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Toxic strains of the *Alexandrium ostenfeldii* complex in southern South America (Beagle Channel, Argentina)

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ABSTRACT

During phytoplankton monitoring in the Beagle Channel (≈54°52′ S, 67°32′ W) a previously undetected *Alexandrium* species was observed in coincidence with mouse bioassay toxicity. Detailed thecal plates analysis using epifluorescence and scanning electron microscopy revealed the presence of the *Alexandrium ostenfeldii* species complex, showing a mixture of the diagnostic features usually used to discriminate between the morphospecies *A. ostenfeldii* and *A. peruvianum*. Cells of the *A. ostenfeldii* complex were commonly observed during spring after the main annual diatom bloom, when temperatures and salinities were respectively around 7.5–10 °C and 30–30.5 psu, and nutrients showed a seasonal decrease. Toxin analysis by liquid chromatography–mass spectrometry revealed the production of 13-desmethyl spirolide C and 20-methyl spirolide G in cell cultures. The cellular contain of spirolides during exponential phase growth was 0.5906 ± 0.0032 and 0.1577 ± 0.0023 pg cell^{-1} for 13-desMe-C and 20-Me-G, respectively. A third unknown compound, with a structure resembling that of spirolides was also detected in culture. Moreover, an additional compound with a similar *m*/*z* (692) than that of 13-desMe-C but presenting a higher retention time (Rt = 40.5 min) was found in high proportions in mussel samples. PSP toxins were present at low concentration in mussels but were not detected in cultures. These results extend the world-wide distribution of toxic strains of the *A. ostenfeldii* complex to the Beagle Channel (southern South America), where toxic events have been traditionally linked to the presence of *Alexandrium catenella*. This is the first confirmed occurrence of spirolides in mussels and plankton from Argentina, which highlights the importance of monitoring these toxins and their producing organisms to protect public health and improve the management of shellfish resources.

1. Introduction

The occurrence of some species of the marine dinoflagellate genus *Alexandrium* Halim has been usually related to harmful algal bloom events in many parts of the world (Anderson et al., 2012). Most of the harmful species of this genus produces paralytic shellfish poisoning (PSP) toxins, which have shown a high diversity in cultured and natural populations of *Alexandrium* from southern South America (Persich et al., 2006; Montoya et al., 2010). Moreover, the presence of other toxin families has been also recorded (Hu et al., 1996; Cembella et al., 2000; Hsia et al., 2006; Van Wagoner et al., 2011). From the more than 30 described *Alexandrium* species, only *A. ostenfeldii* and *A. peruvianum* have been found to produce cyclic imine neurotoxins called spirolides (SPXs) (Cembella et al., 2000; Franco et al., 2006). In the last years, there have been several reports on the production of spirolides by these two *Alexandrium* species in North America and Europe (*e.g.* Aasen et al., 2005; MacKinnon et al., 2006; Ciminiello et al., 2007; Borkman et al., 2012; Tomas et al., 2012). Likewise, the first record on the presence of spirolides in the Southern Hemisphere has been recently observed in shellfish from northern Chile (Alvarez et al., 2010), suggesting a more widespread distribution than previously thought.

Alexandrium ostenfeldii and *Alexandrium peruvianum* are morphologically very similar species that have been originally

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distinguished mainly by the shape of the 1′ and the s.a. plates (Balech and Tangen, 1985). However, a high variability in the shape of these two diagnostic plates has been recurrently observed in natural populations or strains from several locations (*e.g.* Lim et al., 2005; Touzet et al., 2008; Gu, 2011), making difficult to unambiguously assign specimens to one of the two species. Moreover, molecular studies suggest that *A. ostenfeldii* and *A. peruvianum* could represent a species complex with a considerable cryptic diversity or a single genetically structured species with high morphological variation (Kremp et al., 2009, 2014).

In temperate-cold waters of the Beagle Channel the presence of *Alexandrium catenella* has been linked to one of the most important PSP event observed worldwide (Carreto and Benavides, 1993; Benavides et al., 1995). Since that, the occurrence of such toxic events related to *A. catenella* has been recurrently reported (Goya and Maldonado, 2014). During a HAB monitoring programme carried out in a mussel harvesting area of the Beagle Channel since 2005–2007, the results of toxicity bioassays showed values above the accepted limits when the presence of *A. catenella* in plankton samples was extremely low or undetectable (Hernando et al., 2007; Goya and Maldonado, 2014). By contrast, the presence of solitary cells, usually larger and more globose in shape than *A. catenella* was observed. This fact promoted further research efforts to elucidate the source of toxicity, including cell culture establishment and toxin analysis.

In this study, we provide the first report of spirolide detection in both mussels (*Mytilus edulis*) and cultures of the *Alexandrium ostenfeldii* complex from the Beagle Channel, together with a description of its morphology and occurrence in this sub-Antarctic area that sustains rich aquiculture activities.

2. Materials and methods

2.1. Phytoplankton monitoring programme

Field sampling was carried out between July 7, 2005 and December 6, 2007, at a fixed station (Almanza, Fig. 1) located in the Beagle Channel (54°52′22.85″ S, 67°32′18.72″ W). Sampling frequency was biweekly most of the year and weekly during the phytoplankton spring bloom. Water temperature and salinity were measured *in situ* with a Horiba U-10 multi-parametric sensor (Horiba Ltd., Kyoto, Japan). Seawater was sampled at 2 m depth using a 5 l Niskin bottle from a boat. Aliquots of 250 ml were preserved with 4% borax buffered formalin for quantitative phytoplankton analysis. In addition, sub-surface qualitative phytoplankton samples were taken using a 20 μm mesh net and fixed as previously described. All samples were kept in the dark at room temperature until analysis. For chlorophyll *a* (Chl *a*) determination, 0.5–1 l of seawater were filtered onto Whatman GF/F filters and kept frozen (−20 °C) until analysis, which was performed within one week of sampling. Pigment extracts were read in a Turner 450 Beckman spectrofluorometer and corrected for phaeopigments, following Holm-Hansen and Riemann (1978). Concentrations were calculated according to Holm-Hansen et al., 1965. Samples for nutrient analyses were filtered through Whatman GF/F filters and kept frozen (−20 °C) until analysis, which was done within three months after sampling. Nitrates, phosphates and silicates were measured with an automated analyzer (Autoanalyzer Technicon II), following the methods described in Grasshoff (1969), Grasshoff et al. (1983), Technicon (1977) and Eberlein and Kattner (1987).

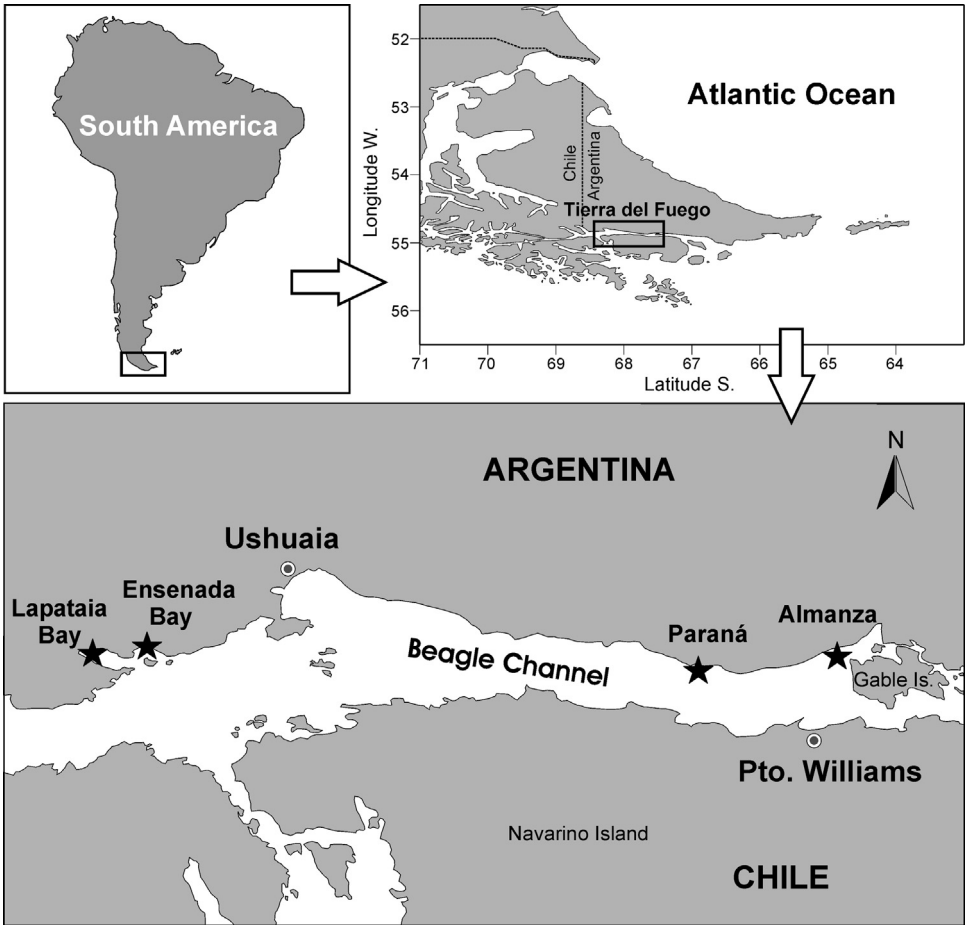


Fig. 1. Map of the study area and sampling sites in the Beagle Channel.

2.2. Microscopic examination

For quantitative estimations, cells were enumerated with a phase contrast Iroscope SI-PH inverted microscope according to the procedures described by Utermöhl (1958). Subsamples of 50 or 100 ml were left to settle for 24 or 48 h respectively in a composite sedimentation chamber. Net samples were examined using phase contrast, differential interference contrast (DIC) and UV epifluorescence microscopy under two Leica DM 2500 microscopes. For dinoflagellates identification, thecal plates were stained with Calcofluor according to Fritz and Triemer (1985). Further scanning electron microscopy observations of selected samples were made with a Jeol JSM-6360 LV (SEM).

2.3. Toxin sampling and cultures of A. ostenfeldii complex

A sampling aimed to toxin analysis in phytoplankton and mussels was carried out during November 2010 at different locations in the Beagle Channel (Fig. 1). Sea water samples (2 l) were filtered through Whatman GF/F filters in order to perform quantitative toxin analysis in the plankton. Net samples were additionally filtered through Whatman GF/F filters to obtain a higher resolution and sensibility of toxin analysis. Mussel samples (*Mytilus edulis*) were taken from the benthic community of each sampling site. Filters and mussels were stored at −20 °C until laboratory analysis.

A live subsample of net sample from Almanza was used for cell culture establishment. Isolation was made using a Nikon Biophot microscope and hand held micropipette. Cells were grown in L1 culture medium at a salinity of 30 psu, a temperature of 11 °C, and a 14/10 h light/dark period.

2.4. Liquid chromatography–mass spectrometry (HPLC–MS) analysis of lipophilic toxins

A culture of the *Alexandrium ostenfeldii* complex in exponential growth phase, with a density of 63×10^6 cells l^{-1}, was used to analyze the toxin profile and the SPXs cell concentration. 800 ml of cell culture were filtered through a Whatman GF/F filter. Toxins were extracted with 100% methanol and sonicated (Vibra Cell, Sonic and Materials) at 0 °C. The extracts were filtered through Whatman GF/F filters to remove cell debris. The obtained extract was purified by ultrafiltration (Micro Vecta-Spin, 100 kDa) before the chromatographic analysis. Toxin extraction from phytoplankton net samples was performed using the same methodology. Lipophilic toxins were extracted from mussels with methanol 100% according to the procedures described by the European Union Reference Laboratory for Marine Biotoxins (EURLMB, 2011). The extracts were centrifuged (3000 rpm), filtered (Whatman GF/F) and cleaned three times with hexane. Finally, additional extract purification was performed by ultrafiltration (Micro Vecta-Spin, 100 kDa).

Toxin extracts were analyzed by liquid chromatography/tandem mass spectrometry (LC–MSn) using a HPLC System (Shimadzu LC20A) interfaced with an ion trap mass spectrometer (Esquire 6000, Bruker Daltonics, Billerica, MA, USA) equipped with an electrospray source. Separation and quantitation of SPXs was performed according to a modification of the method of Ciminiello et al. (2006) using a Lichrosphere C8 column (3 μm, 4.6 mm × 150 mm). The flow rate was 0.5 ml min^{-1}. A gradient elution, 35–80% B in 3 min followed by 80% B for 43 min, was used. Eluent A was a solution of 2 mM ammonium formate and 50 mM formic acid in water, while eluent B was a solution of acetonitrile:eluent A (95:5, v/v).

The product ion spectra were acquired in the positive ion mode with the source and analyzer parameters optimized for the protonated molecules using infusion (180 μl h^{-1}) of 13-desmethyl spirolide-C (13-desMe-C) standard. Analyses were done using nitrogen as a nebulizing (30 psi) and drying gas (10 L min^{-1}, 300 °C). Capillary high voltage was set to 4500 V. Isolation width was 2.0 Da and collision energy of 30% was used for product ion spectra acquisition. Seven scans were averaged for each spectrum. Conditions for tandem mass spectrometry analysis of standard were the same as described for natural and culture samples. Full scan mass spectra (m/z 100–750) were acquired for each sample. Fragmentation studies were used to confirm the identity of cyclic imines. The characteristic loss of water molecules and the presence of ions at m/z = 164 were used as indicators of identity of cyclic imines (Sleno et al., 2004; Aasen et al., 2005; Krock and Cembella, 2007). A 13-desMe-C standard, provided by Dr. Bernd Krock from the Alfred Wegener Institute (AWI), was used to conduct SPXs quantification. Relative molar response factors for other spirolides were assumed to be the same.

2.5. Paralytic shellfish poisoning (PSP) toxins analysis

Subsamples of *Alexandrium ostenfeldii* complex in exponential growth phase were concentrated on Whatman GF/F filters and extracted with acetic acid 0.5 M, sonicated and filtered, before analysis. Mussel extracts according to AOAC (1984) were obtained from each sample for toxin content.

PSP toxin composition of extracts of *Alexandrium ostenfeldii* culture and mussel samples were analyzed by the post-column derivatization HPLC method of Oshima (1995). Two different columns were used: a YMC-Pack ODS-AQ, 3 μm, 150 mm × 4.6 mm (Waters Corp., MA, USA) for N-sulfocarbamoyl toxins and an Alltima C18, 3 μm, 150 mm × 4.6 mm (Alltech, IL, USA) for the other PSP. Toxins were identified by comparison of their retention times and fluorescence emission maxima with those of standards, by the disappearance of peaks by eliminating post-column oxidation, and by spiking experiments (Onodera et al., 1996).

3. Results

3.1. Occurrence of the A. ostenfeldii complex in the Beagle Channel

The annual phytoplankton cycle was characterized by a low density and biomass, estimated as Chl *a*, during the autumn-winter period and a significant increase during spring and summer (Fig. 2a). Phytoplankton biomass peaks were observed during spring, reaching 6.6 (October 2005), 8.6 (October 2006) and 3.2 μg Chl *a* l^{-1} (November 2007). These peaks were recurrently associated to diatom blooms (Fig. 2b). Towards the end of spring and during summer, diatom density decreased and assemblages were enriched by unidentified tiny phytoflagellates and dinoflagellates.

Along the study period (July 2005–December 2007), the *Alexandrium ostenfeldii* complex was observed in 50% of total samples analyzed and represented, on average, 11.1% and <0.05% of total dinoflagellate and total phytoplankton abundance, respectively. During winter, when phytoplankton assemblages were dominated by small phytoflagellates (<5 μm), *A. ostenfeldii* was almost absent. By contrast, it was commonly observed during spring, reaching maximum abundances of 5860 (2005), 3850 (2006) and 200 cells l^{-1} (2007). These highest densities were recurrently observed after the main annual diatom bloom (Fig. 2b), when temperatures and salinities were respectively around 7.5–10 °C and 30–30.5 psu (Fig. 2c), and nutrients showed a seasonal decrease (Fig. 2d). Finally, it abundances started to decrease in summer, and it was only occasionally observed during autumn, with relatively low densities (<100 cells l^{-1}).

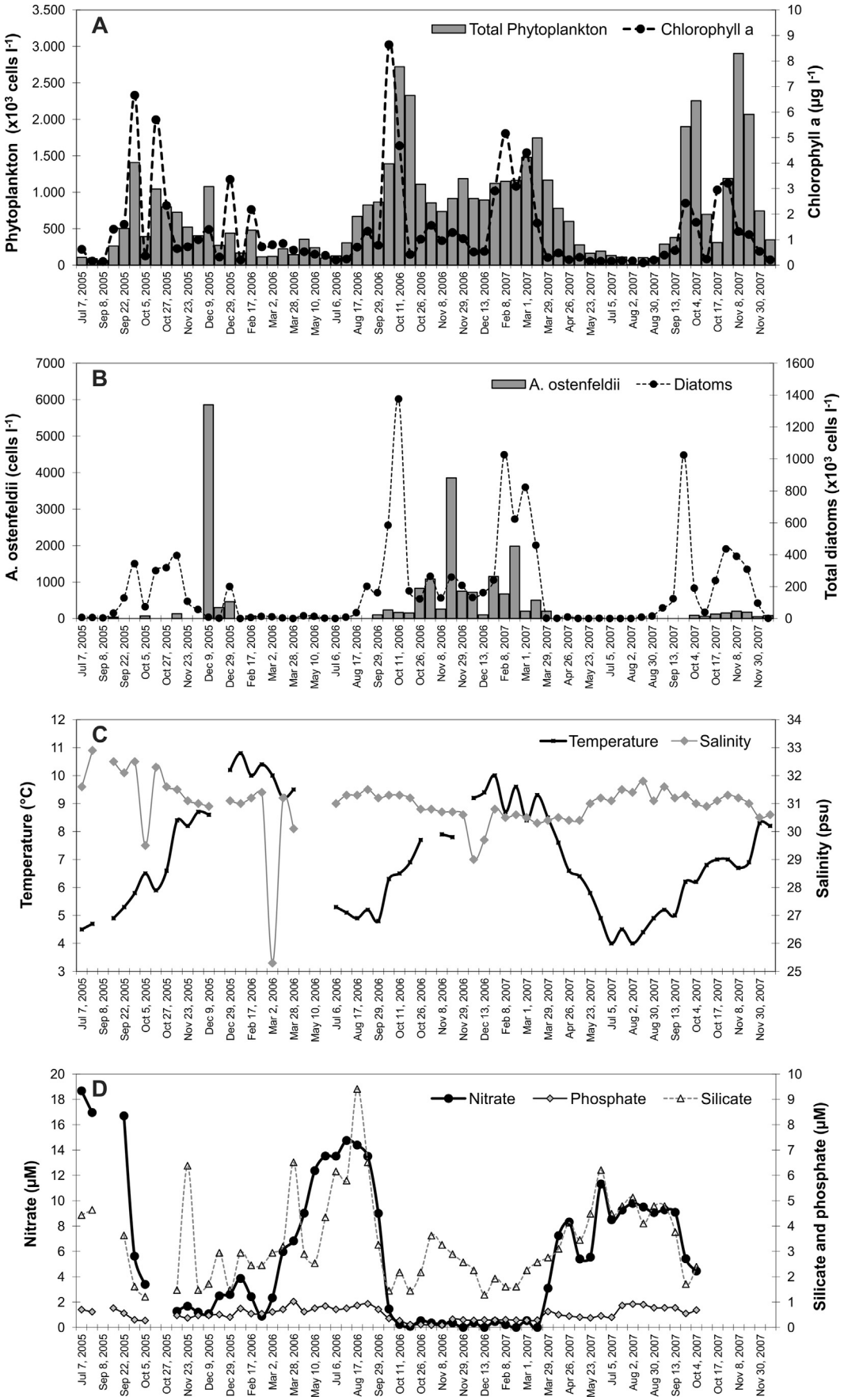


Fig. 2. Temporal distribution of (A) total phytoplankton abundance and chlorophyll a concentration, (B) abundance of the *A. ostenfeldii* complex and total diatoms; (C) temperature and salinity, and (D) nitrates, phosphates and silicates concentrations during sampling period in the Beagle Channel.

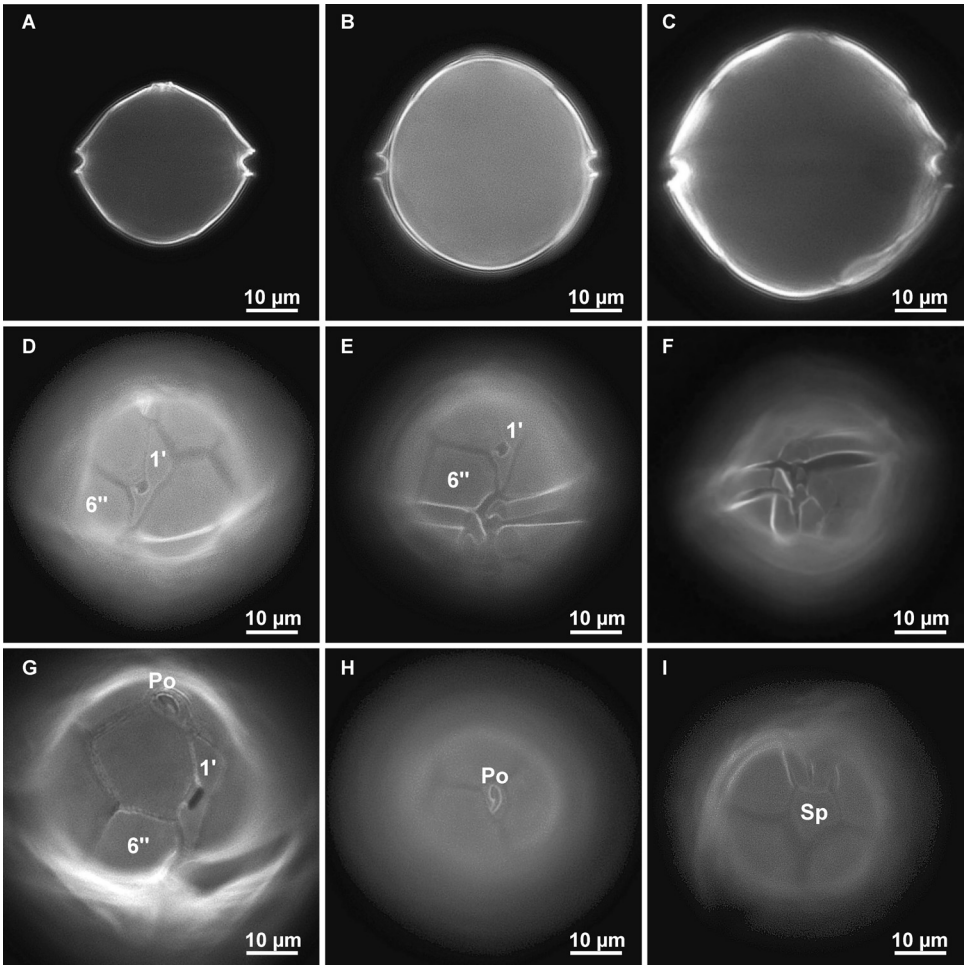


Fig. 3. Specimens of the *Alexandrium ostenfeldii* complex in field samples from the Beagle Channel. Calcofluor stained cells viewed with epifluorescence. (A–C) Cells of different size; (D and E) morphology of Plates 1′ (with a prominent ventral pore) and 6′′; (F) Detail of cingulum and sulcal plates; (G) Epitheca showing the connection between the apical pore plate (Po) and 1′; (H) apical view showing a detail of the apical pore plate (Po); (I) antapical view showing the sulcal posterior plate (Sp).

3.2. Morphology

Field specimens from the Beagle Channel were observed as solitary cells, ranging from 32 to 60 µm length and 34 to 57 µm wide. Cells presented a globose appearance, with both epi- and hipotheca margins lightly rounded (Fig. 3a–c). The cingulum was little excavated and slightly displaced (<1). The first apical (1′) plate was narrow and elongated, and it was arranged in two

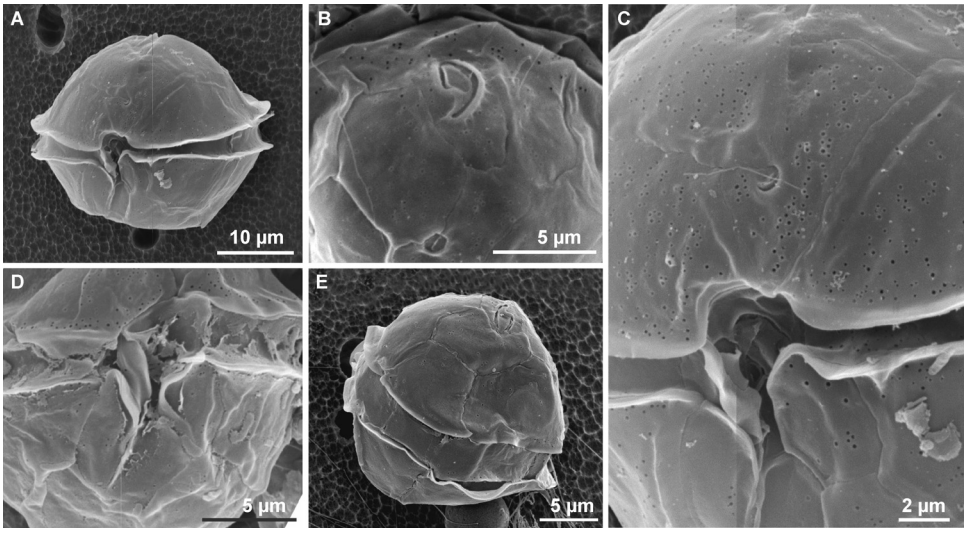


Fig. 4. Scanning electron images of specimens of the *Alexandrium ostenfeldii* complex in cell cultures isolated from the Beagle Channel. (A) Whole theca in ventral view; (B) detail of the apical pore plate; (C) detail of Plate 1′; (D) detail of sulcal plates; (E) whole theca in dorsal view.

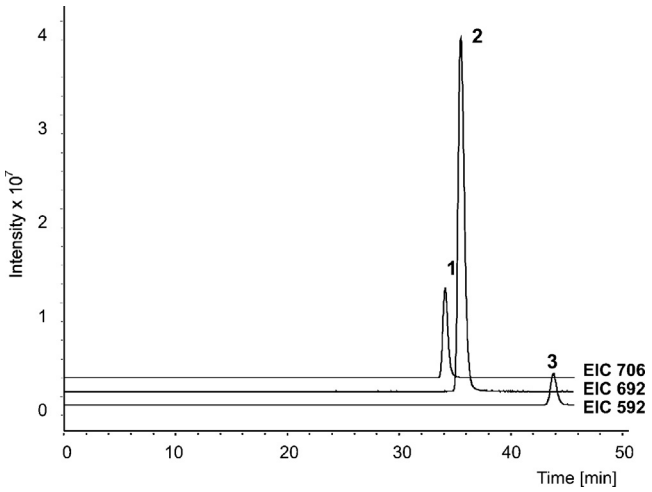


Fig. 5. High performance liquid chromatography/mass spectrometry (HPLC–MS) extracted-ion chromatogram of the *Alexandrium ostenfeldii* extracts. Peak identification (1) 20-methylspirolide G; (2) 13-desmethylspirolide C; (3) unknown spirolide $m/z = 592$.

straight segments with a prominent ventral pore located at its inflexion point (Fig. 3d, e and g). The sulcal anterior (s.a.) was approximately as long as wide, and usually triangular in shape (Fig. 3e and f). The sixth precingular (6″) was wider than tall (Fig. 3e and g). The apical pore plate (Po) was almost completely occupied by a big comma (Fig. 3h). Both, this last plate and the sulcal posterior (sp) lacked a connection pore (Fig. 3h and i).

In culture, specimens were relatively smaller (less than 35 μm long) and presented a more delicate structure (Fig. 4a–e). Under SEM, they showed several small scattered pores on the thecal plates (Fig. 4c). The pore plate (Po) revealed a prominent comma shaped apical pore, surrounded also by small pores (Fig. 4b).

3.3. Spirolide and PSP analyses

HPLC–MS analyses showed detectable amounts of spirolides in both cultures and net phytoplankton samples from Almanza. By contrast, SPXs were not detected in water samples, in which phytoplankton cells are less abundant.

Chromatographic analyses revealed the presence of three different SPXs in cultures, with retention times (Rt) of 33.8, 35.5 and 43.6 min respectively (Figs. 5 and 6). The compound with Rt = 35.5 min ($m/z = 692$) was identified as 13-desmethyl spirolide C (13-desMe-C). The identity of 13-desMe-C was confirmed by its retention time and daughter ion spectra, which were identical to those of authentic standards. Likewise, the compound with Rt = 33.8 min ($m/z = 706$) was identified as 20-methyl spirolide G (20-Me-G). In the absence of authentic standards, the identity of 20-Me-G was tentatively established from their daughter ion spectra (Fig. 7), which closely corresponded with those reported previously for these compounds (Hu et al., 2001; Sleno et al., 2004; Aasen et al., 2005). Finally, a third compound with Rt = 43.6 min ($m/z = 592$) that does not coincide with previously described SPXs, presented a product ion spectra (Fig. 8) similar to those of cyclic imines (Takada et al., 2001; Selwood et al., 2010).

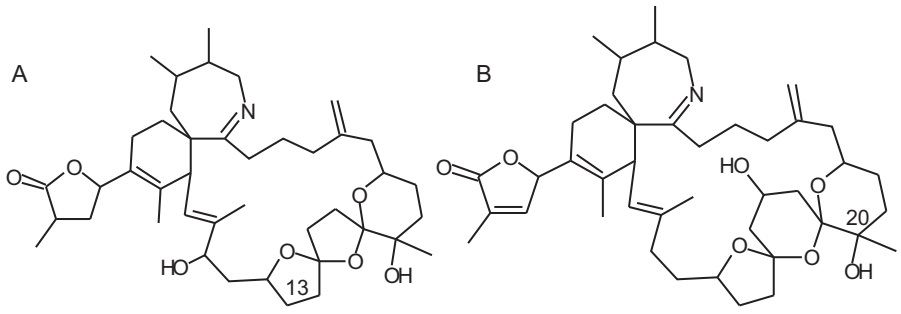
Total spirolide concentration during exponential growth phase was 0.74 pg cell^{-1}, of which 0.5906 ± 0.0032 pg cell^{-1} corresponded to 13-desMe-C and 0.1577 ± 0.0023 pg cell^{-1} to 20-Me-G. The net phytoplankton samples presented a similar toxin profile than that of cultures of *Alexandrium ostenfeldii*.

The presence of 13-desMe-C was detected in all mussel samples analyzed whereas 20-Me-G was only detected in the mussel sample from Lapataia Bay (Fig. 9). Moreover, an additional compound, which was neither detected in cultures nor in phytoplankton samples, was found in high proportions in mussel samples. This compound showed the same m/z ratio (692) than that of 13-desMe-C but presented a higher retention time (Rt = 40.5 min, Fig. 10) and a product ion spectra similar to that obtained for the standard of 13-desMe-C (Fig. 11).

Maximum concentration of SPXs (249.6 μg kg^{-1}) was found in mussels from Almanza, corresponding 68.46 ± 1.82 μg kg^{-1} to 13-desMe-C and 181.21 ± 1.99 μg kg^{-1} for the unknown SPX (Fig. 9).

HPLC PSP analysis showed low toxicity (2.5–1.7 μmol kg^{-1}) in all mussel samples. The maximum PSP toxin content was 2.5 μmol kg^{-1} (GTX2/3 = 55%, STX = 30% and neoSTX = 15%) corresponding to mussels from Ensenada Bay (Fig. 9).

No peak corresponding to PSP toxins was detected in the chromatogram from *A. ostenfeldii* culture.



Spirolides	RT (min) peak n°( )	$[M+H]^+$	Ion observed ion product spectra m/z	Organism
20-methyl G (A)	33.8 (1)	706	688, 670, 652, 392, 374, 346	*A. ostenfeldii*, mussel
13-desmethyl-C (B)	35.5 (2)	692	674, 656, 638, 444, 164	*A. ostenfeldii*, mussel
Unknown 692 (U692)	40.5 (4)	692	674, 656, 638, 444	mussel
Unknown 592 (U592)	43.6 (3)	592	574, 556, 344, 206, 164	*A. ostenfeldii*

Fig. 6. Molecular structure and list of compounds observed in *Alexandrium ostenfeldii* and mussel sample, peak number (), retention times (RT) and MS/MS data.

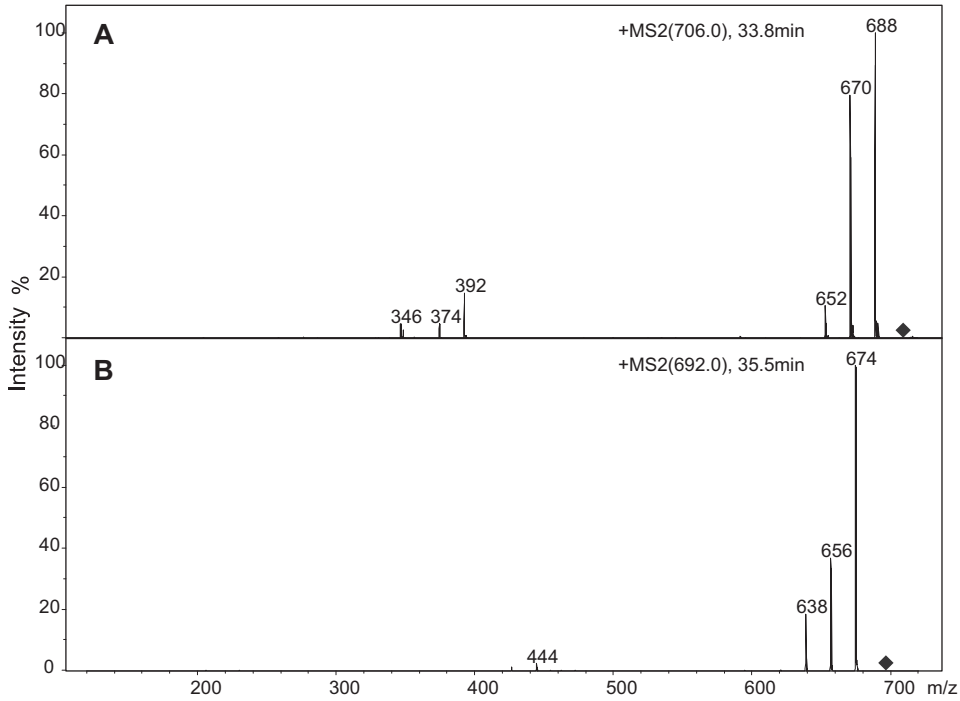


Fig. 7. MS/MS product ion spectra of (a) 20-methylspirolide G and (b) 13-desmethyl spirolide C from *Alexandrium ostenfeldii* extracts.

4. Discussion

4.1. Occurrence and distribution

Even when *Alexandrium peruvianum* was originally described from Peru (Balech and Rojas de Mendiola, 1977), little is known about the distribution of the *Alexandrium ostenfeldii* complex in southern South America. In the southern Pacific coast of Chile, its presence has been occasionally mentioned in southern channels and fjords (Avaria et al., 2003; Lembeye, 2008; Haro et al., 2013). By contrast, and as far as we know, there is no record of the *A. ostenfeldii* complex in the South Atlantic coast of Argentina. From a biogeographical point of view, the *A. ostenfeldii* complex appears to have a cosmopolitan distribution, since it occurs from the Arctic to warm waters (Lundholm and Moestrup, 2006). In this sense, and considering the apparently absence of records of this species complex from Antarctic waters, the Beagle Channel (≈55°S) could be regarded as its southernmost distribution.

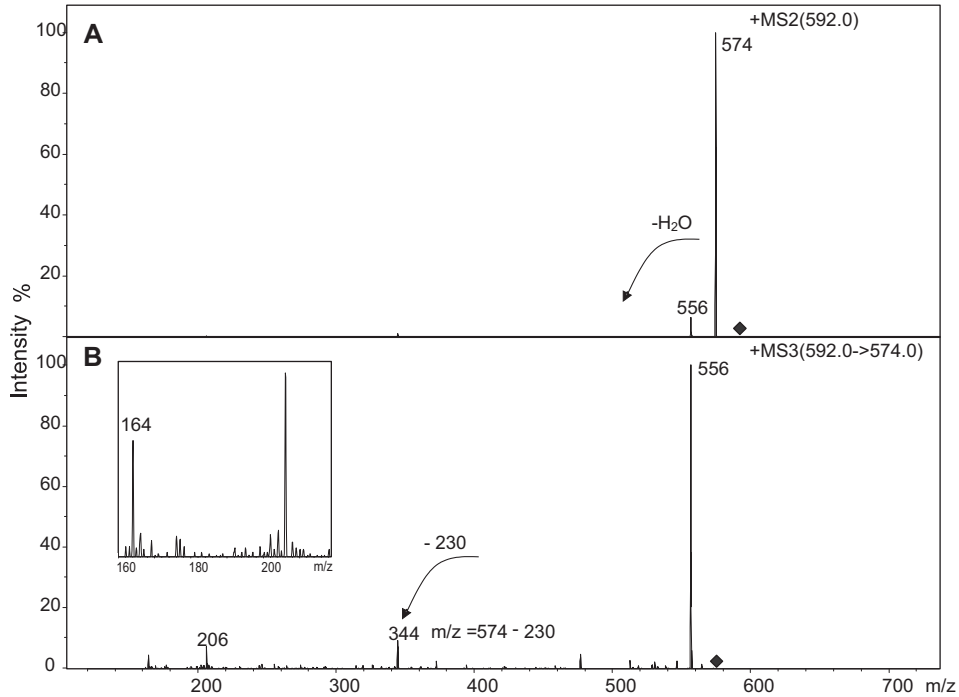


Fig. 8. MS/MS product ion spectra of (a) unknown compound *m*/*z* = 592 from *Alexandrium ostenfeldii* extracts, (b) fragmentation spectrum (MS3) of *m*/*z* = 574. Arrows indicate fragmentation pattern. Insert shows low-mass fragments.

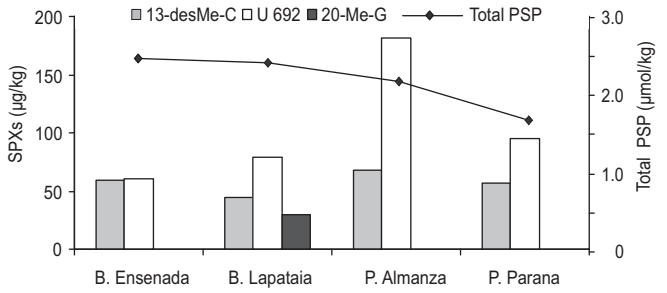


Fig. 9. Concentration of spirolides and paralytic shellfish toxins in mussels (*Mytilus edulis*) in the four sampled locations.

In the present study, the *Alexandrium ostenfeldii* complex was frequently observed during spring, after the main annual diatom bloom. However, it was never observed as a conspicuous component of phytoplankton assemblages during the study period. This pattern agrees with that observed by Avaria et al. (2003), who mentioned *A. ostenfeldii* as one of the most frequently occurring but not abundant species during a cruise conducted between the Strait of Magellan and Cape Horn from 9 to 23 October 1998. Likewise, it was observed in November at the inner-fjord of Seno Ballena (Strait of Magellan, ≈54°S) with a density of only 16.5 cells l^{-1} (Haro et al., 2013).

The range of temperature in which the *Alexandrium ostenfeldii* complex mainly occurred in the Beagle Channel (7.5–10 °C) is low when compared with field results from other latitudes or experimental studies. For example, in the northern Baltic Sea blooms of *A. ostenfeldii* occurs at water temperatures around 20 °C (Hakanen et al., 2012); in the Narragansett Bay (USA) the temperature during the annual maximum of *Alexandrium peruvianum* ranges from 14.8 to 17.8 °C (Borkman et al., 2012), whereas in the Gulf of St. Lawrence (Canada), relatively high concentrations of *A. ostenfeldii* were found at 10.0–12.4 °C (Levasseur et al., 1998). In batch culture experiments from Danish waters, growth occurred at 11.3–23.7 °C and maximum division rates took place at 20 °C (Jensen and Moestrup, 1997), while Chinese isolates showed obvious growth at temperatures between 12 and 24 °C, with the highest exponential division rates around 24 °C (Gu, 2011). On the other hand, the *A. ostenfeldii* complex was observed in the present study in a salinity range between 29.5 and 32.5 psu. A wide range of salinity tolerance from 2 to 35 psu has been globally observed for *A. ostenfeldii* and *A. peruvianum* (Suikkanen et al., 2013, and references therein).

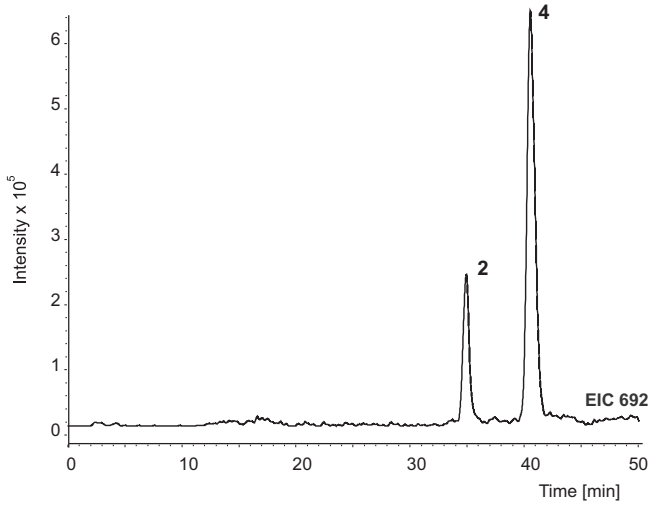


Fig. 10. High performance liquid chromatography/mass spectrometry (HPLC–MS) extracted-ion chromatogram of the mussels (*Mytilus edulis*) extracts. Peak identification, (2) 13-desmethylspirolide C; (4) unknown spirolide $m/z = 692$.

4.2. Morphological features

According to the morphological revision of the genus *Alexandrium* by Balech (1995), *A. ostenfeldii* and *A. peruvianum* are very similar, and the main distinction among them is based on the shape of the s.a. and 1′ plates. In *A. ostenfeldii* the right sides of the 1′ are nearly straight and broken by a large ventral pore forming a distinct angle, whereas in *A. peruvianum* they are curved and interrupted by a relatively smaller ventral pore (Balech and Tangen, 1985). Likewise, the shape of the s.a. is approximately triangular with a pointed anterior end in *A. peruvianum*, while is more rectangular and with a flattened anterior side in *A. ostenfeldii* (Balech and Rojas de Mendiola, 1977; Balech and Tangen, 1985). Other studies, however, have shown a marked variation in the shape of these diagnostic plates, creating uncertainties about the delimitation of both species (Kremp et al., 2009; Gu, 2011). Moreover, recent phylogenetic and morphological data suggest that the *A. ostenfeldii* complex should be regarded as a single genetically structured species (Kremp et al., 2014). After examining cultured isolates from different geographic regions, these authors showed that the traditional morphological delineation of *A. peruvianum* from *A. ostenfeldii* is not well supported, suggesting that *A. peruvianum* should be discontinued as species name and consider a synonym of *A. ostenfeldii*. Even when no material from southern South America was included in the global analysis by Kremp et al. (2014), the specimens from the Beagle Channel are in agreement with their proposal, since they presented a 1′ plate that is in accordance with that described in *A. ostenfeldii* but usually showed a triangular s.a. typical of *A. peruvianum*.

4.3. Toxin analysis

Strains isolated from the Beagle Channel produced mainly spirolide 13-desMe-C and a lower concentration (≈20%) of 20-Me-G. The simultaneous production of these two spirolides has been previously reported in strains isolated from the North Sea (Suikkanen et al., 2013). However, in such cultures 20-Me-G was the most abundant spirolide. A third compound, with an $m/z = 592$ that do not coincide with previously described SPXs, was also detected in our cultures. The neutral loss of water, the loss of a fragment with $m/z = 230$ and the presence of ions $m/z = 164$ and 206 in the sequential product ion spectrum of this compound (Fig. 8), is indicative of its structural similarity with cyclic imines (Sleno et al., 2004; Aasen et al., 2005; Krock and Cembella, 2007; Selwood et al., 2010). However, more spectroscopy studies (high resolution MS and 1D and 2D Nuclear Magnetic Resonance experiments) are necessary to resolve the chemical structure of this unknown moiety. The total spirolide concentration during exponential growth phase of *A. ostenfeldii* cultures (0.74 pg SPX $cell^{-1}$) was similar to those recorded in the North Sea (0.97 pg $cell^{-1}$, Suikkanen et al., 2013) but lower than those observed in the Adriatic Sea (3.7 pg $cell^{-1}$) by Ciminiello et al. (2006).

In mussels, the total SPX concentration was 249.6 µg kg^{-1} meat, which results similar to those recorded by several authors (Aasen et al., 2005; Villar Gonzalez et al., 2006; Amzil et al., 2007). Both 13-desMe-C and 20-Me-G were also found in mussel samples. However, the major compound was a spirolide analogue, which was not observed in cell cultures. This compound showed the same protonated molecule ($[M+H]^+$, $m/z = 692$) than that observed for 13-desMe-C, but showed a longer retention time (Rt = 40.5 min) in HPLC, indicating a less polar closely related molecular structure. The comparative analysis of the product ion spectra of this

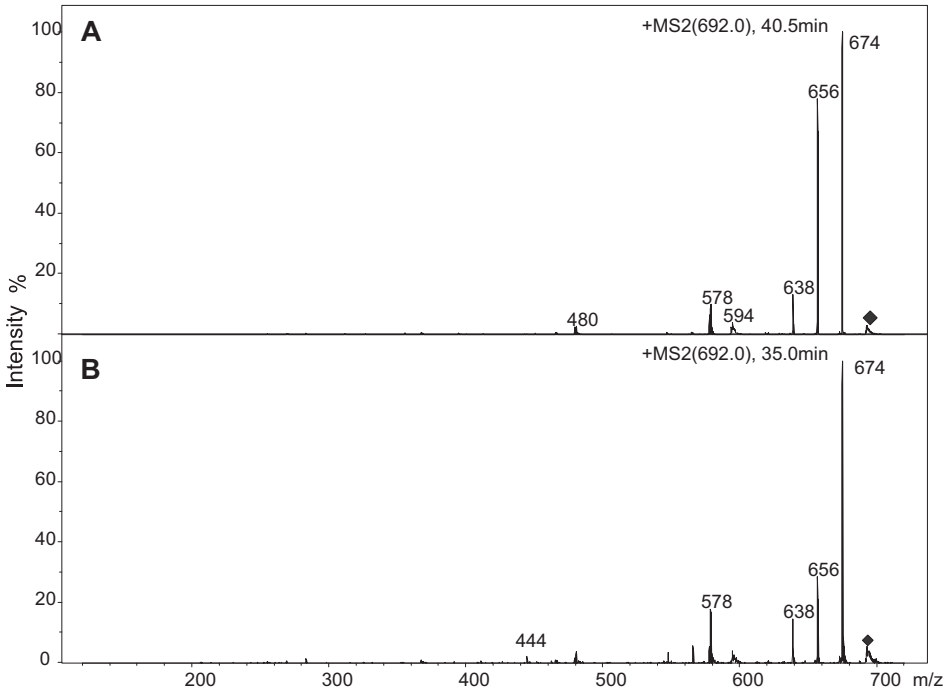


Fig. 11. MS/MS product ion spectra of (a) unknown compound and (b) 13-desmethyl spirolide C of a mussel sample (*Mytilus edulis*) from Puerto Almanza, Beagle Channel.

compound, showed great similarity to that obtained for the 13-desMe-C standard, but with different peak intensities (Fig. 10). It is possible that this compound was formed in the mussel, due either to metabolism or the steaming step used in the preparation of the mussels (Aasen et al., 2005). However, the occurrence of analogues with structural similarity to spirolides has been noted previously in other strains of the *A. ostenfeldii* complex (Van Wagoner et al., 2011). Further conclusion about the structure of this compound cannot be made at this time.

The widespread toxic dinoflagellate *Alexandrium ostenfeldii* complex is the producer of different classes of marine biotoxins depending on its geographical origin (Kremp et al., 2014). The *A. ostenfeldii* strain of the Beagle Channel seems to present a simple spirolide composition, although trace levels of the other spirolides is not discarded. By contrast, the production of PSP toxins was not detected in culture during exponential growth phase.

HPLC analyses for PSP showed low concentration values in all mussel samples. This was in accordance with mouse bioassay results reported for a PSP monitoring programme in this area by Goya and Maldonado (2014). These low PSP values could be the result of a detoxification process that began after a high toxicity event (5600 μg STX equiv. 100 g^{-1}) observed in this region in late February 2010 (Goya and Maldonado, 2014), associated to high abundances of *Alexandrium catenella* (Hernando, unpublished results).

Spirolides are known to display "fast-acting" toxicity in the traditional bioassay, causing rapid death in mice after injection (Cembella et al., 2000; Richard et al., 2001). This could explain the rapid death times in the mouse bioassay observed in the Beagle Channel during December 2005 that led to closure of harvesting areas (Hernando et al., 2007), when the *Alexandrium ostenfeldii* complex reached abundances of $\approx 6 \times 10^3$ cells l^{-1} and *Alexandrium catenella* was almost undetectable.

5. Conclusions

Spirolide production by the *Alexandrium ostenfeldii* complex is reported for the first time in the Beagle Channel (southern Argentina). Even when relatively low abundances of this taxon were observed during the study period, spirolide accumulation in mussels was also detected. SPXs are known to accumulate in shellfish from several regions of the world and be toxic to animals, but at present, there is no conclusive evidence linking them to human intoxications, and there are not regulatory limits for these toxins in shellfish (Munday and Reeve, 2013). This highlights the importance of assessing the risk of such toxins as the basis for establishing tolerance levels in shellfish. PSP toxins, which are recurrently detected in the Beagle Channel associated to *Alexandrium catenella*, were not produced by strains of the *A. ostenfeldii* complex. Further studies will be needed to reveal the genetic identity of the analyzed specimens as wells as to elucidate the structure of the new spirolide derivatives.

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References

Aasen, J., MacKinnon, S.L., LeBlanc, P., Walter, J.A., Hovgaard, P., Aune, T., Quilliam, M.A., 2005. Detection and identification of spirolides in Norwegian shellfish and plankton. Chem. Res. Toxicol. 18, 509–515.

Alvarez, G., Uribe, E., Paulo, A., Marino, C., Blanco, J., 2010. First identification of azaspiracid and spirolides in *Mesodesma donacium* and *Mulinia edulis* from Northern Chile. Toxicon 55, 638–641.

Amzil, Z., Sibat, M., Royer, F., Masson, N., Abadie, E., 2007. Report on the first detection of pectenotoxin-2, spirolide-A and their derivatives in French shellfish. Mar. Drugs 5, 168–179.

Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M., 2012. The globally distributed genus *Alexandrium*: multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae 14, 10–35.

AOAC, 1984. Official Methods of Analysis, 14th ed. Association of Official Analytical Chemists, Arlington.

Avaria, S., Cáceres, C., Castillo, P., Muñoz, P., 2003. Distribución del microfitoplancton marino en la zona Estrecho de Magallanes-Cabo de Hornos, Chile, en la primavera de 1998 (crucero CIMAR 3 fiordos). Rev. Cienc. Tecnol. Mar 26, 79–96.

Balech, E., Tangen, K., 1985. Morphology and taxonomy of toxic species in the Tamarensis Group (Dinophyceae): *Alexandrium excavatum* (Braarud) comb. nov. and *Alexandrium ostenfeldii* (Paulsen) comb. nov. Sarsia 70, 333–343.

Balech, E., Rojas de Mendiola, B., 1977. Un nuevo *Gonyaulax* productor de hemotalasia en Perú (Protozoa, Dinoflagellata). Neotropica 23, 49–54.

Balech, E., 1995. The Genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine Station, Ireland.

Benavides, H., Prado, L., Diaz, S., Carreto, J.J., 1995. An exceptional bloom of *Alexandrium catenella* in the Beagle Channel, Argentina. In: Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Lavoiser Science Publishers, Paris, pp. 113–119.

Borkman, D.G., Smayda, T.J., Tomas, C.R., York, R., Strangman, W., Wright, J., 2012. Toxic *Alexandrium peruvianum* (Balech and de Mendiola) Balech and Tangen in Narragansett Bay, Rhode Island (USA). Harmful Algae 19, 92–100.

Carreto, J.I., Benavides, H.R., 1993. World record of PSP in southern Argentina. Harmful Algae News 5, 2.

Cembella, A.D., Lewis, N.I., Quilliam, M.A., 2000. The marine dinoflagellate *Alexandrium ostenfeldii* (Dinophyceae) as the causative organism of spirolide shellfish toxins. Phycologia 39, 67–74.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Grauso, L., Tartaglione, L., Guerrini, F., Pistocchi, R., 2007. Spirolide toxin profile of Adriatic *Alexandrium ostenfeldii* cultures and structure elucidation of 27-hydroxy-13,19-didesmethyl spirolide C. J. Nat. Prod. 70, 1878–1883.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Magno, S., Tartaglione, L., Cangini, M., Pompei, M., Guerrini, F., Boni, L., Pistocchi, R., 2006. Toxin profile of *Alexandrium ostenfeldii* (Dinophyceae) from the Northern Adriatic Sea revealed by liquid chromatography–mass spectrometry. Toxicon 47, 597–604.

Eberlein, K., Kattner, G., 1987. Automatic method for the determination of orthophospate and total dissolved phosphorus in the marine environment. Fresenius J. Anal. Chem. 326, 354–357.

EURLMB, 2011. EU-Harmonised Standard Operating Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC–MS/MS. European Union Reference Laboratory for Marine Biotoxins. http://aesan.msps.es/en/CRLMB/web/home.shtml.

Franco, J.M., Paz, B., Riobo, P., Pizarro, G., Figueroa, R., Fraga, S., Bravo, I., 2006. First report of the production of spirolides by *Alexandrium peruvianum* (Dinophyceae) from the Mediterranean Sea. In: 12th International Conference on Harmful Algae. Copenhagen, Denmark, September 4–8, 2006, (Programs and Abstracts), p. 174.

Fritz, L., Triemer, R.E., 1985. A rapid simple technique utilizing Calcoflour White M2R for the visualization of dinoflagellate thecal plates. J. Phycol. 21, 662–664.

Goya, A.B., Maldonado, S., 2014. Evolution of PSP toxicity in shellfish from the Beagle Channel (Tierra del Fuego, Argentina): an overview. In: Sauvé, E. (Ed.), Molluscan Shellfish Safety. Springer, Netherlands, pp. 15–23.

Grasshoff, K., 1969. A simultaneous multiple channel system for nutrient analyses in seawater with analog and digital data record. In: Technicon International Congress, Chicago. Technicon Publication Board, Chicago, pp. 133–145.

Grasshoff, K., Ehrhardt, M., Kremling, K., 1983. Methods of Seawater Analysis. Verlag Chemie, Weinheim.

Gu, H., 2011. Morphology, phylogenetic position, and ecophysiology of *Alexandrium ostenfeldii* (Dinophyceae) from the Bohai Sea, China. J. Syst. Evol. 49, 606–616.

Hakanen, P., Suikkanen, S., Franzén, J., Franzén, H., Kankaanpää, H., Kremp, A., 2012. Bloom and toxin dynamics of *Alexandrium ostenfeldii* in a shallow embayment at the SW coast of Finland, northern Baltic Sea. Harmful Algae 15, 91–99.

Haro, D., Aguayo-Lobo, A., Acevedo, J., 2013. Características oceanográficas y biológicas de las comunidades del plancton y necton del Área marina costera Protegida Francisco Coloane: una revisión. An. Inst. Patagonia (Chile) 41, 77–90.

Hernando, M., Torres, E., San Román, N., Hoffmeyer, M., 2007. Monitoreo planctónico y ambiental para el desarrollo sustentable del cultivo comercial de mejillón (Mytilus edulis chilensis) en la zona de Almanza, Canal Beagle (Tierra del Fuego). Subproyecto GEF A-B-62, Proyecto PNUD Arg/02/018. Informe técnico final, pp. 1–43.

Holm-Hansen, O., Riemann, B., 1978. Chlorophyll a determination: improvements in methodology. Oikos 30, 438–447.

Holm-Hansen, O., Lorenzen, C.J., Holmes, R.W., Strickland, J.D., 1965. Fluorometric determination of chlorophyll. J. Cons. Int. Explor. Mer. 30, 3–15.

Hsia, M.H., Morton, S.L., Smith, L.L., Beauchesne, K.R., Huncik, K.M., Moeller, P.D., 2006. Production of goniodomin A by the planktonic, chain-forming dinoflagellate *Alexandrium monilatum* (Howell) Balech isolated from the Gulf Coast of the United States. Harmful Algae 5, 290–299.

Hu, T., Curtis, J.M., Walter, J.A., Wright, J.L.C., 1996. Characterization of biologically inactive spirolides E and F: identification of the spirolide pharmacophore. Tetrahedron Lett. 37, 7671–7674.

Hu, T., Burton, I.W., Cembella, A.D., Curtis, J.M., Quilliam, M.A., Walter, J.A., Wright, J.L.C., 2001. Characterization of spirolides A, C and 13-desmethyl C, New marine toxins isolated from toxic plankton and contaminated shellfish. J. Nat.Prod. 64, 308–312.

Jensen, M.Ø., Moestrup, Ø., 1997. Autecology of the toxic dinoflagellate *Alexandrium ostenfeldii*: life history and growth at different temperatures and salinities. Eur. J. Phycol. 32, 9–18.

Kremp, A., Tahvanainen, P., Litaker, W., Krock, B., Suikkanen, S., Leaw, C.P., Tomas, C., 2014. Phylogenetic relationships, morphological variation, and toxin patterns in the *Alexandrium ostenfeldii* (Dinophyceae) complex: implications for species boundaries and identities. J. Phycol. 50, 81–100.

Kremp, A., Lundholm, T., Dreßler, N., Erler, K., Gerdts, G., Eirtovaara, S., Leskinen, E., 2009. Bloom forming *Alexandrium ostenfeldii* (Dinophyceae) in shallow waters of the Åland Archipelago, Northern Baltic Sea. Harmful Algae 8, 318–328.

Krock, B., Cembella, A., 2007. Mass spectral characterization of undescribed spirolides in an isolate of the dinoflagellate Alexandrium ostenfeldii from Atlantic Canada. In: Proceedings of the 6th International Conference on Molluscan Shellfish Safety. pp. 316–321.

Lembeye, G., 2008. Harmful algal blooms in the austral Chilean channels and fjords. In: Silva, N., Palma, S. (Eds.), Progress in the Oceanographic Knowledge of Chilean Interior Waters, from Puerto Montt to Cape Horn. Comité Oceanográfico Nacional – Pontificia Universidad Católica de Valparaíso, Valparaíso, pp. 99–103.

Levasseur, M., Berard-Therriault, L., Bonneau, E., Roy, S., 1998. Distribution of the toxic dinoflagellate *Alexandrium ostenfeldii* in the Gulf of St. Lawrence. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission (UNESCO), Santiago de Compostela, Spain, pp. 54–57.

Lim, P.T., Usup, G., Leaw, C.P., Ogata, T., 2005. First report of *Alexandrium taylori* and *Alexandrium peruvianum* (Dinophyceae) in Malaysia waters. Harmful Algae 4, 391–400.

Lundholm, N., Moestrup, Ø., 2006. The biogeography of harmful algae. In: Granéli, E., Turner, J.T. (Eds.), Ecology of Harmful Algae. Ecological Studies, vol. 189. Springer-Verlag, Berlin Heidelberg, pp. 23–35.

MacKinnon, S.L., Walter, J.A., Quilliam, M.A., Cembella, A.D., LeBlanc, P., Hardstaff, I.W., Burton, I.W.R., Lewis, N.I., 2006. Spirolides isolated from Danish strains of the toxigenic dinoflagellate *Alexandrium ostenfeldii*. J. Nat. Prod. 69, 983–987.

Montoya, N.G., Fulco, K.V., Carignan, M.O., Carreto, J.I., 2010. Toxin variability in cultured and natural populations of *Alexandrium tamarense* from southern South America – evidences of diversity and environmental regulation. Toxicon 56, 1408–1418.

Munday, R., Reeve, J., 2013. Risk assessment of shellfish toxins. Toxins 5, 2109–2137.

Onodera, H., Oshima, Y., Watanabe, M.F., Watanabe, M., Bolch, C.J., Blackburn, S., Yasumoto, T., 1996. Screening of paralytic shellfish toxins in freshwater cyanobacteria and chemical confirmation of the toxins in culture *Anabaena circinalis* Australia. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. IOC UNESCO, pp. 563–566.

Oshima, Y., 1995. Postcolumn derivatization liquid chromatography method for paralytic shellfish toxins. J. AOAC Int. 78, 528–532.

Persich, G.R., Kulisb, D.M., Lilly, E.L., Anderson, D.M., Garcia, V.M.T., 2006. Probable origin and toxin profile of *Alexandrium tamarense* (Lebour) Balech from southern Brazil. Harmful Algae 5, 36–44.

Richard, D., Arsenault, E., Cembella, A.D., Quilliam, M., 2001. Investigations into the toxicology and pharmacology of spirolides, a novel group of shellfish toxins. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J.S., Lewis, R.J. (Eds.), Ninth International Conference on Harmful Algal Blooms. IOC of UNESCO, Hobart, Australia, pp. 383–386.

Selwood, A.I., Miles, C.O., Wilkins, A.L., Van Ginkel, R., Munday, R., McNabb, P., 2010. Isolation, Structural Determination and Acute Toxicity of Pinnatoxins E, F and G. J. Agric. Food Chem. 58, 6532–6542.

Sleno, L., Chalmers, M.J., Volmer, D.A., 2004. Structural study of spirolide marine toxins by mass spectrometry. Part II. Mass spectrometric characterization of unknown spirolides and related compounds in a cultured phytoplankton extract. Anal. Bioanal. Chem. 378, 977–986.

Suikkanen, S., Kremp, A., Hautala, H., Krock, B., 2013. Paralytic shellfish toxins or spirolides? The role of environmental and genetic factors in toxin production of the *Alexandrium ostenfeldii* complex. Harmful Algae 26, 52–59.

Takada, N., Umemura, N., Suenaga, K., Uemara, D., 2001. Structural determination of pteriatoxins A, B and C, extremely potent toxins from the bivalve *Pteria penguin*. Tetrahedron Lett. 42, 3495–3497.

Technicon, 1977. Silicates in water and seawater. Technicon AutoAnalyzer II Industrial Method No. 186-72W/B Technicon Industrial Systems, Tarrytown, New York.

Tomas, C.R., van Wagoner, R., Tatters, A.O., White, K.D., Hall, S., Wright, J.L., 2012. *Alexandrium peruvianum* (Balech and Mendiola) Balech and Tangen a new toxic species for coastal North Carolina. Harmful Algae 17, 54–63.

Touzet, N., Franco, J.M., Raine, R., 2008. Morphogenetic diversity and biotoxin composition of Alexandrium (Dinophyceae) in Irish coastal waters. Harmful Algae 7, 782–797.

Utermöhl, H., 1958. Zur vervollkommnung der quantitativen phytoplankton-methodik. Mitt. Int. Vereinigung Theor. Angew. Limnol. 9, 1–38.

Van Wagoner, R.M., Misner, I., Tomas, C.R., Wright, J.L., 2011. Occurrence of 12-methylgymnodimine in a spirolide-producing dinoflagellate *Alexandrium peruvianum* and the biogenetic implications. Tetrahedron Lett. 52, 4243–4246.

Villar Gonzalez, A., Rodriguez-Velasco, M.L., Ben-Gigirey, B., Botana, L.M., 2006. First evidence of spirolides in Spanish shellfish. Toxicon 48, 1068–1074.