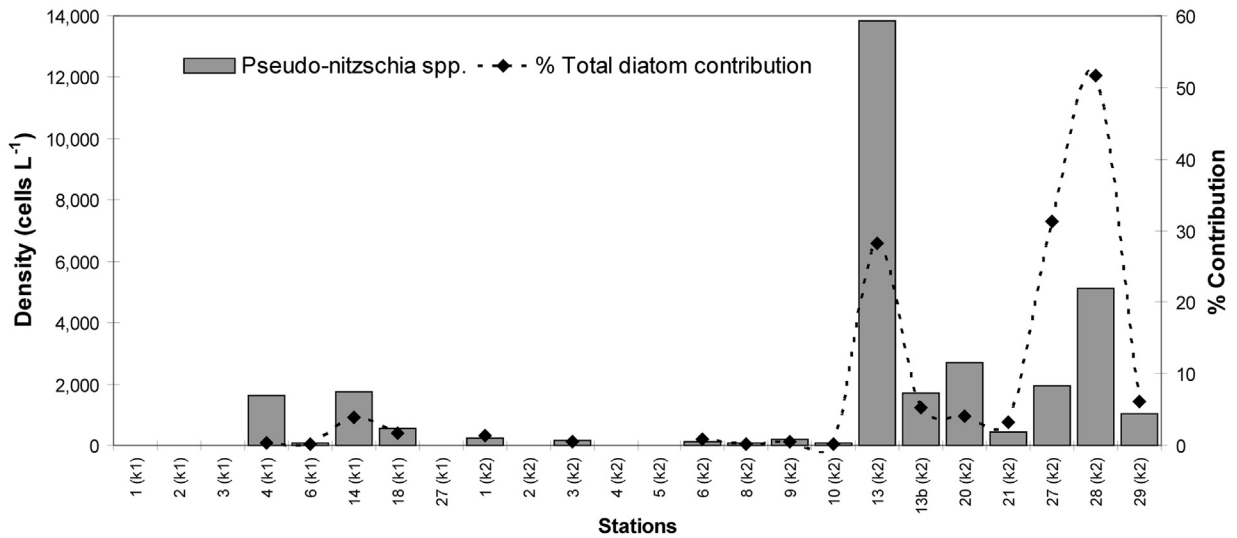


Fig. 4. Density of *Pseudo-nitzschia* (cells L⁻¹) and the relative contribution of this genus to total diatom cell densities based upon cell counts from Niskin bottle samples collected at surface waters during the summer expedition. K1 and k2 refers to different cruise legs.

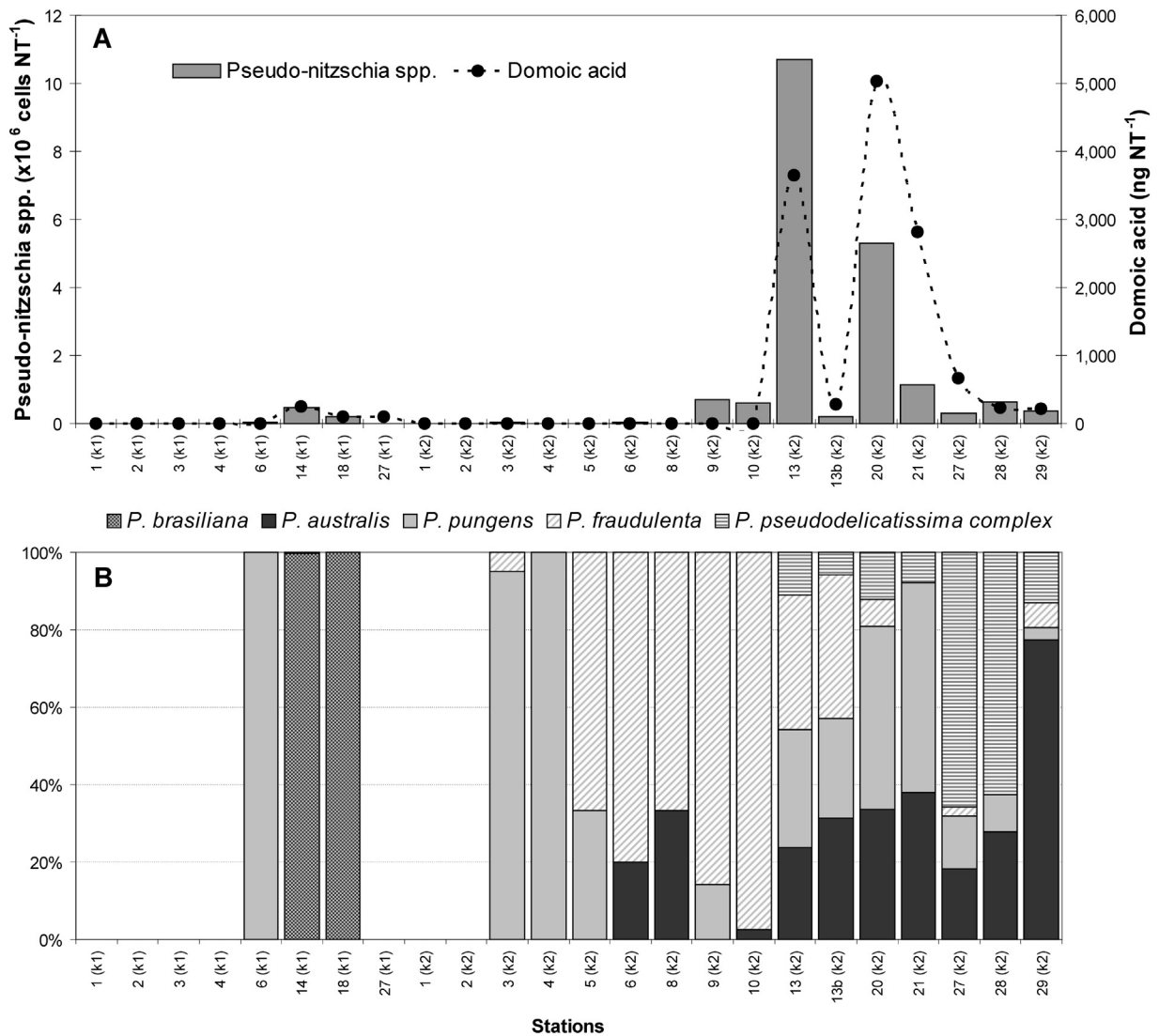


Fig. 5. Occurrence of *Pseudo-nitzschia* species and domoic acid in net tow (NT) samples collected during the summer expedition. (A) Cell densities of *Pseudo-nitzschia* species and concentration of domoic acid; (B) Relative cell abundance of *Pseudo-nitzschia* species. K1 and k2 refers to different cruise legs.

were also found. The most commonly occurring species were *P. pungens*, *P. fraudulenta* and *P. australis*, represented, respectively, in 54.2, 45.8 and 41.7% of net samples. Among these *Pseudo-nitzschia* species, *P. fraudulenta* and *P. pungens* were the most abundant in seven and five samples, respectively (Fig. 5B). In addition, these two species reached high percentages in those samples with the highest *Pseudo-nitzschia* cell densities recorded along this expedition (i.e., St 13, 20 and 21). Likewise, *P. brasiliana* and the unidentified species of the *P. pseudodelicatissima* complex represented the highest percentage of *Pseudo-nitzschia* taxa in two samples each, whereas *P. australis* predominated in only one sample.

Domoic acid was detected in 41.7% of net samples, with concentrations between 96 and 5041 ng NT⁻¹ (Fig. 5A). The highest DA concentrations were detected at St 13 and 20, which were also the stations where the highest cell densities of *Pseudo-nitzschia* species were found. In fact, DA concentrations and *Pseudo-nitzschia* species densities showed a significant positive correlation ($r_s=0.67$; $p < 0.05$). Nevertheless, low DA concentration was recorded at St 27 (K1), but no *Pseudo-nitzschia* cells were detected in either Niskin bottle or phytoplankton net samples.

In the northern area (≈ 39 – 41°S), low DA concentrations were detected together with the occurrence of *P. brasiliana* (St 14 and 18), which represented >99% of total *Pseudo-nitzschia* cells at both stations (Fig. 5B). Estimate cellular DA content in these samples was 0.45–0.56 pg cell⁻¹ if this species is considered as the unique DA source.

In the southern area, at St 13–29, south of San Matías Gulf and Valdés Peninsula, the DA concentrations were considerably higher (Fig. 5A). Domoic acid was detected in seven samples, in which the co-occurrence of *P. australis*, *P. pungens* and specimens of the *P. pseudodelicatissima* complex was always observed (Fig. 5). In addition, *P. fraudulenta* was also observed in five of those samples.

3.2.2. Spring expedition

The genus *Pseudo-nitzschia* was observed in 95.3% of the Niskin bottle samples, with densities ranging between 40 and 5.5×10^5 cells L⁻¹ (mean: 72,113 cells L⁻¹; $n=41$), and accounting for 0.003 to 99.3% of total diatom cell densities, with an average contribution of 32.0% (Fig. 6). The highest *Pseudo-nitzschia* densities ($>2.5 \times 10^5$ cells L⁻¹) were recorded in San Matías Gulf (St 19–24). The genus was mainly represented by *P. fraudulenta*, which accounted for

86.8–97.4% of total diatom density. In net samples, the genus *Pseudo-nitzschia* was observed at 86.0% of stations, with densities ranging between 40 and 98.6×10^6 cells NT⁻¹ (Fig. 7A). Densities estimated in bottle and net samples showed a positive significant correlation ($r_s=0.77$; $p < 0.05$).

At least eight *Pseudo-nitzschia* species were observed during this expedition: *P. australis*, *P. brasiliana*, *P. dolorosa*, *P. fraudulenta*, *P. lineola*, *P. pungens*, *P. turgidula*, *P. turgiduloides* and these were accompanied by unidentified specimens of the *P. pseudodelicatissima* complex. The most commonly occurring species were *P. fraudulenta* and *P. pungens*, present respectively in 79.1 and 74.4% of all net samples examined. In addition, *P. fraudulenta* was the most abundant species found during this expedition (Fig. 7B), reaching densities of up to 10^5 cells L⁻¹ and representing an average of 74.1% ($n=34$) of total *Pseudo-nitzschia* cells. By contrast, *P. pungens*, which also had a widespread distribution, represented an average of 10.1% ($n=32$) of the genus cell density.

Domoic acid was detected in 20.9% of net samples, with concentrations between 69 and 54,805 ng NT⁻¹ (Fig. 7A). High concentrations were found at only two sampling stations located in shelf waters south of Valdés Peninsula (St. 46) and in Nuevo Gulf (St. 47), where *Pseudo-nitzschia* cell densities were relatively low. By contrast, at stations 19–24, where high *Pseudo-nitzschia* cell densities were recorded, DA was almost completely absent (Fig. 7A). In fact, DA concentrations and *Pseudo-nitzschia* densities showed only a weak and non-significant correlation for the spring expedition ($r_s=0.27$; $p > 0.05$).

At stations 46 and 47, with high DA concentrations, *Pseudo-nitzschia* cell densities were dominated by *P. australis* (Fig. 7), which reached densities of up to 1.7×10^6 cells NT⁻¹. *Pseudo-nitzschia australis* was observed in other four samples during this expedition but at low densities. At St. 42, where low DA values were also recorded, the species density reached 5.6×10^3 cells NT⁻¹. In the other three samples (St 43–45), densities were lower than 100 cells NT⁻¹ and DA was not detected.

Rather low DA values were measured from other stations, but no clear association with a single *Pseudo-nitzschia* species could be detected. For example, at St 1, located in northern shelf-break waters, relatively low DA values (281 ng NT⁻¹) were detected. At this sampling station, *Pseudo-nitzschia* cells were dominated by specimens of the *P. pseudodelicatissima* complex, but *P. dolorosa*, *P. pungens* and *P. fraudulenta* were also observed. At St 3–5, in which a

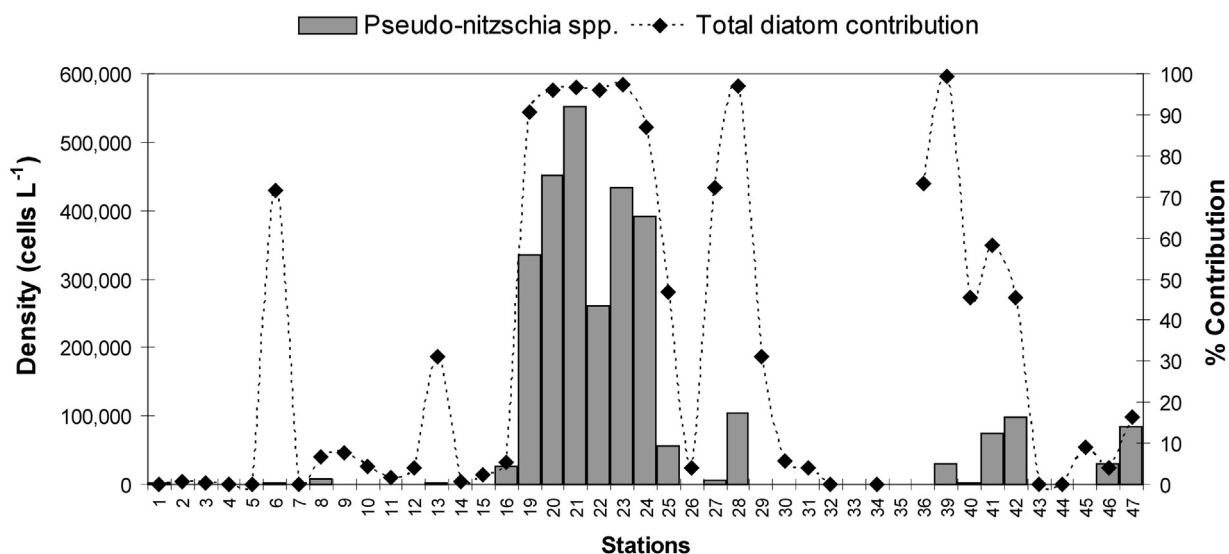


Fig. 6. Density of *Pseudo-nitzschia* (cells L⁻¹) and the relative contribution of this genus to total diatom cell densities based upon cell counts from Niskin bottle samples collected at surface waters during the spring expedition.

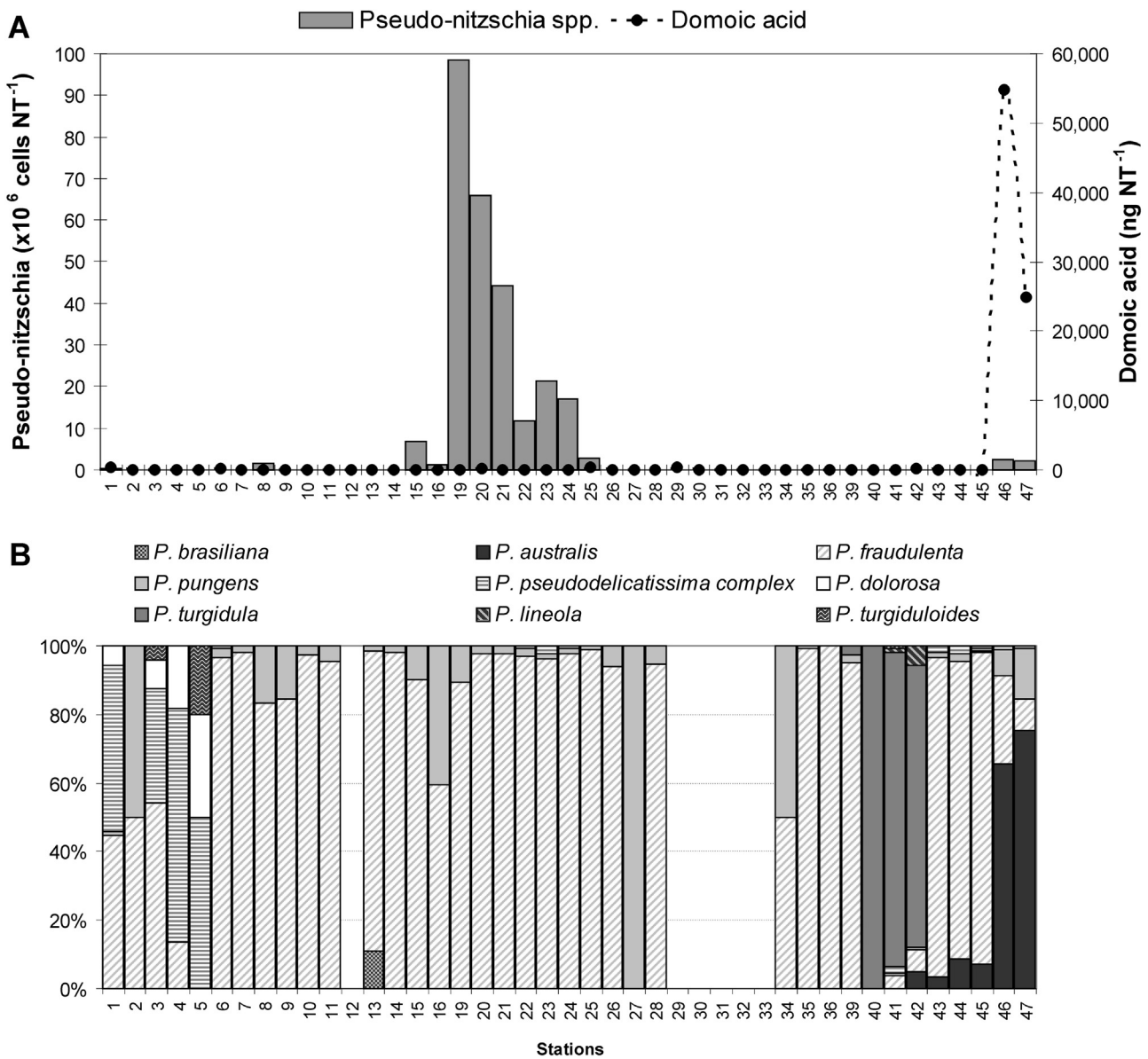


Fig. 7. Occurrence of *Pseudo-nitzschia* species and domoic acid in net tow (NT) samples collected during the spring expedition. (A) Cell densities of *Pseudo-nitzschia* species and concentration of domoic acid; (B) Relative cell abundance of *Pseudo-nitzschia* species.

large contribution of specimens of the *P. pseudodelicatissima* complex was observed, DA was not detected. At stations 6, 20 and 25, low DA values were recorded ($116\text{--}345\text{ ng NT}^{-1}$). In these samples, *P. fraudulenta* was the dominant *Pseudo-nitzschia* species, accounting for 96.5–99.0% of total cells and reaching densities from 8.8×10^4 to 6.4×10^6 cells NT⁻¹. This finding does not match with that for several other samples in which *P. fraudulenta* reached even higher densities (up to 9.9×10^6 cells NT⁻¹) but no DA was detected (Fig. 7). Very low DA values (69 ng NT^{-1}) were detected at St 27, from which *P. pungens* was the only *Pseudo-nitzschia* species recorded. Finally, at St 29, DA values of 323 ng NT^{-1} were detected, but no *Pseudo-nitzschia* cells were observed in net samples.

3.3. Correlation analysis

Spearman's correlation analysis showed that DA concentration was significantly correlated with cell densities of *P. australis* ($r_s = 0.55$), *P. pungens* ($r_s = 0.43$) and *P. pseudodelicatissima* complex ($r_s = 0.47$) (Table 1). Regarding the relationships between

Pseudo-nitzschia species and environmental factors, the correlation of *P. fraudulenta* with temperature was significantly positive ($r_s = 0.30$) and significantly negative ($r_s = -0.37$) for *P. australis*. In addition, densities of *P. fraudulenta* ($r_s = 0.62$) and *P. pungens* ($r_s = 0.60$) were positively correlated with salinity. No significant

Table 1

Spearman rank correlation coefficients for *Pseudo-nitzschia* species, domoic acid (DA), temperature (Temp) and salinity (Sal).

Species	DA	Temp	Sal
<i>P. brasiliiana</i>	0.13	-0.07	-0.16
<i>P. australis</i>	0.55**	-0.37**	0.01
<i>P. fraudulenta</i>	0.17	0.30**	0.62**
<i>P. pungens</i>	0.43**	0.15	0.50**
<i>P. pseudodelicatissima</i> complex	0.47**	-0.20	0.22
<i>P. dolorosa</i>	-0.02	-0.10	0.07
<i>P. turgidula</i>	-0.07	-0.02	0.25
<i>P. lineola</i>	0.06	-0.05	0.16
<i>P. turgiduloides</i>	-0.13	-0.03	0.14

** $p < 0.05$, $n = 67$.

correlations between DA concentration and temperature or salinity were found ($r_s = -0.23$ and -0.13 , respectively; $p > 0.05$).

4. Discussion

The number of described *Pseudo-nitzschia* species, as well as the number of confirmed DA producers in culture, has notably increased in the last few years (e.g., Lelong et al., 2012; Lim et al., 2013; Orive et al., 2013; Teng et al., 2016; Percopo et al., 2016). By contrast, the worldwide distribution of most of these species and their toxicity in natural populations is still poorly known. In the present work, we focused on the occurrence of *Pseudo-nitzschia* species and domoic acid throughout the Argentine Sea. In this region, DA has been previously detected but the identity of the planktonic producers remains unclear.

The genus *Pseudo-nitzschia* was widespread during this study, observed in $\approx 80\%$ of all examined samples with concentrations up to 5.5×10^6 cells L^{-1} . By contrast, domoic acid was detected in only $\approx 28\%$ of samples, which indicates that not all *Pseudo-nitzschia* species are consistently toxigenic in the Argentine Sea. This is not surprising given that only five of the nine identified taxa, *P. australis*, *P. brasiliana*, *P. fraudulenta*, *P. pungens* and *P. turgidula*, are considered as potentially toxic species (Lelong et al., 2012). However, these potentially toxic species represented an average of 96% of total *Pseudo-nitzschia* density. It is therefore possible that some of these species mentioned as DA producers in other parts of the world are represented by non-toxigenic genotypes in the Argentine Sea, or produce DA only under specific environmental conditions, such as nutrient depletion, elevated irradiance and pH (reviewed in Lelong et al., 2012) or is inducible by exposure to copepod grazing (Tammilehto et al., 2015).

Previous studies have revealed that numerous species of the genus *Pseudo-nitzschia* can usually be found in the Argentine Sea (Negri and Inza, 1998; Ferrario et al., 1999; Almandoz et al., 2007). Considering the first report of two species in the present study, the total number of *Pseudo-nitzschia* species found in the Argentine Sea has been raised to 13 (Table 2). This number is based only on detailed morphological examination of field specimens and no molecular approaches have yet been applied to examine species diversity. Given that a great number of new cryptic or pseudo-cryptic species have been described in the last 20 years, species richness in the Argentine Sea could even be considerably higher.

The usual co-occurrence of more than one (and up to five) *Pseudo-nitzschia* species in field samples makes it difficult if not impossible to associate DA production to individual species. Nevertheless, *P. australis* showed the highest correlation with DA concentration, followed by the complex *P. pseudodelicatissima* and then *P. pungens*, suggesting the importance of the former species as

a major DA producer in the Argentine Sea. Considering both cruises, DA was detected in all stations where *P. australis* was present at densities higher than 50,000 cells NT^{-1} . By contrast, high concentrations ($>500,000$ cells NT^{-1}) of *P. pungens* and the *P. pseudodelicatissima* complex were found in samples without detectable DA.

The key role of *P. australis* in the global production and distribution of DA has been confirmed in many studies. The production of DA by field populations of *P. australis* was first confirmed in Monterey Bay, California (USA) in 1991, during an ASP outbreak that caused massive deaths of pelicans (*Pelecanus occidentalis*) and cormorants (*Phalacrocorax penicillatus*) (Fritz et al., 1992; Garrison et al., 1992). Since then, numerous studies have confirmed DA production of this species in culture, as well as its association with poisoning episodes in marine birds and mammals (e.g., Míguez et al., 1996; Sierra-Beltrán et al., 1997; Fraga et al., 1998; Scholin et al., 2000; Cusack et al., 2002; Schnetzer et al., 2007; Fire et al., 2010; McCabe et al., 2016).

In Argentina, DA was first recorded in July 2000 from continental shelf waters next to the city of Mar del Plata ($\approx 38^\circ S$). At that time, during a bloom of *Pseudo-nitzschia australis*, DA was detected in phytoplankton, mussels (*Mytilus edulis*) and anchovies (*Engraulis anchoita*) (Negri et al., 2004). Moreover, DA was detected in phytoplankton samples from San Jorge Gulf, associated with the presence of both *P. australis* and *P. pungens* (Krock et al., 2015). In our study, the association of DA and high cells densities of *P. australis* was mainly evident in shelf waters south of Valdés Peninsula and in Nuevo Gulf during spring. Assuming *P. australis* as the only DA producer in these samples, cellular DA content was 14.6–32.3 pg cell $^{-1}$, whereas if total *Pseudo-nitzschia* cells are considered these cell quotas would be reduced to 11.0–21.2 pg cell $^{-1}$. In either case, these quotas represent the higher values observed during our study and are in the range of the highest DA cell content measured in *P. australis* cultures (22, 27 and 37 pg cell $^{-1}$) by Guannel et al. (2011), Cusack et al. (2002) and Garrison et al. (1992), respectively.

Pseudo-nitzschia fraudulenta, together with *P. pungens*, was the most commonly observed species during this study. In addition, it bloomed in San Matías Gulf (St 19–24) during spring, accounting for more than 95% of total diatom abundances. However, only low DA values were detected in two of the seven samples with high densities of *P. fraudulenta* ($\approx 50,000$ – $500,000$ cells L^{-1}). This suggests that this species may be only weakly toxigenic in the Argentine Sea, as was previously found in New Zealand coastal waters (Rhodes et al., 1998a, 1998b). The DA cell quotas estimated from the two samples in which DA was detected together with a predominance of *P. fraudulenta* ($\approx 98\%$ of total *Pseudo-nitzschia*) were 0.002 and 0.120 pg cell $^{-1}$. These values are comparable to DA cell quotas obtained in cultures of *P. fraudulenta* by Rhodes et al. (1998a,b) (maximum 0.03 pg cell $^{-1}$) and Thessen et al. (2009) (maximum 0.16 pg cell $^{-1}$). Despite these low toxin levels, DA has been previously detected in phytoplankton net samples (up to 6.6 $\mu g L^{-1}$) collected in San José Gulf and Nuevo Gulf, coinciding with high cell densities of *P. fraudulenta* (Sastre et al., 2007). During the period when DA was detected, Sastre et al. (2007) observed *P. fraudulenta* at densities up to 24,200 cells L^{-1} in a period of strong nutrient depletion that reduced nitrate and silicate concentration to below detection limits. This is consistent with the finding that DA production by *P. fraudulenta* notably increases under silicate-limited conditions (Tatters et al., 2012).

Pseudo-nitzschia brasiliana, a known warm water species, has been observed in Brazil (Sepetiba Bay), Gulf of Panama, Gulf of Mexico, Gulf of California, Vietnam, Indonesia, Thailand and South Korea (Lundholm et al., 2002), and more recently also in Spain (Quijano-Scheggia et al., 2005), China (Li et al., 2010), Tunisia (Sahraoui et al., 2011) and Malaysia (Lim et al., 2012a,b). In the

Table 2

List of *Pseudo-nitzschia* species recorded from the Argentine Sea, including first reports from the present study.

Species	Reference
<i>P. americana</i>	Almandoz (2008)
<i>P. australis</i>	Negri and Inza (1998)
<i>P. brasiliana</i>	this study, first report
<i>P. dolorosa</i>	this study, first report
<i>P. fraudulenta</i>	Negri and Inza (1998)
<i>P. heimii</i>	Almandoz et al. (2007)
<i>P. lineola</i>	Almandoz et al. (2007)
<i>P. multiseriis</i>	Ferrario et al. (1999)
<i>P. pseudodelicatissima</i> complex	Ferrario et al. (1999)
<i>P. pungens</i>	Negri and Inza (1998)
<i>P. subcurvata</i> ^a	Almandoz et al. (2007)
<i>P. turgidula</i>	Negri and Inza (1998)
<i>P. turgiduloides</i>	Almandoz et al. (2007)

^a Identification not confirmed by electron microscopy.

present study, *P. brasiliensis* was recorded for the first time in the Argentine Sea, extending its known worldwide distribution southwards. This species was only observed in a few samples collected in the northern part ($\approx 39\text{--}40^\circ\text{S}$) of both sampling expeditions, at seawater temperatures around 13°C (spring) and 16°C (summer). During summer, when it reached higher densities, it was associated with DA occurrence, at estimated cell quotas of $0.45\text{--}0.56\text{ pg cell}^{-1}$. This is an interesting finding because the first toxin analyses of strains of *P. brasiliensis* isolated from Sepetiba Bay (Brazil) did not detect the presence of DA (Lundholm et al., 2002). The lack of DA production was then confirmed in several strains isolated from the Mediterranean Sea (Quijano-Scheggia et al., 2010), south-eastern coast of China (Wang et al., 2012) and South China Sea of Malaysian Borneo (Lim et al., 2012a). Nevertheless, the toxigenicity of *P. brasiliensis* was detected by Sahraoui et al. (2011), based upon the analysis of strains isolated from Bizerte Lagoon, Tunisia. In addition, the same authors also associated the occurrence of DA in field samples with the presence of *P. brasiliensis* (Sahraoui et al., 2012). Thus, our current results agree with the recent findings of *P. brasiliensis* as a DA producer. The estimated cell quotas of $0.45\text{--}0.56\text{ pg cell}^{-1}$ in field samples are higher than those previously detected in culture (8.9 fg cell^{-1}) by Sahraoui et al. (2011), but represent low values when compared with other toxigenic *Pseudo-nitzschia* species (Trainer et al., 2012).

Pseudo-nitzschia turgidula, another potentially toxic species found in this study, showed a restricted distribution, being confined to colder ($\approx 7\text{--}8^\circ\text{C}$) southern shelf-break waters sampled during spring, which is in accordance with previous results in the Argentine Sea (Almandoz et al., 2007). Toxigenic strains of *P. turgidula* have been isolated in New Zealand and at Ocean Station PAPA (50°N , 145°W) in the north-eastern Pacific, but they presented only low DA cell quotas (5×10^{-6} to 0.03 pg cell^{-1}) (Rhodes et al., 1998b; Trick et al., 2010). During our study, *P. turgidula* was observed in low cell densities and its distribution was not associated with DA occurrence. However, intense blooms of this species (up to $3.3 \times 10^6\text{ cells L}^{-1}$) have been found in northern shelf waters of the Argentine sea by Negri and Inza (1998), although no DA analyses were carried out during this bloom.

Pseudo-nitzschia dolorosa is a recently described species (Lundholm et al., 2006), which morphologically resembles *P. turgidula*. Both species can be mainly differentiated by examination of the number of striae and fibulae, which usually requires analysis by electron microscopy. Besides its original description, which confirmed its occurrence in Europe and Monterey Bay, California (Lundholm et al., 2006), it was also mentioned in a few studies carried out in the Drake Passage (Ferrario et al., 2004), northeast subarctic Pacific (Marchetti et al., 2008), the coasts of Portugal (Churro et al., 2009) and Malaysian Borneo (Lim et al., 2012a). In our study, it was confined to northern shelf-break waters sampled during spring, with temperatures of $8.1\text{--}9.7^\circ\text{C}$ and salinities from 33.7 to 33.9. During its previous finding in the Drake Passage (south of South America), it was observed in very low densities at water temperatures of 6.4°C and salinity of 34.0 (Ferrario et al., 2004).

The finding of DA in two samples in which *Pseudo-nitzschia* cells were not detected could be interpreted in different ways. First, the fact that only low levels of DA were detected, in both samples from summer and spring expeditions, respectively, could indicate that even if *Pseudo-nitzschia* cells were present, they were at densities below the detection limits of microscopic analysis. Second, although *Pseudo-nitzschia* species are considered to be by far the major DA producing diatoms, other species, such as *Halamphora coffeiformis* (Agardh) Levkov (previously known as *Amphora coffeaeformis*), *Nitzschia navis-varingica* (Lundholm and Moestrup, 2000; Kotaki et al., 2004; Romero et al., 2011; Tan et al., 2016) and *N. bizertensis* (Smida et al., 2014) have also been found to produce

DA. In any case, these benthic species are mainly found in brackish waters and were not detected in the present study in the Argentine Sea. Finally, DA could have been accumulated in zooplankton feeding on toxic *Pseudo-nitzschia* (Tester et al., 2001; Maneiro et al., 2005), such that intact diatom cells were no longer identifiable. Thus, even when plankton samples were pre-filtered through a $200\text{ }\mu\text{m}$ mesh to remove large zooplankton before toxin analysis, the presence of DA accumulated in early life stages of zooplankton, their eggs or fecal pellets cannot be excluded.

Mariculture production in Argentina is an increasing activity with great future potential, given the large extension of its coast line (Kapetzki et al., 2013). Currently, there are several shellfish harvesting areas and also some shellfish culture sites, mainly located in the North Patagonian gulfs (RMCP, 2013). The wide distribution of several potentially toxic *Pseudo-nitzschia* species and particularly *P. australis* in Argentina, from northern waters of the Buenos Aires Province to southern waters of the Beagle Channel (e.g. Almandoz et al., 2007, 2011), could represent a potential risk for DA accumulation in shellfish. It is expected that the information here provided on the association of *Pseudo-nitzschia* species and DA occurrence provides a useful dataset to future monitoring programs.

5. Conclusions

Despite the wide distribution of *Pseudo-nitzschia* species observed in the Argentine Sea during spring and summer, the occurrence of DA was confined to a low percentage of samples. From the at least nine *Pseudo-nitzschia* species found throughout this study, DA occurrence was mainly associated with the presence of *P. australis*. By contrast, other potentially toxic species, such as *P. fraudulenta*, *P. pungens* and *P. turgidula* did not show a clear association with DA or showed low toxin levels. The complete biogeographical association of toxin production and species composition remains unresolved for the Argentine Sea, and additional field and culture studies are required to elucidate the pattern of DA production in this region.

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