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MORPHOLOGICAL, MOLECULAR, AND TOXIN ANALYSIS OF FIELD POPULATIONS OF *ALEXANDRIUM* GENUS FROM THE ARGENTINE SEA[1]

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In the Argentine Sea, blooms of toxigenic dinoflagellates of the *Alexandrium tamarense* species complex have led to fish and bird mortalities and human deaths as a consequence of paralytic shellfish poisoning (PSP). Yet little is known about the occurrence of other toxigenic species of the genus *Alexandrium*, or of their toxin composition beyond coastal waters. The distribution of *Alexandrium* species and related toxins in the Argentine Sea was determined by sampling surface waters on an oceanographic expedition during austral spring from ~39°S to 48°S. Light microscope and SEM analysis for species identification and enumeration was supplemented by confirmatory PCR analysis from field samples. The most frequent *Alexandrium* taxon identified by microscopy corresponded to the classical description of *A. tamarense*. Only weak signals of Group I from the *A. tamarense* species complex were detected by PCR of bulk field samples, but phylogenetic reconstruction of rDNA sequences from single cells from one station assigned them to ribotype Group I (*Alexandrium catenella*). PCR probes for *Alexandrium minutum* and *Alexandrium ostenfeldii* yielded a positive signal, although *A. minutum* morphology did not completely match the classical description. Analysis of PSP toxin composition of plankton samples revealed toxin profiles dominated by gonyautoxins (GTX1/4). The main toxic cyclic imine detected was 13-desMe-spirolide C and this supported the association with *A. ostenfeldii* in the field. This study represents the first integrated molecular, morphological and toxinological analysis of field populations of the genus *Alexandrium* in the Argentine Sea.

***Key index words:* *Alexandrium minutum*; *Alexandrium ostenfeldii*; LC-FD; LC-MS/MS; PCR; PSP; southwestern Atlantic; spirolides**

***Abbreviations*: APC, apical pore complex; GTX, gonyautoxin(s); LC-FD, liquid chromatography with fluorescence detection; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; NeoSTX, neosaxitoxin; Po, apical pore plate; PSP, paralytic shellfish poisoning; PST, paralytic shellfish toxin(s); Sa, anterior sulcal plate; Sp, posterior sulcal plate; SPX, spirolide(s); STX, saxitoxin; vp, ventral pore**

Among toxic marine dinoflagellates, the genus *Alexandrium* is one of the best studied because of its tendency to form harmful algal blooms and its broad biogeographical distribution from polar, temperate, and subtropical waters worldwide (Anderson et al. 2012). From a morphological perspective, the genus *Alexandrium* is rather homogeneous, therefore detailed analysis of several characters such as cell size and shape, chain formation, sulcal list development, sulcal excavation, and size and shape of the thecal plates (mainly Po, 1′, 6″, Sp and Sa) are needed for species identification (Balech 1995). Nevertheless, attempts to define consistent morphospecies within the genus have not always been successful and remain controversial (John et al. 2014).

With the advent of molecular techniques, the classification of *Alexandrium* species has been revised by several authors. Phylogenetic analyses identified well-supported clades within the genus, although these were not always consistent with classical species descriptors (Penna et al. 2008, Anderson et al. 2012). Phylogenetic analyses inferred by maximum likelihood analysis of partial sequences of the LSU rDNA gene showed the existence of three well-supported complexes of species: the *Alexandrium ostenfeldii* group, the *Alexandrium minutum* group, and the *Alexandrium tamarense* species complex (John et al. 2003, Lilly et al. 2007, Anderson et al. 2012).

Within the *Alexandrium tamarense* species complex, phylogenetic analysis has revealed distinct clusters

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of ribotypes. A comprehensive phylogeny constructed from a broad data set of ribosomal operon sequences yielded ribosomal phylogenetic trees that always recovered five distinct groups, showing genetic distances separating the Groups as large as those observed between other *Alexandrium* species (Wang et al. 2014). A detailed examination of the morphological criteria previously proposed for taxonomic identification demonstrated that most features are shared or variable among the five respective ribosomal groups, rendering them unsuitable for species definition and discrimination (John et al. 2003, 2014, Lilly et al. 2007, Anderson et al. 2012). In particular, Group I showed intragenomic rDNA variants dispersed across strains in the phylogenetic tree uniting the many different genotypes to the same or closely related populations (Miranda et al. 2012). Recently, named species have been proposed to describe the five ribotypes (John et al. 2014). The nomenclature of Group I has generated debate with different opinions on how to rename the Group I members (John et al. 2014, Fraga et al. 2015); the Nomenclature Committee for Algae (Prud'homme van Reine 2017) has finally established *Alexandrium catenella* as the valid name for this group based upon nomenclatural priority.

Around half of the ~30 morphologically described species of the genus *Alexandrium* are known to produce toxins (Anderson et al. 2012). Most dangerous for health are the paralytic shellfish toxins (PST), which are produced by certain members of the *A. tamarense* species complex and *A. minutum*, and are responsible for human illness and even deaths of human seafood consumers and economic losses in aquaculture and fisheries (Boesch et al. 1997, Lagos 2003, García et al. 2004). This PST family of tetrahydropurine neurotoxins consists of saxitoxin (STX) and more than three dozen naturally occurring analogs. Among paralytic shellfish poisoning (PSP) toxins, the nonsulfated carbamoyl toxins STX and neosaxitoxin (neoSTX) are the most potent blockers of voltage-gated Na^+-ion channels, followed by the sulfated carbamoyl toxins, such as the gonyautoxins (GTX), then the decarbamoyl (dc-) toxins, and finally the N-sulfocarbamoyl (B and C toxins) as the least toxic group (Wiese et al. 2010).

In addition to the frequent presence of PST among populations of *Alexandrium ostenfeldii*, other toxins linked to this species include cyclic imine neurotoxins (Cembella et al. 2001, Franco et al. 2006), particularly spirolides (SPX). Even though to date, no human cases of shellfish poisoning have been associated with SPX, strong neurotoxic symptoms have been observed when SPX analogs are administrated intraperitoneally into laboratory rodents (Guéret and Brimble 2010). Recently, other toxic cyclic imines, including gymnodimines, previously found only in the dinoflagellate *Karenia selliformis*, were detected in *A. ostenfeldii* from U.S. coastal estuaries (reported as *Alexandrium peruvianum* by Van Wagoner et al. 2011 and Borkman et al. 2012), from the Netherlands (Van de Waal et al. 2015) and from the Baltic Sea (Salgado et al. 2015, Harju et al. 2016).

Particularly in western South Atlantic waters, PSP events and the presence of several toxic species of *Alexandrium* have been known for many decades (Balech 1995). Toxic outbreaks are documented from Argentina, Uruguay, and southern Brazil (e.g., reported by Davison and Yentsch 1985, Carreto et al. 1986, Brazeiro et al. 1997, Odebrecht et al. 2002). In the Argentine Sea, blooms of the *A. tamarense* species complex have been frequently detected during spring since 1980 (Carreto et al. 1981, Esteves et al. 1992, Gayoso and Fulco 2006), leading in some cases to fish and bird mortalities and also to human deaths (Elbusto et al. 1981, Vecchio et al. 1986, Montoya et al. 1996, 1998, Montoya and Carreto 2007). In southern Brazil coastal waters, the first *Alexandrium* bloom was recorded in 1996 (Odebrecht et al. 2002). Specimens of the *A. tamarense* species complex were reported as belonging to Group I, now recognized as *A. catenella* (Prud'homme van Reine 2017), in Argentina (Penna et al. 2008) and Brazil (Persich et al. 2006). The PST producer *Gymnodinium catenatum* was also recorded in northern Argentinean waters (Balech 1995) and it was recently associated with PSP events (Sunesen et al. 2014). The causative species of a toxic bloom reported from southeastern Brazil near Rio de Janeiro (Menezes et al. 2007) was identified at the time as *A. minutum*, although the authors observed the presence of a strong reticulation on the hypotheca that is not found in *A. minutum*. Further analysis has suggested that this may represent a new species with the proposed name *Alexandrium fragae* (Branco et al. 2016). Along the Uruguayan coast, members of the *A. tamarense* species complex have also been frequently found since 1980 (Davison and Yentsch 1985, Brazeiro et al. 1997, Méndez et al. 2000). The more distantly related, *Alexandrium fraterculus* is typically nontoxic but has been associated with PSP events from Uruguayan waters (Méndez 1993).

Toxic strains of *Alexandrium ostenfeldii* have been obtained from the Beagle Channel, southern Argentina (Almandoz et al. 2014), but *A. ostenfeldii* has not been previously reported from open shelf and slope waters of the Argentine Sea. Nevertheless, an associated spirolide toxin (SPX-1) has been detected in very low concentrations in shellfish harvested from Santa Cruz, Chubut, Rio Negro, and Buenos Aires Provinces (Turner and Goya 2015), suggesting a widespread distribution of this dinoflagellate in the study area.

Until now, the only genetic characterization of *Alexandrium* species from the Argentine Sea comes from a unique strain of *A. catenella* (Group I of the *A. tamarense* species complex; Penna et al. 2008), isolated from northern coastal waters (Carreto et al.

2001). Toxin profiles were established for field populations from coastal waters adjacent to Mar del Plata (~38°S) and Patagonia (~43°S–50°S; Montoya et al. 2010) and for cultured strains of the *A. tamarense* species complex (Carreto et al. 2001, Montoya et al. 2010, Krock et al. 2015). By contrast, little is known about the occurrence of other toxigenic species of the genus *Alexandrium*, or about their toxin content and composition in natural populations outside of coastal waters. The shelf margin of the Argentine Sea is characterized as a very productive zone due to its specific oceanographic and ecological conditions (Acha et al. 2004, Matano et al. 2010, Piola et al. 2010, Carreto et al. 2016). The presence of other species of toxigenic dinoflagellates has been reported along the shelf margin (Akselman et al. 2014, Tillmann and Akselman 2016). The present study addresses two main objectives: (i) to establish the general pattern of diversity and geographical distribution of *Alexandrium* species in the Argentine Sea between 39°S and 48°S; and (ii) to determine PST and SPX composition in natural plankton populations and their association with *Alexandrium* species in this region. This work represents the first holistic attempt to integrate molecular genetics and morphological identification of *Alexandrium* species with toxin composition in field populations from the Argentine Sea.

MATERIALS AND METHODS

Plankton sampling of field populations. Plankton from the continental shelf and slope waters of the Argentine Sea were sampled during an expedition onboard R/V Puerto Deseado carried out in austral spring from October 26 to November 9, 2013. A total of 44 sampling stations were located between ~39°S and 47°S. Physical data on conductivity (salinity)/temperature/depth were determined with a profiling conductivity-temperature-density device (Sea Bird SBE 911 plus; Bellevue, WA, USA) for each sampling station. Niskin bottles were deployed at 3 and 10 m depth; 2.5 L of seawater from each depth were pooled, from which 250 mL aliquots were taken for determination of total plankton community composition and for qualitative and quantitative analysis of potentially toxic species.

Plankton net samples were collected by vertical net tows (NT) through the upper 20 m of the water column with a 20 μm-mesh Nitex net. Net hauls were taken for analysis of bulk toxin concentrations and to correlate cell abundances of *Alexandrium* species with the respective toxin content and composition in field samples. Each net haul concentrate was adjusted to 1 L with 0.2 μm-filtered seawater, of which 100 mL was fixed with acidic Lugol's iodine solution for species identification by microscopy. The rest was sequentially size-fractionated through Nitex mesh of 200, 50, and 20 μm by gravity filtration. The particulate material retained on each mesh was resuspended in 40 mL of filtered seawater and transferred into 50 mL centrifuge tubes. Each size-fraction concentrate was split into four 10 mL aliquots. Three aliquots were centrifuged at 3,220*g* for 15 min at room temperature. The pellets were stored at −20°C for later analysis of PST and cyclic imine toxins and rDNA sequences. The remaining 10 mL of cell concentrate from each size-fraction was fixed with 1.5 mL of acidic Lugol's iodine solution for enumeration of putatively toxic species for the comparison of cell densities and toxin concentration.

Phytoplankton cell enumeration and morphological identification. Morphological examination was conducted by phase-contrast, differential interference contrast and UV epifluorescence microscopy with a Leica DM2500 microscope equipped with a DFC420C camera (Leica Microsystems GmbH, Wetzlar, Germany). The cells were crushed with an awl to release the cytoplasm from the thecae for plate pattern and plate shape descriptions by light microscopy. Additionally, cells were stained with the optical brightener calcofluor-white for epifluorescence microscopic observations of the thecal plates (Fritz and Triemer 1985).

For SEM analysis, samples were filtered onto 0.2 μm polyamide filters or onto 3 μm polycarbonate filters and dehydrated through an ethanol dilution series (25%, 50%, 75%, 100%) with final critical point dehydration. Specimens were sputter-coated with Au-Pd prior to examination under two electron microscopes, a Jeol JSM-6360 LV SEM (JEOL, Tokyo, Japan) and an FEI Quanta (FEG 200, Eindhoven, the Netherlands).

Cell abundance of *Alexandrium* in fractionated NT concentrates was determined by counting 1 mL of Lugol's iodine-fixed samples from each fraction size in a Sedgewick-Rafter chamber (LeGresley and McDermott 2010) with an inverted phase-contrast optical microscope (Leica DMIL LED; Leica Microsystems GmbH). These data (cells · NT^{-1}) served for semi-quantitative comparison of cell densities and toxin concentrations. The limit of detection of the counting method was 40 cells · NT^{-1}.

Cell abundance of *Alexandrium* species in water samples collected by Niskin bottles was determined according to the Utermöhl (1958) inverted microscope counting method. Subsamples (50 mL) from the Niskin bottles, fixed with acidic Lugol's iodine solution, were left to settle for 24 h in a composite sedimentation chamber prior to counting. The limit of detection of this method was 20 cells · L^{-1}.

Given the difficulty to differentiate between certain *Alexandrium* species by routine microscopic analysis, *A. tamarense* species complex and *A.* aff. *minutum* were pooled during cell counting of net and bottle samples. By contrast, *A. ostenfeldii* was more readily discriminated as a distinct species based on its globose shape and typically larger cells than for *A.* aff. *minutum* and members of the *A. tamarense* species complex. The identity of *A. ostenfeldii* cells was additionally confirmed by detailed microscopic observations of the first apical (1′) thecal plate, including the presence, size, and shape of the characteristic ventral pore (vp), from 5 to 15 cells from each bottle or net sample. The relative contribution of *A. tamarense* and *A.* aff. *minutum* cells in net samples was estimated by detailed analysis of the shape and size of the sixth precingular (6″) and posterior sulcal (Sp) plates of all *Alexandrium* cells present in a drop of subsample cell concentrate (2–15 cells for each station).

Toxin extraction and analysis. Analysis of multiple lipophilic toxins, with focus on the cyclic imine toxins including spirolides, was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS; AB Sciex 4000 with Q-Trap, Darmstadt, Germany), as described in Krock et al. (2008). Spirolides detectable by this method were SPX-A (*m/z* 692 > 150), SPX-1 and SPX-G (*m/z* 692 > 164), SPX-B (*m/z* 694 > 150), 13-desmethyl SPX-D (*m/z* 694 > 164), SPX-C and 20-methyl SPX-G) (*m/z* 706 > 164) and SPX-D (*m/z* 694 > 164). Cell pellets from the plankton NT 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*g* at 4°C for 15 min. The supernatant was transferred to a 0.45 μm pore-size spin-filter (Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at 800*g*, followed by transfer of the filtrate to autosampler vials for LC-MS/MS analysis.

Analysis of PSP toxins was performed after separation of target analytes in reversed-phase mode by high-performance liquid chromatography with postcolumn derivatization and fluorescence detection (LC-FD) according to Krock et al. (2007). The LC-FD analysis was carried out on a LC1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a PCX 2500 postcolumn derivatization system (Pickering Laboratories, Mountain View, CA, USA). The LC-system consisted of a G1379A degasser, a G1311A quaternary pump, a G1229A autosampler, a G1330B autosampler thermostat, a G1316A column thermostat and a G1321A fluorescence detector. The limit of detection by this fluorescence method for the PST with the highest detection limit, i.e., the N1-OH carbamoyl derivatives (GTX1/4), was 1.5 ng · NT^{-1}.

Toxin cell quotas. Cell quotas of spirolides and PST were estimated as total toxin content from two size-fractions (20–50 μm and 50–200 μm) divided by the total number of *Alexandrium* cells in the same pooled fractions. The >200 μm size-fraction was not considered for combined analysis of cell densities and toxin concentration in the net samples, as this size-fraction represented <0.7% of both *Alexandrium* cells and total toxin content of NTs. Only those samples with cell abundances >10,000 cells · NT^{-1}, based on the precision and accuracy estimates of cell counting methods reported in ICES (2006), were considered for cell quota calculations. Given the difficulties in distinguishing between *A. tamarense* and *A.* aff. *minutum* in routine cell counting, these species were considered together for PSP toxin cell quota estimates. In the case of spirolides, the calculations were based only on abundance of *A. ostenfeldii*, which was assumed to be the exclusive source of these toxins.

Molecular analysis by polymerase chain reaction of Alexandrium *in bulk field plankton samples.* Deficiencies in sample acquisition, handling, and archiving led to considerable degradation of plankton DNA. Accordingly, reliable molecular analysis could only be performed for a total of 11 net samples containing sufficient intact DNA, corresponding to the 20–50 μm size-fractions of St 2, 3, 4, 5, 6, 7, 9, 14, 27, 43, and 44. Although sample DNA quality was not adequate for full quantitative analyses, qPCR assays were performed to detect and differentiate *Alexandrium* species within the plankton communities, as described in Toebe et al. (2013). DNA extracted from plankton samples was diluted to 1 ng DNA per reaction. All qPCR experiments were performed in triplicate on a StepOnePlus Real Time PCR System (Applied Biosystems, Darmstadt, Germany) in a final volume of 10 μL reaction mixture, containing 5 μL of a 2× TaqMan Fast Universal PCR Master Mix with AmpliTaq Fast DNA polymerase and dNTPs and the passive reference dye ROX (Applied Biosystems). The qPCR cycle conditions were as follows: Stage 1, Step 1: hold 50°C for 2 min, Stage 2, Step 2: hold 95°C for 20 s, followed by 40 cycles of Stage 3, Step 1: hold 95°C for 1 s, Step 2: hold 60°C for 20 s.

A standard dilution series for *Alexandrium* species was prepared from genomic DNA of a known cell concentration, diluted from 10 ng to 10 fg. Primers were added at a final concentration of 900 nM each and TaqMan MGB probes labeled with 6FAM (6-carboxyfluorescein) were at a final concentration of 200 nmol. The TaqMan assays specific for three *Alexandrium* species sensu John et al. (2014) (*A. catenella*=*A. fundyense*, Group I; *A. mediterraneum*, Group II; *A. tamarense*, Group III) of the *A. tamarense* species complex, *A. tamutum*, and *A. minutum* were applied as described in Toebe et al. (2013). The assay for *A. ostenfeldii* and *A. pacificum*, Group IV of the *A. tamarense* species complex, was performed as in Elferink et al. (2016).

Single-cell PCR and phylogenetic analyses of the A. tamarense *species complex.* Single cell picking of members of *Alexandrium* species by micropipette isolation was performed with an inverted microscope (Axiovert 200M; Zeiss, Göttingen, Germany) under 200× magnification. A total of sixteen cells were randomly selected from the 20–50 μm size-fraction collected by NT from St 27. For PCR amplification, cells were incubated for 10 min at 95°C, then frozen again and thawed. The PCR mix included the following primers for the forward and reverse LSU rDNA amplification: D1R-F (5-ACC CGC TGA ATT TAA GCA TA-3) and D2C-R (5-CCT TGG TCC GTG TTT CAA GA-3), respectively. The PCR reaction conditions were as follows: HotMasterTaq® (5Prime, Hamburg, Germany) buffer 1×, 0.1 mM of dNTPs, 0.1 mM each of forward and reverse primer and 1.25 units of Taq polymerase were added to selected cells, in total reaction volumes of 50 mL. The reactions were subjected to the following thermo-cycling conditions: one cycle of 95°C for 7 min; 35 cycles 94°C for 45 s, 54°C for 2 min, 70°C for 1.5 min, plus a final extension at 70°C for 5 min.

The resulting PCR amplicons were subsequently cloned into a pCR®2.1-TOPO® vector according to the protocol of the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA). Three to six clones per PCR amplicon was picked and sequenced from both ends by standard sequencing chemistry on an ABI 3130 XL capillary sequencer (Applied Biosystems), with the universal M13 forward and reverse primers supplied with the cloning kit. After removal of vector and primer sequences, the forward and backward sequences were assembled with CLC Main Workbench 7 (www.clcbio.com) and sequences assigned to species/genera by blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Confirmed *Alexandrium* sequences were included into the alignment from John et al. (2014). Maximum Likelihood phylogenetic analyses of the D1-D2 LSU rDNA data set were conducted with MEGA version 6 (Tamura et al. 2013) based on the following parameter (GTR G+I and 1,000 bootstrap replications) utilizing the alignment from John et al. (2014). The alignment comprised representative sequences from several *Alexandrium* species. The alignment consisted of filtered sequence of *Alexandrium* strains/species to remove sequences with differences of ≤2 bp; one representative sequence from each was selected for inclusion in the phylogenetic analysis. This filtering procedure captured a most of the within-species variation while reducing the total number of sequences in the analysis for all species from 638 to 45, indicated in the tree as "ver. 1, ver. 2…" (for details see John et al. 2014).

Data analysis. Nonparametric Spearman's correlation analyses were employed to determine correlations between cell abundance of *A. tamarense* species complex plus *A.* aff. *minutum* and PST concentration; and between *A. ostenfeldii* cell abundance and SPX concentration.

RESULTS

Alexandrium *species diversity.* Morphological analyses of *Alexandrium* specimens from field populations from the Argentine Sea were consistent with the presence of at least three different morphospecies, two of them generally conforming to classical species descriptions (i.e., Balech 1995) of *A. tamarense* and *A. ostenfeldii*. In addition, cells resembling

A. minutum were also found, but the morphological features presented by those cells did not completely agree with the classical description from Balech (1995).

Alexandrium tamarense *species complex*: The majority of cells (40 of 50 examined) that belonged to this species complex were slightly flattened and wider than long (Fig. 1A), but 10 cells were longer than wide, with an epitheca taller than the hypothecae and a more or less oval shape in ventral view (Fig. 1B). Cells varied from 25 to 37 μm in length and from 25 to 38 μm width (n = 30). The Po bore a distinct comma-shaped apical pore and scattered marginal pores. A connecting pore was also present on a few cells (Fig. 1C). The first apical plate (1′) presented an almost rhomboidal shape with two

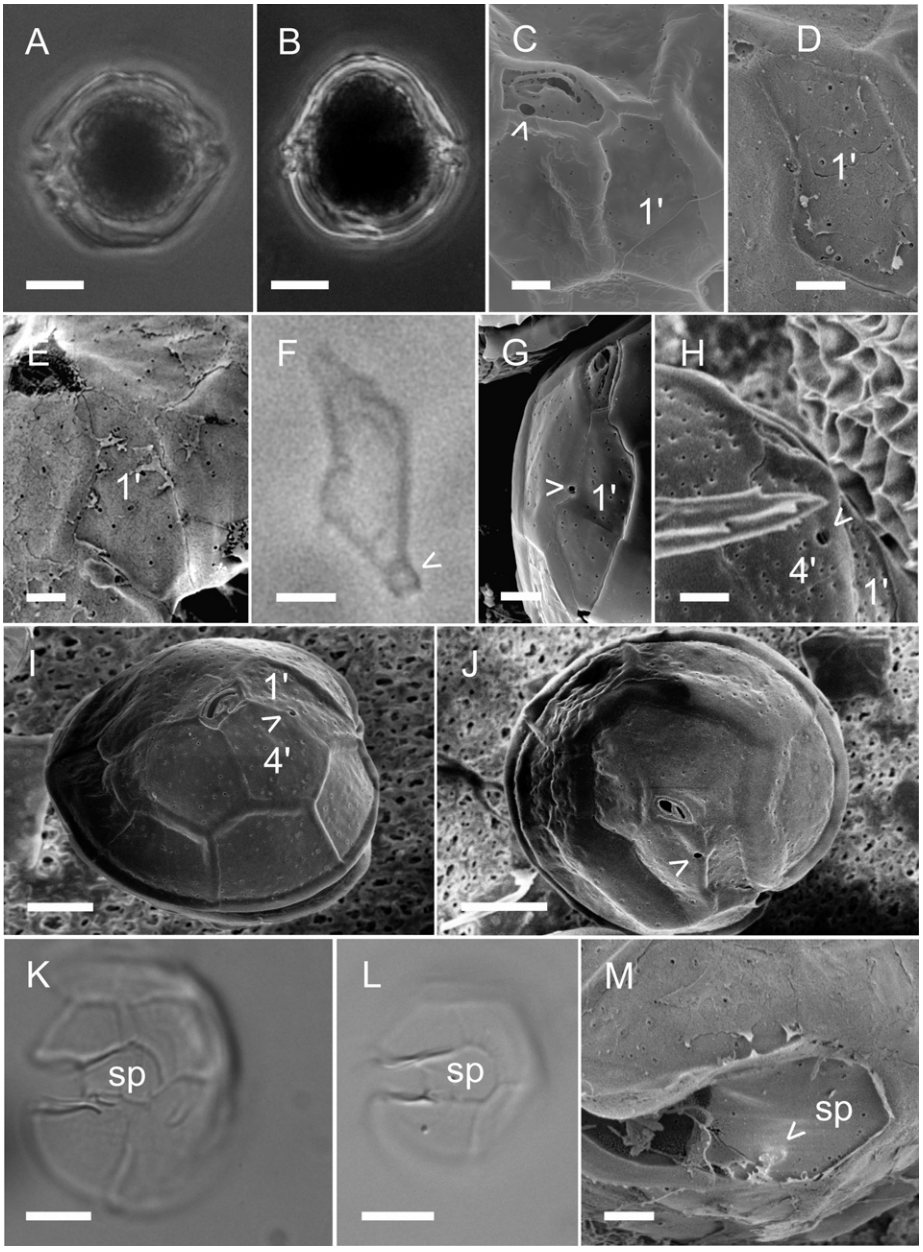

FIG. 1. Light microscope (LM; A, B, F, K, L) and scanning electron microscope (SEM; C–E, G–J, M) images of cells of the *Alexandrium tamarense* species complex from the Argentine Sea. (A, B) General views showing a flattened (A) or oval (B) cell shape; (C) apical view, with the presence of a connective pore in Po (arrow); (D–F) variation in shape of first apical (1′) plates from almost rhomboidal (D, F) to highly asymmetric (E); note the presence of a small ridge at the posterior margin (F, arrow); (G, H) location of the ventral pore: in the suture between the 1′ and 4′ plates (G, arrow), or in the 4′ plate (I, arrow); (G–J) size variation of the ventral pore (arrows); (K-M) posterior sulcal plate (sp): longer than wide without connective pore (K, L) or almost isodiametric (M); note the presence of a connective pore (arrow). Scale bar = 10 μm (A, B, I–L) or 2 μm (C–H, M).

straight right and two straight left margins (Fig. 1D), or a concave right anterior margin bordering the fourth precingular (4′) plate (Fig. 1E). Some specimens (~5%) had a small ridge in the posterior margin of the 1′ plate (Fig. 1F). A vp, a species descriptor of *A. tamarense* sensu Balech (1995), was located between the 1′ and 4′ plates (Fig. 1G) in all cells or in one unique case on the 4′ plate (Fig. 1H). The size of the pore varied from rather small to a distinctly larger in diameter, but was always circular (Fig. 1, G–J). The Sp was ~1.6 times longer than wide ($n = 15$; Fig. 1, K and L). In a few specimens, a conspicuous antapical connective pore was observed and the Sp plate was more isodiametric (Fig. 1M).

Alexandrium *aff.* minutum: Field specimens presented a general oval shape (Fig. 2A). Cells varied from 23 to 35 μm in length and from 20 to 33 μm in width ($n = 15$). The Po plate had a distinct comma-shaped pore, but no connecting pore was observed (Fig. 2C). The first apical plate (1′) was elongated and presented a vp at the right mid-margin of the plate (Fig. 3B) or along the posterior half (Fig. 2C). The 1′ plate was always connected to Po by a narrow extension (Fig. 2D). The 6″ plate was narrow, ~1.2–1.75 times longer than wide ($n = 18$) (Fig. 2, B, C, E and F). The Sp was short, ~1.5 times wider than long ($n = 12$; Fig. 2, G and H).

Alexandrium ostenfeldii: Field specimens exhibited a globose cell shape and rounded margins, characteristic of *Alexandrium ostenfeldii* (Fig. 3A). Cells varied from 30 to 45 μm in length and from 25 to 45 μm in width ($n = 20$). A total of 25 cells designated as *A. ostenfeldii* based on size and shape were exemplarily examined in more detail; all of them showed an elongated 1′ plate with straight margins, and presented a large kidney-shaped vp (Fig. 3, B and C).

The qPCR analysis of *Alexandrium* taxa from NT samples confirmed the presence of both *A. minutum* and *A. ostenfeldii*, representing the first records of these species in the sampling area. The DNA of members of the *A. tamarense* species complex was apparently too degraded in the archived bulk samples for species confirmation, thus the molecular signals were near the detection limit, but successful single-cell PCR amplification was achieved with micropipette-picked cells from St 27 (Valdés Peninsula). Confirmed *Alexandrium* sequences of the D1-D2 LSU rDNA from this species complex, with alignment and phylogenetic placement of other *Alexandrium* taxa from John et al. (2014), provided a Maximum Likelihood phylogenetic tree (Fig. 4). The sequences from the Argentine Sea clustered within *Alexandrium* ribotype Group I, consistent with *A. catenella*.

Cell abundance and distribution of Alexandrium *species.* *Alexandrium* cells were found in 78% of the Niskin bottle samples, in abundances ranging from 20 to 2.75×10^4 cells $\cdot$ L^{-1}. Maximum cell densities were found in San Matías Gulf (1.32×10^3 cells $\cdot$ L^{-1}), along the Valdés Peninsula (1.08×10^3

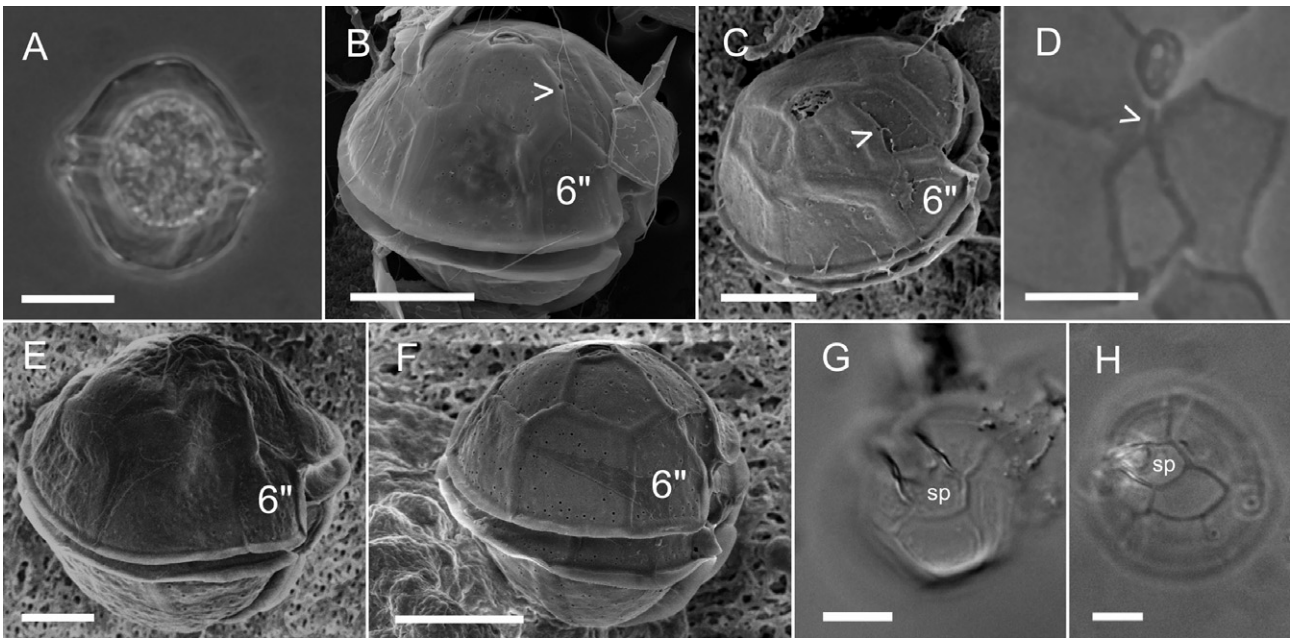

FIG. 2. LM (A, D, G, H) and SEM (B, C, E, F) images of cells of *Alexandrium* aff. *minutum* from the Argentine Sea. (A) general view showing an oval cell shape; (B, C) general views showing a ventral pore in the middle of the first apical (1′) plate (B) or in the posterior half of the plate (C); note the dimensions of the 6″ plate (longer than wide); (D) detailed view of the 1′ plate showing a narrow extension connecting to the pore plate (arrow), the image is optically reversed; (E, F) general views showing a narrow 6″ plate; (G, H) antapical view showing a posterior sulcal (sp) plate wider than long. Scale bar = 10 μm.

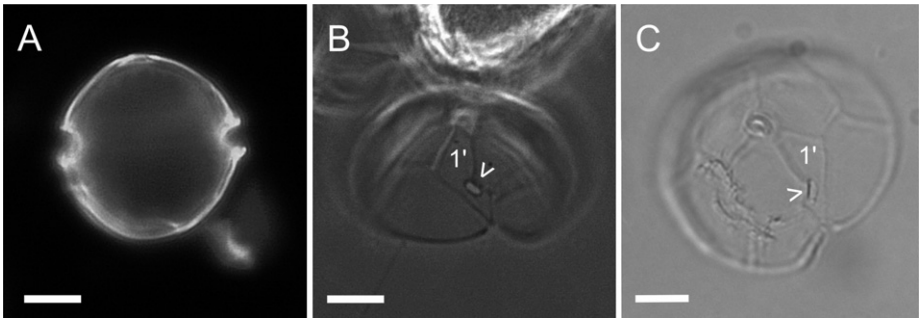

FIG. 3. Light microscopy images of *Alexandrium ostenfeldii* from the Argentine Sea. (A) epifluorescence image of calcofluor-stained cell showing a globose shape and rounded margins; (B, C) mirror inverted ventral view (B) and apical view (C) of the epithecal plates showing an elongated first apical plate (1′) with straight margins and a conspicuous ventral pore (arrow). Scale bar = 10 μm.

cells · L^{-1}), in San Jorge Gulf (16.5×10^4 cells · L^{-1}) and in shelf waters at ~44°S (2.75×10^4 cells · L^{-1}; Fig. 5), where surface (2 m) water temperature was around 11°C and salinity ranged between 33.2 and 33.7.

Throughout the Argentine Sea, microscopic analysis of Niskin bottle samples assigned most *Alexandrium* cells to the *A. tamarense* species complex. Cells corresponding to *A. tamarense* species complex were observed in net samples from 22 stations, occurring mostly in shelf waters from ~39°S to 47°S (Fig. 6A). These cells were found within a surface (2 m) temperature range from 8.3°C to 13.4°C, and in a salinity range from 32.9 to 34.2. The relative cell abundance of the *A. tamarense* species complex ranged between 29% and 100%, with an average of 94% ($n = 22$).

Alexandrium minutum was found in slope waters at ~40°S and ~44°S (St 2, 3, 4, 6, 7, 43 and 44), in shelf waters south of Buenos Aires Province (St 14), and east of the Valdés Peninsula (St 27). The species was detected in nine of 11 NT samples analyzed by qPCR, but microscopic analysis confirmed the presence of cells with morphological characteristics similar to *A. minutum* in only two of the 11 samples analyzed by qPCR (St 5 and 27), and in other two additional net samples, one north of Valdés Peninsula (St 25) and the other from slope waters at ~44°S (St 45; Fig. 6B). In these samples, however, *A.* aff. *minutum* represented only a low percentage of total *Alexandrium* cells analyzed (~10% at St 5, 6% at St 25, 12% at St 27, and 20% at St 45). *Alexandrium* aff. *minutum* cells were found within a surface (2 m) temperature range between 7.8°C and 13.5°C, and a salinity range from 33.2 to 34.0.

Alexandrium ostenfeldii was present in only three Niskin bottle samples from slope waters, in low abundances ranging from 60 to 200 cells · L^{-1}, and with the maximum cell density at 44°S, in surface (2 m) waters at 9.4°C with a salinity of 33.7. *Alexandrium ostenfeldii* was detected in 10 of the 11 net samples analyzed by qPCR. The detection of *A. ostenfeldii* signatures by qPCR occurred with samples from slope waters at ~40°S and ~44°S (St 3 to 7, 43 and 44), in "El Rincón" (St 9), in shelf waters south of Buenos Aires Province (St 14) and east of the Valdés Peninsula (St 27). Microscopic observations confirmed the presence of this species at most stations where the species was detected by qPCR, except for St 4, 9, and 27 from which no corresponding vegetative cells were found. Additionally, *A. ostenfeldii* was observed by microscopic analysis of plankton samples from St 1 (slope waters) and St 25 (Valdés Peninsula), which were not analyzed by qPCR (Fig. 6C). Relative abundance of *A. ostenfeldii* cells ranged between 0.5% and 100% of total *Alexandrium* cells, with an average of 52% ($n = 9$). Cells of *A. ostenfeldii* were found in waters with a surface (2 m) temperature range from 8.1°C to 13.4°C, and in a salinity range from 33.2 to 34.1.

In addition to the common *Alexandrium* motile cells, pellicle cysts were found in 23% of the net samples. They were distinguished from motile cells by a spherical cell shape, a larger size (~50 μm), a granulose cytoplasm, and by a very thin external cell covering, after loss of the thecae. Some cysts still showed remnants of the theca attached to the outer layer.

Toxin composition. The PSP toxins were detected in ~64% of net samples, and were consistently associated with the presence of *Alexandrium tamarense* species complex plus *A.* aff. *minutum* cells ($r = 0.88$, $P < 0.05$), with total concentrations ranging between 0.1 and 1,156 ng · NT^{-1} (Fig. 7). Gonyautoxins reached the highest concentrations among all PST analogs (Fig. 8). The GTX1/4 concentrations ranged between 0.3 and 1,104 ng · NT^{-1} and GTX2/3 between 0.1 and 31.7 ng · NT^{-1} (Table S1 in the Supporting Information). Maximum concentrations of both toxins were detected in the San Jorge Gulf and adjacent open waters. Cell quotas of total GTX estimated for the *A. tamarense* species complex plus *A. minutum* were all below 0.8 pg · $cell^{-1}$. The N-sulfocarbamoyl toxins C1/2 were detected in 61% of net samples, although at low concentrations (<8 ng · NT^{-1}), except for St 31 where a small peak was detected (13 ng · NT^{-1}). The high potency carbamoyl analogs STX and neoSTX were the least

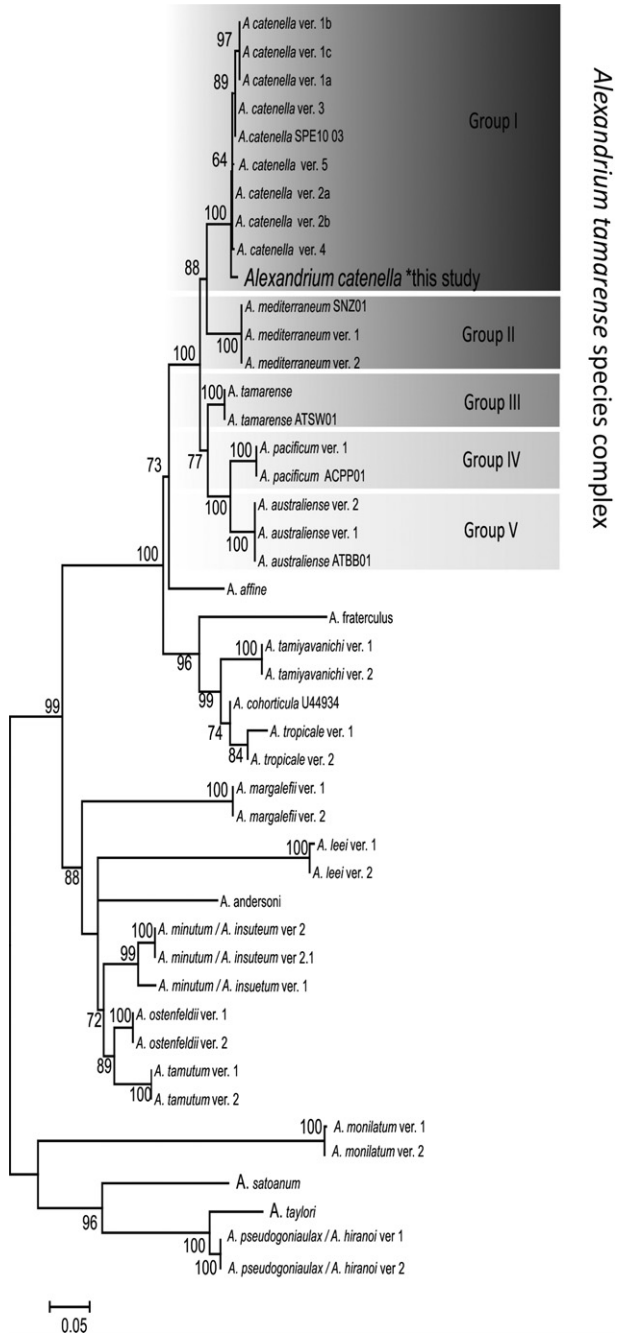

FIG. 4. Maximum Likelihood phylogenetic tree constructed from D1–D2 LSU rDNA sequence analysis from the genus *Alexandrium*. The alignment consists of representative sequences from several *Alexandrium* species filtered to simplify the analysis as indicated in John et al. (2014). The tree is supplemented by alignments derived from sequence analysis of selected single cells of the *Alexandrium tamarense* species complex (indicated as Group 1 member *Alexandrium catenella*) from St 27 (Valdés Peninsula).

common toxins; they were found in only three and seven samples, respectively, and at concentrations <7 ng · NT^{-1}.

The PST composition profiles were estimated only from samples with total PST content above

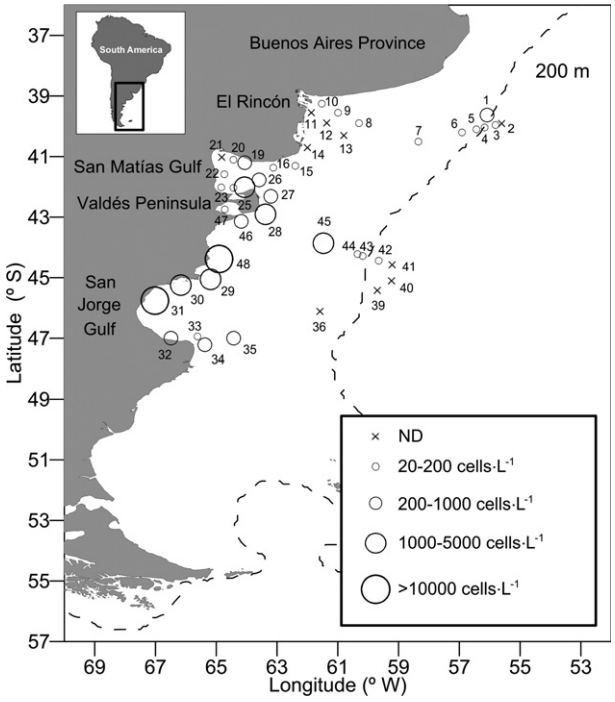

FIG. 5. Distribution and cell abundance of the genus *Alexandrium* in the study area based on Niskin bottle samples. Next to the symbols are the station numbers. ND: not detected.

1.5 ng · NT^{-1} ($n = 13$) to avoid biased toxin profiles due to different fluorescence detection limits of the individual toxins. All profiles were dominated by GTX1/4, which represented between 74% and 97% of total PST content (Fig. 8).

Results of lipophilic toxin analysis showed the presence of two cyclic imines, spirolides SPX-1 and 20-Me-SPX-G, together with the presence of *Alexandrium ostenfeldii* cells ($r = 0.81$, $P < 0.05$; Fig. 9). *Alexandrium ostenfeldii* cell quotas estimated for total SPX ranged between 15 and 36 pg · $cell^{-1}$. In general, SPX-1 was more widely distributed and abundant than 20-Me-SPX-G. The former toxin was found at 23% of stations in concentrations ranging between 28.3 and 2,747.5 ng · NT^{-1} (Table S1), with maximum values in slope waters at 40°S and 44°S and in southern coastal waters of Buenos Aires Province. Most SPX-1 (84%–100%) was detected in the 20–50 μm size-fractions, except at St 1 (50%) and St 4 (38%) where higher proportions of SPX-1 were detected from the 50–200 μm size-fraction. The analog 20-Me-SPX-G was detected in only two samples at low concentrations (0.5 ng · NT^{-1}), representing only 0.02% and 1.7%, respectively, of total spirolide content.

DISCUSSION

In the Argentine Sea, as in many other temperate coastal and shelf seas around the world, dinoflagellates of the genus *Alexandrium* are primarily

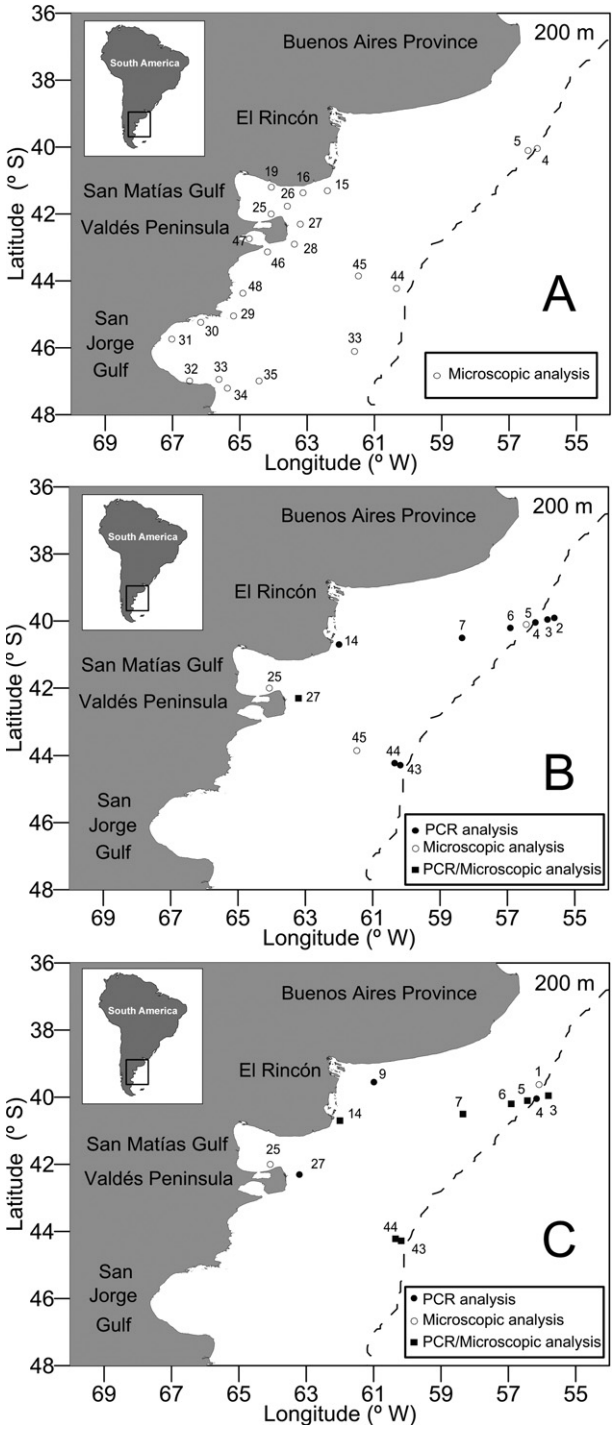

FIG. 6. Distribution of *Alexandrium tamarense* species complex (A), *Alexandrium* aff. *minutum* (B) and *Alexandrium ostenfeldii* (C) based on detection (presence/absence) from net samples by microscopic and/or molecular analysis. Next to the symbols are station numbers; for a full plot of all stations see Figure 5.

responsible for the toxic blooms, causing shellfish toxicity and associated human illness and fatalities (Vecchio et al. 1986). Previous *Alexandrium* blooms have always been associated with the *A. tamarense* species complex (Carreto et al. 2002). From our current analysis, specimens from the *A. tamarense* species complex were again confirmed by microscopy as the most abundant and widespread *Alexandrium* taxon in the Argentine Sea. The PCR analyses from net samples, however, detected only weak signals for Group I from the *A. tamarense* species complex that were all near the detection limit. In any case, positive results by PCR probes were obtained for *A. minutum* and *A. ostenfeldii*, two species that were never recorded in the study area. *Alexandrium ostenfeldii* was unambiguously identified by detailed microscopic observations and compatible rDNA sequences were confirmed by PCR analysis, whereas *A. minutum* was identified by confirmatory PCR but microscopic analysis showed the presence of cells with morphological features that did not completely match with the classical description of *A. minutum* by Balech (1995).

Cells from the *Alexandrium tamarense* species complex observed in this study presented variable morphological characteristics; such as the shape of the 1′ plate, the size and location of the vp (at the margin of the 1′ plate, between the 1′ and 4′ plate or within the 4′ plate), and the shape of the Sp plate (clearly longer than wide or almost isodiametric). Similarly, ribotype Group 1 strains of the *A. tamarense* species complex from the west coast of Greenland showed a highly variable suite of morphological features, including the Sp plate morphology, the presence or absence of the connecting pore, size of the vp and shape of the 1′ and 6″ plates (Tillman et al. 2016).

Some specimens assigned in this work to the *Alexandrium tamarense* complex by microscopic analysis presented an oval shape and an almost isodiametric Sp plate (Fig. 1, B and M), resembling the description of *Alexandrium acatenella* from Japan (Balech 1995, Yoshida et al. 2003). Balech (1995) reported *A. acatenella*, from rare records in the San Matías Gulf and found that this species can be distinguished from *A. tamarense* by a more oval shape, an epitheca longer than the hypotheca and a wider Sp plate bearing a connecting pore. However, *A. acatenella* was questionable as a separate species, with only minor differences from *A. tamarense* (Balech 1995).

Molecular phylogenetic analysis by qPCR performed on single isolated cells of the *Alexandrium tamarense* complex from St 27, clearly assigned them to the toxigenic ribotype Group I (i.e., *A. catenella*; Prud'homme van Reine 2017), as presented in Figure 4. The genetic characterization of picked *Alexandrium* cells performed in the current work is in accordance with previous sequencing data on the only other strain of the *A. tamarense* complex (MDQ1096, Mar del Plata, Argentina) from Argentine waters analyzed by molecular techniques (Penna et al. 2008). Genetic analysis has also shown that the Argentine strain MDQ1096 has the same

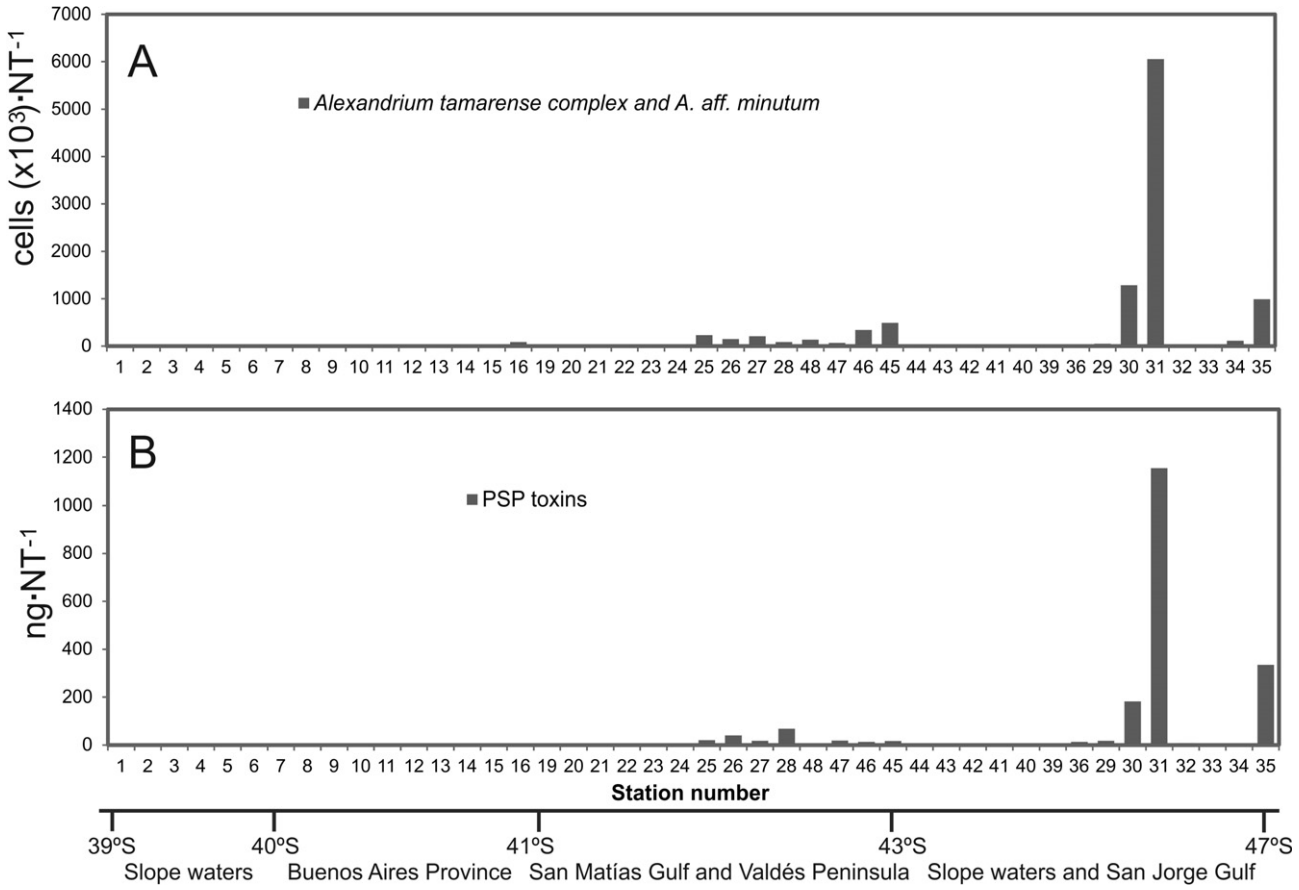

FIG. 7. Combined cell abundances of the *Alexandrium tamarense* species complex and *Alexandrium* aff. *minutum* (A) and paralytic shellfish poisoning (PSP) toxin concentrations (B) in 20–200 size-fractions of the net samples.

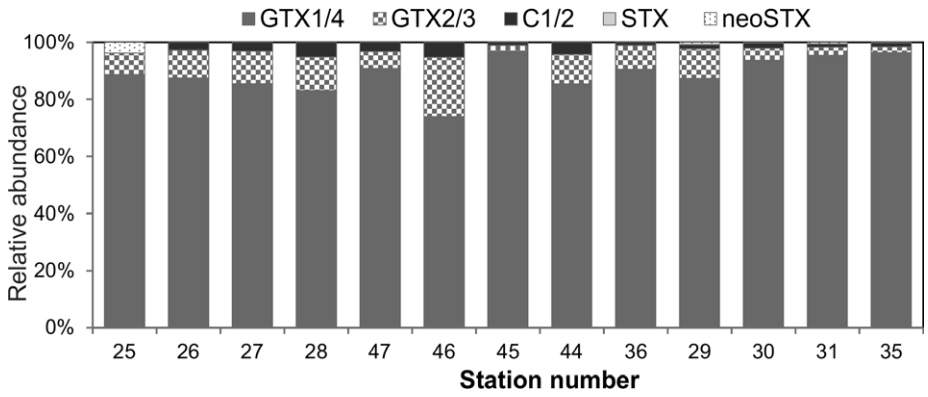

FIG. 8. Paralytic shellfish poisoning toxin profiles (% total composition in ng) determined from plankton net samples.

D1–D2 LSU rRNA gene sequences as the *A. catenella* from Chilean waters and from the Southeast Pacific (Lilly et al. 2007, Aguilera-Belmonte et al. 2011).

The failure to confirm the *Alexandrium tamarense* species complex (Group I) taxon by PCR analyses of net samples from the Argentine Sea may be attributable to alternative explanations. One possibility is that the low signal and reduced sensitivity of this assay is merely due to poor DNA quality in terms of purity and integrity of the field samples. When environmental samples are examined with molecular tools, false-negative results caused by qPCR inhibitors coextracted with target DNA may occur (Wilson 1997, Park et al. 2007). Alternatively, cells with morphological characteristics according to the *A. tamarense* species complex could belong to the cryptic

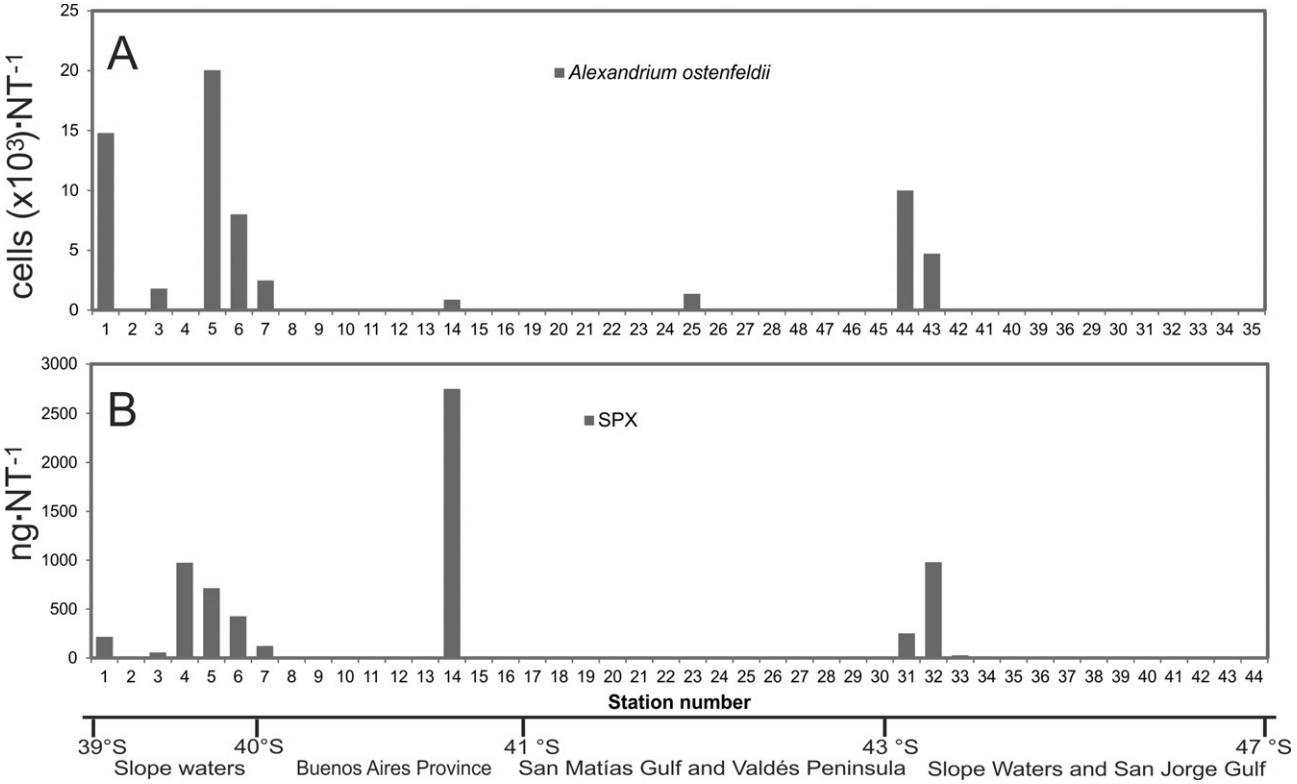

FIG. 9. Cell abundances of *Alexandrium ostenfeldii* (A) and spirolide toxin concentrations (B) in 20–200 size-fractions of the net samples.

species *Alexandrium australiense* (= Group V; John et al. 2014). This was the only ribotype from the *A. tamarense* species complex for which primers were not applied in this study. Another alternative explanation is that the *A. tamarense* species complex populations from Argentina comprise specimens with very variable SSU rDNA and LSU rDNA regions and thus they cannot be detected with primers designed for other populations. The possibility that variability in rDNA content might affect the efficiency of the qPCR approaches (Toebe et al. 2013), as well as that high variability of *Alexandrium* taxa may be inherent within populations at the local or regional scale, should be considered if qPCR is to be effectively applied to environmental samples (Galluzzi et al. 2010).

Although the presence of *Alexandrium minutum* was not unambiguously proven by morphological or sequence DNA analysis, its novel detection by specific primers in bulk field samples from Argentine waters is an important fact to consider and underlines the need for enhanced temporal-spatial field sampling to increase our knowledge of the diversity and distribution of toxic phytoplankton in the Southwest Atlantic. However, it is important to consider that qPCR applied to field samples can display uncertainty in the assay specificity as a result from the potential presence of unknown related species with similar sequences to the primer sites (Park et al. 2007). In any case, this toxigenic species generally displays a toxin profile comprising mostly or exclusively high potency sulfated carbamoyl toxins (=GTX), as characterized from a "tamarensoid" culture of this species from Laguna Obidos, Portugal (NEPCC 253; Cembella et al. 1987). Morphological analysis of our material showed cells with similar features to *A. minutum* but not completely matching the classic morphological characterization of this species (Balech 1995), mainly because cells analyzed in this study were bigger (23–35 μm) than those reported in the literature (15.5–29 μm; Balech 1995). The most distinctive morphological character of *A. minutum* is the shape of the 6″ plate, which is longer than wide (Balech 1995). In our samples, a narrow 6″ plate, similar to that described for *A. minutum* from New Zealand (MacKenzie and Berkett 1997) was observed, although the sutural connection between this plate and the 4″ plate was more prominent in samples from this study than in the original description. In any case, considerable variation was observed in the suture between 4″ and 6″ in cultures from Denmark (Hansen et al. 2003). Another morphological feature of *A. minutum* is an apparent disconnection between the 1′ plate and Po, caused by the presence of a thin thread-like anterior part of the 1′ plate in most specimens (Balech 1995). In our material, a more or less thin anterior part of the 1′ plate in contact with Po was always observed, which is in accordance with strains from France analyzed by Lilly et al. (2005) and with

field specimens from Jamaica (Ranston et al. 2007). The presence of a vp in the suture between the 1′ and 4′ plates was detected in all specimens analyzed from the Argentine Sea. This feature was used to distinguish between *A. minutum* and *Alexandrium angustitabulatum* (Balech 1995), but further morphological and molecular analyses have shown that this character is not stable and varies within clonal cultures of *A. minutum* (Kim et al. 2002). According to the original description, the vp in *A. minutum* is located in the posterior right margin of the 1′ plate, but this was not the case for all specimens analyzed in this study, as the pore was located in the mid-part of this plate in some cells.

Alexandrium ostenfeldii, a species that was previously found in the Beagle Channel (Almandoz et al. 2014), was detected for the first time in slope and shelf waters of the Argentine Sea. The most distinctive morphological characteristic of this species is the presence of a large vp and a narrow and very oblique 1′ plate (Balech 1995). In accordance with *A. ostenfeldii* from the Beagle Channel (Almandoz et al. 2014), Argentine Sea specimens exhibited a 1′ plate with straight right margins.

The highest cell abundances of the genus *Alexandrium* were detected in San Matías and San Jorge Gulfs and shelf waters close to Valdés Peninsula. Those areas present particular oceanographic conditions defined by strongly stratified water columns in the Gulfs (Gagliardini and Rivas 2004, Krock et al. 2015) and the presence of the Valdés Peninsula tidal front (Carreto et al. 1986, Acha et al. 2004), both characteristics that are known to favor dinoflagellate cell accumulation (Cloern et al. 2005, Jephson and Carlsson 2009). Our data show that members of the *A. tamarense* species complex were the most abundant and widely distributed from the genus, being found more often in shelf waters from ~39°S to 47°S. In contrast, *A.* aff. *minutum* and *A. ostenfeldii* were mainly found across slope waters. The presence of *A. minutum* in offshore waters is unusual as this species used to occur generally in coastal enriched environments as estuaries or lagoons (Belin 1993, Giacobbe and Maimone 1994, Vila et al. 2004), or even small harbors (Garcés et al. 2004, Bravo et al. 2010), frequently with freshwater influence. However, it is important to consider that *A.* aff. *minutum* abundances from the shelf edge front observed in this work were low.

Analysis of PSP toxins revealed GTX and their analogs in all samples where the *Alexandrium tamarense* species complex and/or *A.* aff. *minutum* was present, except for a few samples in which low cell abundances were found (below 19×10^3 cells $\cdot$ NT^{-1}) and these toxins were not detected. In these rare cases, lack of toxin detection could be due to the presence of a nontoxigenic *Alexandrium* population, but it is more likely that PSP toxins were present but at levels below the limit of detection of the fluorescence method. Cell disruption or even minor physical stress can also lead to toxin leakage from the plankton slurries collected on meshes during the collection processes (Johansen and Rundberget 2006). Although plankton collection and filtration for toxin analyses during this study were carried out as rapidly and carefully as possible, and in the same manner for each sampling station, some losses of toxins cannot be ruled out.

Different toxin profiles have been used as a biochemical marker to discriminate among *Alexandrium* species and even among geographical populations within a species (Anderson et al. 2012). This approach must be interpreted carefully, however, because some *Alexandrium* species, including members of the *A. tamarense* species complex, can exhibit a highly variable suite of profiles depending on strain-specific differences, even within a morphologically homogeneous population (Alpermann et al. 2010, Anderson et al. 2012). In this work, we found a profile dominated by GTX, with GTX1/4 as main variants, across all stations in which total PST were detected above 1.5 ng $\cdot$ NT^{-1}. This level was chosen to take into account that different limits of detection of the individual PSP toxins can influence to the PSP profile, especially since the method has a distinctly lower sensitivity for detection of GTX1/4. This dominance of GTX1/4 is in accordance with previous analysis of field plankton samples (Andrinolo et al. 1999) and profiles from two cultured *A. tamarense* morphospecific strains isolated from the San Jorge Gulf (Krock et al. 2015) and in phytoplankton collected along the Argentine Sea (Montoya et al. 2010).

In contrast, toxin profiles from cultured members of the *Alexandrium tamarense* species complex, including the strain MDQ1096, isolated from different areas in the Argentine Sea showed dominance of N-sulfocarbamoyl derivatives C1/2 and contained less GTX1/4 (Carreto et al. 2001). A similar toxin profile was found by Montoya et al. (2010) in strains of the *A. tamarense* species complex isolated from various locations in Argentine coastal and open waters. These isolates always contained a high relative amount of C1/2, with the exception of a more toxic strain from Valdés Peninsula, which presented a profile dominated by GTX1/4. A profile similar to Argentine strain MDQ1096, with dominance of *N*-sulfocarbamoyl derivatives, was found in *A. catenella* from natural populations from Chilean waters (Persich et al. 2006, Krock et al. 2007, Varela et al. 2012). However, seven *A. catenella* strains isolated from the Chilean regions Aysén and Los Lagos, and all placed within *A. tamarense* Group I by internal transcribed spacer sequencing, presented a remarkable variability in toxin content and profiles. Six of these strains showed high proportions of GTX (Aguilera-Belmonte et al. 2011), whereas only one presented C1/2 as the major toxins. This high variability in toxin profiles among populations of the *A. tamarense* species complex from Argentine waters

seems therefore to be a recurrent pattern for ribotype Group I of the species complex in other locations as well, e.g., from the North Sea (Alpermann et al. 2010) and the Chilean coast (Aguilera-Belmonte et al. 2013).

Nevertheless, even though members of the *Alexandrium tamarense* species complex were most abundant and widespread in the Argentine Sea, they were not the only potentially toxigenic species represented in the study area. In particular, PST profiles of the *A. minutum* group are also typically dominated by carbamoyl toxins, particularly GTX (Cembella et al. 1987), and thus might contribute to and even bias the composition of the field samples. Previous toxin analyses on *A. minutum* showed relatively stable profiles that do not include the production of *N*-sulfocarbamoyl toxins, such as C1/2 (Franco et al. 1994, Chang et al. 1997, Chou et al. 2004). In this sense, the detection of C1/2 toxins in our samples cannot be explained by the presence of *A. minutum*. Even though *A. minutum* could contribute to the high proportion of GTX1/4 (up to 80%) found in our field samples, cells corresponding to the *A. minutum* group on a morphological basis were detected in only three of 13 samples in which GTX1/4 was the main PST component. Even in these samples, the species was found only in low cell abundances. Moreover, while *A. minutum* was detected by PCR analysis, its presence in the samples could not be unambiguously confirmed by morphological analysis, presumably due to low cell densities. It is therefore unlikely that this species is a major contributor to PST levels in shellfish from the study area. However, considering that *A. minutum* has been described as a temperate species with optimal growth conditions above 15°C (Chapelle et al. 2015), it could play a more significant role in future PST events in the Argentine Sea, especially under a global warming scenario.

The other *Alexandrium* species present in the field samples, *A. ostenfeldii*, is also known as a PST toxin producer in some regions (Hansen et al. 1992, MacKenzie et al. 1995, Van de Waal et al. 2015). The fact that this species is capable of synthesizing more than one group of toxins raises a potentially confusing issue in interpreting patterns of toxin distribution in the plankton in association with specific *Alexandrium* taxa. The PST-producing populations of *A. ostenfeldii* tend to produce these toxins only in brackish waters or in inner coastal areas (Tomas et al. 2012, Kremp et al. 2013, Burson et al. 2014, Salgado et al. 2015). Our data do not discern a definitive relationship between the occurrence of PST and *A. ostenfeldii*. Nevertheless, in samples in which *A. ostenfeldii* was the dominant species of the genus, PST were almost absent, whereas *A. ostenfeldii* was present in only one of the 13 samples with significant amounts of these toxins. Collectively, this evidence suggests, as is the case of *A. minutum*, that *A. ostenfeldii* can also be excluded as a major source of PST in the field samples. However, the conditions under which populations of *A. ostenfeldii* produce these toxins are still not understood (Suikkanen et al. 2013). Full characterization of the toxin profiles and association with the biosynthetic genes must be interpreted with respect to the phylogenetic relationships (i.e., rDNA sequences) of *A. ostenfeldii* from the Argentine Sea with known populations from the Beagle Channel (Almandoz et al. 2014) and from the Chilean coast (Salgado et al. 2015). Regarding PST association with species in the field is important to note that *Gymnodinium catenatum*, the only other PST producer besides *Alexandrium* species recorded in the area (Sunesen et al. 2014), was not observed in the samples from this study.

Analysis of lipophilic toxins showed the presence of relatively high amounts of cyclic imine toxins, namely SPX, co-occurring with *Alexandrium ostenfeldii*. Spirolides have been associated with *A. ostenfeldii* as the only known source (Cembella et al. 2001, Franco et al. 2006). Spirolide analysis of our samples showed a toxin profile similar to that obtained from the Beagle Channel (Almandoz et al. 2014), in both cases dominated by SPX-1 and less 20-Me-SPX-G. The marked dominance of SPX-1 in the profiles found in this study matches the profile of two of the five phylogenetic groups of *A. ostenfeldii* as defined by Kremp et al. (2013), which comprise strains from USA, Spain, UK, and Ireland.

There was co-occurrence of *Alexandrium ostenfeldii* cells and SPX except for only three field samples. In one sample no toxins but low abundances (1.4×10^3 cells $\cdot$ NT^{-1}) of *A. ostenfeldii* were detected, whereas for two samples there was detectable SPX-1 but no *A. ostenfeldii* cells. In the first case, the undetected toxins might be due to SPX concentrations below the detection limit, either because of nontoxigenic strains or to toxin losses in the sampling and processing methods. In the second case, the detection of SPX and the apparent absence of *A. ostenfeldii* cells may be accounted for by transfer of these toxins through the food web. The trophic transfer of lipophilic toxins from dinoflagellates to zooplankton is well known (Tester et al. 2000) and evidenced here by two stations where high SPX concentrations (270–587 ng $\cdot$ NT^{-1}) were detected in the 50–200 μm size-fraction.

Low cyclic imine (SPX) concentrations were previously reported from shellfish from Buenos Aires Province (Turner and Goya 2015), and from mussels and plankton from the Beagle Channel (Almandoz et al. 2014), but this is the first report of SPX in plankton samples from the Argentine shelf and slope waters. The three SPX cell quotas estimated from field samples in this study (15–36 pg $\cdot$ cell^{-1}) were ~50 times higher than those estimated for the cultured Beagle Channel strain (0.74 pg $\cdot$ cell^{-1}; Almandoz et al. 2014) and were also higher than those for strains from the Mediterranean Sea (Salgado et al. 2015) and Denmark (Otero et al. 2010,

Medhioub et al. 2011). By contrast, higher quotas than estimated in this study were found for cultured isolates from Nova Scotia, Canada (John et al. 2001) and North Carolina, USA (Tatters et al. 2012). In any case, cell SPX quota estimates based on field samples are prone to various methodological biases and assumptions. Analysis of SPX toxin content and composition in cultured isolates of *A. ostenfeldii* from the Argentine Sea are necessary to unambiguously determinate the toxin biogeographical patterns for this region, in relation to natural populations.

The presence of pellicle cysts of dinoflagellates in several samples also contributes to uncertainty in the correspondence between particular toxins characteristic of *Alexandrium* and taxonomic identification. Pellicle cysts are directly derived from vegetative cells that have shed their thecae, and hence provide no thecal plate structures or any other morphological characteristics for microscopic identification (Balech 1995). For species such as *A. ostenfeldii*, with a thin fragile theca, pellicle cysts can be readily formed as artifacts during NTs and subsequent handling (Cembella et al. 2001) or occur naturally as part of the life cycle (Figueroa et al. 2006, 2008). The pellicle cysts found in this study cannot be unequivocally attributed to the genus *Alexandrium*, but no other genus with tendencies to produce pellicle cysts resembling those of *Alexandrium* (e.g., *Fragilidium*) were found in this study. The presence of pellicle cysts of *A. ostenfeldii* thus may account for the detection of rDNA signatures corresponding to this species at St 4, 9 and 27 where motile cells were not detected. This may also explain the detection of *A. minutum* by qPCR at St 43, but where no motile cells of the genus *Alexandrium* were found.

Morphological differentiation between *Alexandrium* species is often difficult and molecular techniques are therefore important to assist and to complement species identification (Groben et al. 2004, John et al. 2005, 2014). Further integrative studies are needed to interpret the discrepancy between molecular techniques and microscopic analysis for the *A. tamarense* complex and the *A. minutum* group. For example, a full length rDNA sequencing of the specimens resembling *A. minutum* found during the present study would confirm the presence and affiliation of the species in the Argentine Sea. Moreover, results of this work suggest the importance of applying alternative methods in a complementary way to assess the diversity and distribution of *Alexandrium* species in field studies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. *Alexandrium* species and lipophilic and PSP toxins detected from plankton net samples along the Argentine Sea. nd, not detected.