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Seasonal variability of *Dinophysis* spp. and *Protoceratium reticulatum* associated to lipophilic shellfish toxins in a strongly stratified Chilean fjord

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ABSTRACT

The fine scale vertical distribution of *Dinophysis* spp. and *Protoceratium reticulatum* (potential producers of lipophilic shellfish toxins, LSTs) and its relation with LSTs in shellfish was studied in Reloncaví fjord, a strongly stratified system in Southern Chile. Samples were taken over two years from late spring to early autumn (2007–2008 period) and from early spring to late summer (2008–2009 period). *Dinophysis* spp., in particular *Dinophysis acuminata*, were always detected, often forming thin layers in the region of the salinity driven pycnocline, with cell maxima for *D. acuminata* of 28.5×10^3 cells L^{-1} in March 2008 and 17.1×10^3 cells L^{-1} in November 2008. During the 2008–2009 sampling period, blooms of *D. acuminata* co-occurred with high densities of cryptophyceans and the ciliate *Mesodinium* spp. The highest levels of pectenotoxin-2 (PTX-2; 2.2 ng L^{-1}) were found in the plankton in February 2009, associated with moderate densities of *D. acuminata*, *Dinophysis tripos* and *Dinophysis subcircularis* ($0.1–0.6 \times 10^3$ cells L^{-1}). However, only trace levels of PTX-2 were observed in bivalves at that time. Dinophysistoxin (DTX-1 and DTX-3) levels in bivalves and densities of *Dinophysis* spp. were not well correlated. Low DTX levels in bivalves observed during a major bloom of *D. acuminata* in March 2008 suggested that there is a large seasonal intraspecific variability in toxin content of *Dinophysis* spp. driven by changes in population structure associated with distinct LST toxin profiles in Reloncaví fjord during the study period. A heterogeneous vertical distribution was also observed for *P. reticulatum*, whose presence was restricted to summer months. A bloom of this species of 2.2×10^3 cells L^{-1} at 14 m depth in February 2009 was positively correlated with high concentrations of yessotoxins in bivalves (51–496 ng g^{-1}) and plankton samples (3.2 ng L^{-1}). Our results suggest that a review of monitoring strategies for *Dinophysis* spp. in strongly stratified fjord systems should be carried out. They also indicate that early warning of LST events based on *Dinophysis* cell numbers are not reliable for seafood control.

1. Introduction

The occurrence of lipophilic shellfish toxins (LSTs) causes important economic losses in coastal areas with human health consequences in seafood consumers (van Dolah, 2000). Among these toxins, okadaic acid (OA) and dinophysistoxins (DTXs) are of special concern because they cause diarrheic shellfish poisoning (DSP), a gastrointestinal syndrome which includes symptoms such as intense diarrhea, vomiting and strong abdominal pain (Quiliam and Wright, 1995). OA and DTXs (mainly DTX-1 and DTX-2) are produced by dinoflagellate species of the genera *Dinophysis* and *Prorocentrum* while dinophysistoxin-3 (DTX-3) comprises the fatty acid acyl esters of these toxins resulting from shellfish metabolism (Quilliam, 2003). Although only OA and DTXs have been shown to inhibit phosphatase activity, which is linked to inflammation of the intestinal tract and diarrhea (Hamano et al., 1986), other LSTs such as the pectenotoxins (PTXs) and yessotoxins (YTXs) have been included in seafood safety regulations because they are toxic to

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mice following intraperitoneal injection of lipophilic shellfish extracts. PTXs are known to be produced only by *Dinophysis* species while YTXs are related to the dinoflagellates *Protoceratium reticulatum*, *Lingulodinium polyedrum* and *Gonyaulax spinifera* (Blanco et al., 2005; Tubaro et al., 2010).

In Chile, LSTs show a distinct pattern in their latitudinal distribution. DTX-1 and DTX-3 seem to be restricted to southern Chile (53–41°S) where they are the predominant DSP toxins (García et al., 2004a, 2010, 2012; Zhao et al., 1993). Chronic occurrence of DTXs in bivalves during spring–summer in this area is usually associated with *Dinophysis acuta* (Lembeye et al., 1993) and less frequently with *Dinophysis acuminata* (Garcia et al., 2012; Uribe et al., 2001). Although DTX-1 has been detected in plankton samples from this region, the causative organism has not been identified (Trefault et al., 2011). On the other hand, pectenotoxin-2 (PTX-2), pectenotoxin-2 seco acid (PTX-2sa) and pectenotoxin-11 (PTX-11) have been found in plankton samples collected along a latitudinal transect (27–54°S) (Trefault et al., 2011) and at 18°S (Krock et al., 2009). PTX2 was also detected in passive samplers (DIAION resin) and filter feeders from southern (Garcia et al., 2012; Goto et al., 2000; Pizarro et al., 2011) and northern Chile (Blanco et al., 2007), respectively. Production of PTX-2 by *D. acuminata* strains from northern and southern Chile was confirmed in extracts of isolated cells (Blanco et al., 2007) and in cultures (Fux et al., 2011). YTXs were recorded in bivalves from southern Chile (Garcia et al., 2012; Villarroel, 2004; Yasumoto and Takizawa, 1997) and in plankton samples from northern Chile (Álvarez et al., 2011; Krock et al., 2009) containing *P. reticulatum*. Hence, the geographical distribution of LSTs in Chile is broadly known, but the seasonal variability of these toxins and their relation with the distribution of the causative dinoflagellates has not been assessed in a quantitative manner.

The fjords region in Southern Chile, which is one of the main shellfish production areas in the world, is subject to endemic DSP outbreaks that pose a serious threat to public health, shellfisheries and aquaculture. Mainstream information on the seasonal occurrence of DSP toxins in this geographic area is usually estimated by mouse bioassay (MBA) (e.g. Lembeye et al., 1997, 1998; Seguel and Sfeir, 2010; Seguel et al., 2005; Uribe et al., 1995). This method may be appropriate for seafood safety control, because it provides an overall response (in OA equivalents) from co-extracted LSTs, but it is not selective, sensitivity is low and no information is obtained on toxin profile and content. To gain a better understanding on DSP toxin dynamics in southern Chile, it is necessary to quantify potential LST producers in the plankton using sampling strategies that consider the frequent low density of *Dinophysis* spp., their heterogeneous vertical distribution in the water column (Reguera et al., 2012), and the presence of their putative prey, *Mesodinium* spp. (Park et al., 2006). The objective of this study was to describe the seasonal distribution of *Dinophysis* spp. and *Protoceratium reticulatum*, potential producers of LSTs in Reloncaví fjord, a strongly stratified fjord from southern Chile, and their relationship to concentrations of LSTs (OA, DTXs, PTXs and YTXs) in plankton populations and bivalves in the area.

2. Materials and methods

2.1. Study area and sample collection

Reloncaví fjord, located in the uppermost region (~41.6°S) of the Chilean fjords area (Fig. 1), is the site of one of the largest production areas of mytilid bivalves in Chile. This 60-km long fjord has a surface of 170 km^2, a maximum depth of 460 m and can be used as a representative model for other fjords in the region. The study was performed during two sampling periods at a fixed (~20-m deep) sampling station located at the head of the fjord (41°29'S, 72°18'W; Fig. 1). Samples were collected every 2 or 3 weeks from December 2007 to April 2008 (late spring to early autumn; 2007–2008 period) and from October 2008 to March 2009 (early spring to late summer; 2008–2009 period); there were seven sampling dates in each period. CTD (Sea Bird 19-plus) casts were used to obtain real time vertical profiles of salinity, temperature and fluorescence. Water transparency

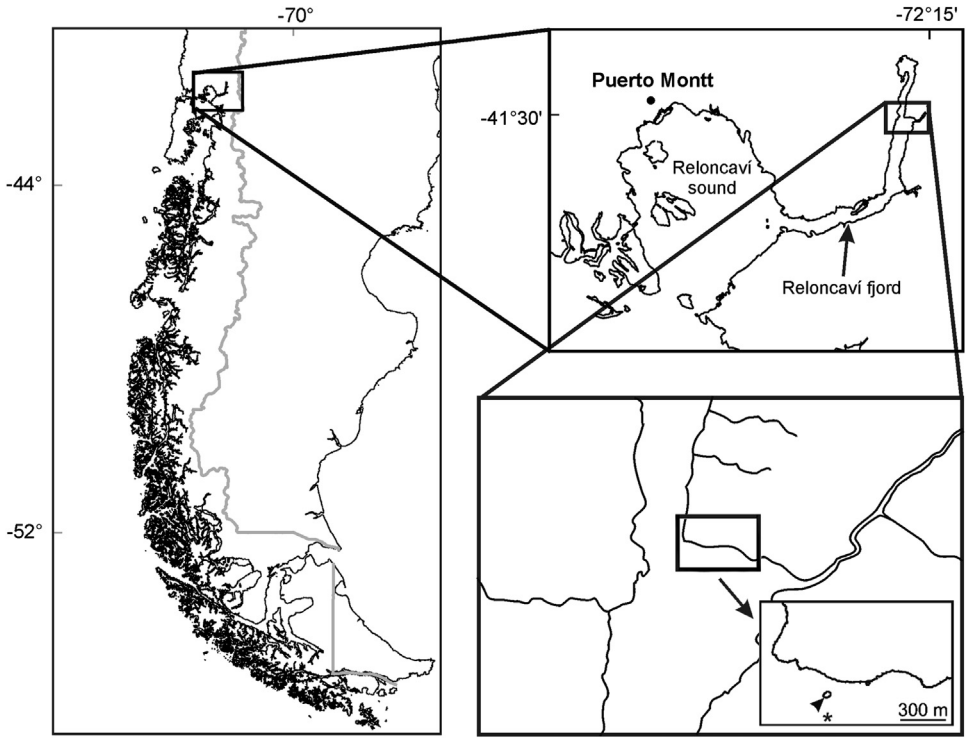

Fig. 1. Location of sampling stations for plankton (asterisk) and shellfish (arrowhead) in Reloncaví fjord, Chile.

was estimated from Secchi depth. Five depths were selected based on the CTD profiles: subsurface (1), upper (2) and lower (3) boundaries of the pycnocline, the fluorescence maximum (4) and 16 m (5). Chlorophyll-*a* (chl-*a*), nitrate (NO_3^-), orthophosphate (PO_4^{3-}) and silicic acid ($Si(OH)_4$) concentrations were determined from triplicate 50 mL water samples collected with a submersible pump (Rule 800 GPH; 7.5 L min^{-1}) connected to a hose. After each depth was sampled, the pump was placed at the next depth and allowed to flush for 2 min to eliminate water left from the previous depth. To obtain a high vertical-resolution distribution of potentially toxic (LST) dinoflagellates, samples (3 replicates of 500 mL) were taken every meter from 1 to 20 m and immediately fixed with Lugol's solution.

Depth-integrated samples for determination of LSTs in the plankton (one replicate) were taken during the 2008–2009 period only at 1–4, 5–8, 9–12 and 13–16 m using the submersible pump. For each depth interval, the pump was placed at the lower depth level and raised for 8 min at a constant speed until the upper depth level was reached. About 60 L of water was pumped, the 88-20 μm size-fraction collected and backwashed in 50 mL of filtered sea water, and this suspension was filtered onto Whatman GF/F glass-fiber filters, which were preserved in methanol and stored at −20 °C until toxin extraction.

For determination of LSTs in bivalves, three replicates of 6–10 adult specimens of *Mytilus chilensis* and *Aulacomya ater* were collected by the steep rocky shore, located at about 150 m from the water sampling station (Fig. 1) by a SCUBA diver at three depths (5, 10 and 15 m). Due to the bathymetric distribution of these two species, *M. chilensis* specimens were taken from 5 and 10 m while those of *A. ater* were taken from 15 m. In addition, 2 kg of *M. chilensis* were taken from 10 m during the 2008–2009 period to estimate DSP toxicity by MBA. All bivalve samples were stored at −20 °C until toxin analyses were performed.

2.2. Determination of nutrients and chl-a

Water samples for determination of NO_3^-, PO_4^{3-} and $Si(OH)_4$ were collected in 50 mL polyethylene bottles and kept frozen (−20 °C) until analysis using colorimetric procedures (Strickland and Parsons, 1972). Water samples (50 mL) for chl-*a* determination were filtered onto Whatman GF/F glass-fiber filters and frozen at −20 °C until analysis. Chl-*a* was extracted overnight in acetone (90%), measured by a digital fluorometer (Turner Design Model PS-700) and its concentration (μg L^{-1}) determined using the equation recommended by Parsons et al. (1984).

2.3. Enumeration of dinoflagellates potential producers of LSTs

The 500-mL samples were left to sediment for two weeks. After this period, water from each sample was carefully siphoned out with a Pasteur pipette attached to a thin tube until a final volume of 50 mL was left. Densities (cells L^{-1}) were estimated from these concentrated samples according to Utermöhl (1958) using 10-mL sedimentation columns. The whole surface of the chamber was examined at a magnification of 400× under an inverted microscope (Nikon Eclipse TS100). The ciliate *Myrionecta rubra* and cryptophycean microalgae, both of them are currently used to support laboratory cultures of *Dinophysis* spp. (Park et al., 2006), were also determined for the 2007–2008 period. For these, cells were quantified in random fields under 400× magnification until at least 100 units ($p < 0.05$) were enumerated

2.4. Extraction of plankton and shellfish for LSTs analysis.

Bivalve samples were analyzed by Liquid Chromatography with Fluorimetric Detection (LC–FD). In addition, plankton (20–88-μm size fraction) and bivalve samples taken during the 2008–2009 period were analyzed by Liquid Chromatography–Mass Spectrometry (LC–MS).

Two grams of hepatopancreas (HP) was removed from bivalve samples, homogenized and extracted twice with 3 mL of chilled 80% methanol, under mechanical stirring using a tissue tearer. Determination of OA, DTX-1, DTX-2 and DTX-3 by LC–FD was performed according to García et al. (2004b). The clean and dry shellfish extracts were used for derivatization with ADAM and then followed at the excitation and emission wavelengths 365 and 415 nm respectively (fluorescence detector, Jasco FP-2020 Plus). The HPLC chemical analyses were performed on a Shimadzu Liquid Chromatography System equipped with a pump (Shimadzu LC-6A), a Rheodyne injector (7725i, Rheodyne, Cotati, CA, USA), a fluorescence detector (Jasco FP-2020 Plus) and a reverse phase column Supelcosil LC-18 (250 × 4 mm; 5 μm particle size) (Supelco, USA). An isocratic mobile phase of CH3CN/CH3OH/H2O 8:1:1 (v/v) with a flow rate of 1 mL min^{-1} was run at room temperature. For LC–MS, determination of OA, DTX-1, DTX-2, PTX-2, YTX and 45-OHYTX followed the method developed by Gerssen et al. (2009), on a Waters XBridge C18 (150 × 2.1 mm, 5 μm particle size) using an ion trap mass spectrometer (Thermo Finnigan LCQ-Advantage) equipped with an electrospray ionization interface (ESI). Full scan spectra were acquired in negative mode in the mass range *m/z* 300–2000 and positive mode in the mass range *m/z* 80–2000. Selected ion chromatograms for YTX and 45-OHYTX were performed in negative ion mode at *m/z* 1141 $[M–2Na+H]^-$ and *m/z* 1157 $[M-2Na+H]^-$ respectively and for PTX2 in positive ion mode at *m/z* 876 $[M+NH_4]^+$. Toxin analytical standards of OA, DTX-1, DTX-2 and PTX-2 were purchased from the Institute for Marine Bioscience of the National Research Council of Canada (IMB-NRCC), YTX from the Institute of Environmental Science and Research Ltd. (New Zealand) and 45-OHYTX was provided by Professor T. Yasumoto.

Plankton samples were evaporated under N_2 stream and extracted with 0.5 mL of 80% methanol in an ultrasonic bath for 10 min. Chromatographic separations were performed on an Agilent 1200 LC (Agilent Technologies, USA) equipped with a Luna C8 column (50 × 1 mm, 3 μm) and a SecurityGuard cartridge (4 × 2 mm, 3 μm; Phenomenex, USA). Separations were carried out at 30 °C and 0.2 mL min^{-1} using a binary gradient elution based on McNabb et al. (2005) and Villar-González et al. (2007) with modifications. In short, mobile phases consisted of 100% water (A) and 95% acetonitrile (B), both containing 2 mM ammonium formate and 50 mM formic acid. The chromatographic gradient was programmed from 90% A up to 100% B in a total run cycle of 25 min including 8.5 min of post-run equilibration. Mass spectrometry detection was carried out with a 3200 QTRAP mass spectrometer (Applied Biosystems, USA) in positive and negative mode. Multiple Reaction Monitoring analysis was performed with two m/z transitions for each compound as quantitative and qualifier ions (parent ion > daughter ion 1/daughter ion 2): ESI positive [M+H or M+Na]+, 508.2 > 202.2/160.2 for GYM, 692.5 > 444.2/426.3 for SPX-1, 881.6 > 539.5/569.5 for PTX-2, 899.5 > 557.5/587.5 for PTX-2 seco acid, 843.5 > 362.4/462.4 for AZA-1; ESI negative [M–H or M-2Na+H]-, 803.5 > 255.2/209.2 for OA and DTX-2, 817.5 > 255.2/209.2 for DTX-1, 1141.5 > 855.2/713.2 for YTX, 1157.5 > 855.2/713.2 for 45-OHYTX, 1155.5 > 869.2/727.2 for homoYTX, and 1171.5 > 869.2/727.2 for 45-OHhomoYTX.

2.5. MBA

A modified version of Yasumoto's method (Yasumoto et al., 1978) was applied to the mouse biological assays for DSPs. Briefly, 20 g of bivalve HP was extracted three times with acetone and evaporated. To eliminate paralytic shellfish poisoning (PSP) toxins from the samples, the obtained residue was extracted with diethyl-ether, evaporated and resuspended in 1 mL of Tween-60 (1%).

Then, 1 mL of this solution was injected intraperitoneally into each of three mice (CF-1), weighting 19–20 g. Bivalves were considered toxic if two out of three mice died within 24 h.

2.6. Data analyses

A Canonical Correspondence Analysis (CCA) was performed to evaluate the relevance of environmental variables on the density of the most abundant species of dinoflagellates potentially producing LSTs and the composition of these toxins in plankton samples during the 2008–2009 period. Given that LST levels were estimated from depth-integrated samples, dinoflagellate densities, salinity and water temperature recorded from 1 to 4, 5 to 8, 9 to 12 and 13 to 16 m depth intervals were averaged. These average values were related to the corresponding average values of chl-*a* and nutrients obtained from discrete depths. Data were logarithmically transformed

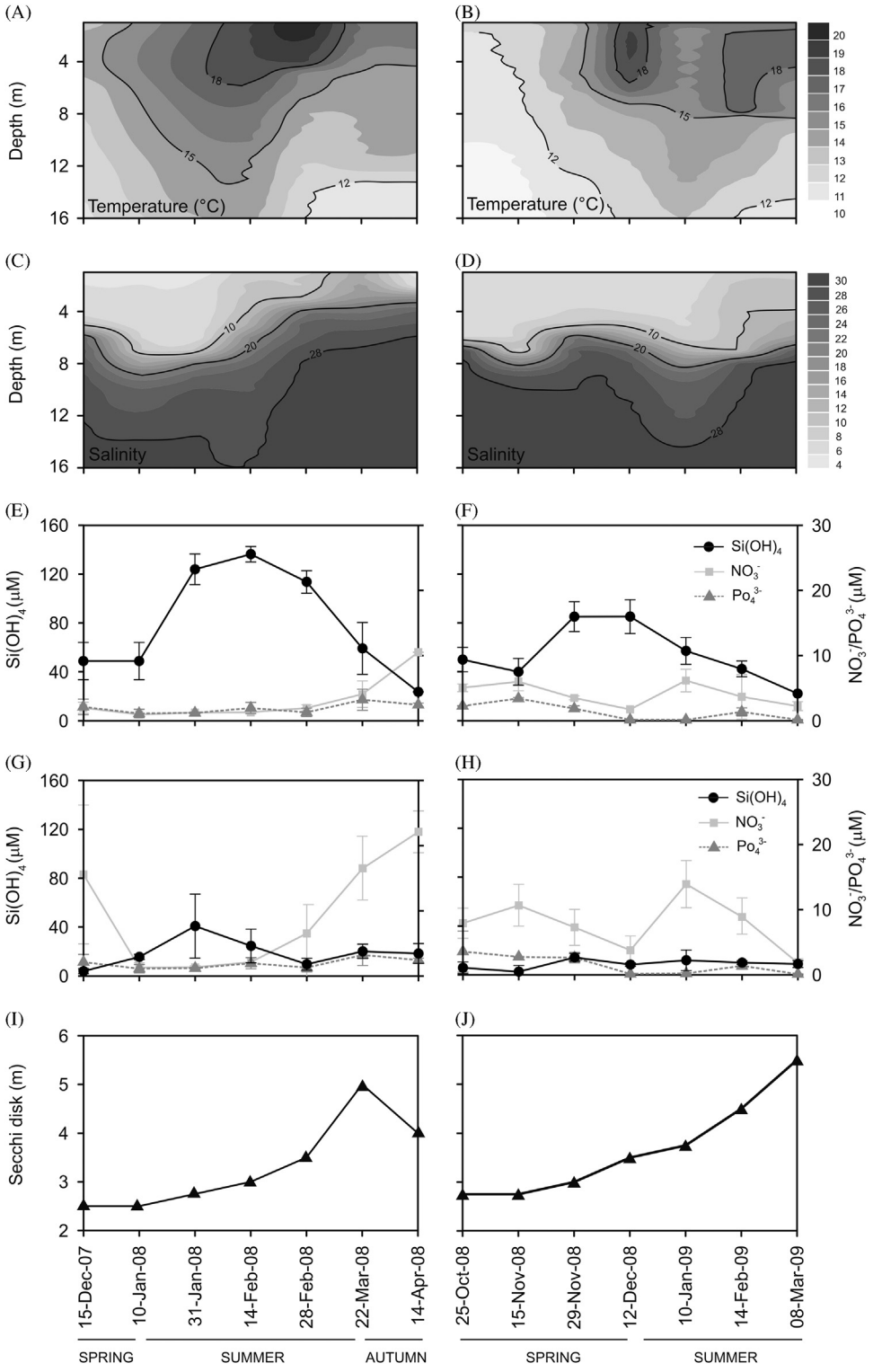

Fig. 2. Vertical distribution of (A, B) temperature (°C) and (C, D) salinity in Reloncaví fjord, Chile. Average concentrations (µM) of nutrients in the upper (E, F) and lower bounds of the pycnocline (G, H) and water transparence (m) as measured by Secchi disk (I, J).

[ln (x+1)] and organized in an "explanatory" matrix that included the measured environmental variables (temperature, salinity, $Si(OH)_4$, NO_3^-, PO_4^{3-}) and a "biological" matrix that included the density of the most abundant potentially toxic (LST) dinoflagellates, *Mesodinium* spp., cryptophyceans, levels of LSTs in the plankton and chl-*a* concentrations ($n=28$). The CCA was performed using CANOCO 4.5 Software (ter Braak, 1995). Monte Carlo permutation testing (500 permutations, CANOCO 4.5) was used to determine the significance of the environmental variables and the first two ordination axes.

3. Results

3.1. Environmental conditions

The top 16 m of the water column was strongly stratified during the whole study period, with an upper layer of warm (14–19 °C) brackish ($4 < S < 12$) water and a lower layer of colder (10–13 °C) and much saltier ($22 < S < 30$) water. This system exhibited a pronounced pycnocline located at 5–8 m (Fig. 2a–d). The upper layer was usually characterized by higher concentrations of $Si(OH)_4$ (22–136 μM), whereas the lower layer had higher values of NO_3^- (1.3–16 μM; Fig. 2e–h). PO_4^{3-} levels (0.1–3.5 μM) were homogeneous throughout the water column. Water transparency (Secchi depth) was lower during spring (2.5 m) and gradually increased towards mid-summer (~5 m; Fig. 2i, j). Large differences in chl-*a* concentrations were observed between the two sampling periods (Fig. 3a, b). Low ($< 0.5\ \mu g\ L^{-1}$) to moderate (1–2.7 $\mu g\ L^{-1}$) concentrations were recorded during most of the 2007–2008 period and maximum values (~4 $\mu g\ L^{-1}$) were observed in early autumn (14 April). In contrast, during the 2008–2009 period, very low ($< 0.3\ \mu g\ L^{-1}$) to moderate (0.5–6.2 $\mu g\ L^{-1}$) values were observed in early spring (from 25 October to 15 November) and very high chl-*a* concentrations (13–23 $\mu g\ L^{-1}$) occurred from mid-spring to late summer (from 29 November to 8 March).

3.2. Vertical distribution and composition of *Dinophysis* spp. and *Protoceratium reticulatum*

Potentially toxic (LST producing) dinoflagellates were represented by five species of the genus *Dinophysis* and by *Protoceratium reticulatum* (Table 1). *Dinophysis* species were detected on every sampling date. *Dinophysis acuminata* was the most abundant species, frequently constituting >70% of total *Dinophysis* spp. densities. Low densities (< 50 cells L^{-1}) of *Dinophysis* spp. were generally distributed throughout the water column, but cells were aggregated in a maximum density ($0.5–3 \times 10^3$ cells L^{-1}) in 1–2 m thin layers, either at the top or the base of the pycnocline (Fig. 3c, d). Exceptions to this pattern were observed on 22 March and 15 November 2008, when moderate to high densities of *Dinophysis* spp. ($1–28.5 \times 10^3$ cells L^{-1}) appeared in thicker layers (5–7 m). Blooms of this species were observed on 22 March 2008 and 15 November 2008 with maximum densities of 28.5×10^3 cells L^{-1} (7 m) and 17.1×10^3 cells L^{-1} (5 m), respectively. During the 2008–2009 period, blooms of *D. acuminata* co-occurred with high densities of cryptophyceans and the ciliate *Mesodinium* spp. (Fig. 4).

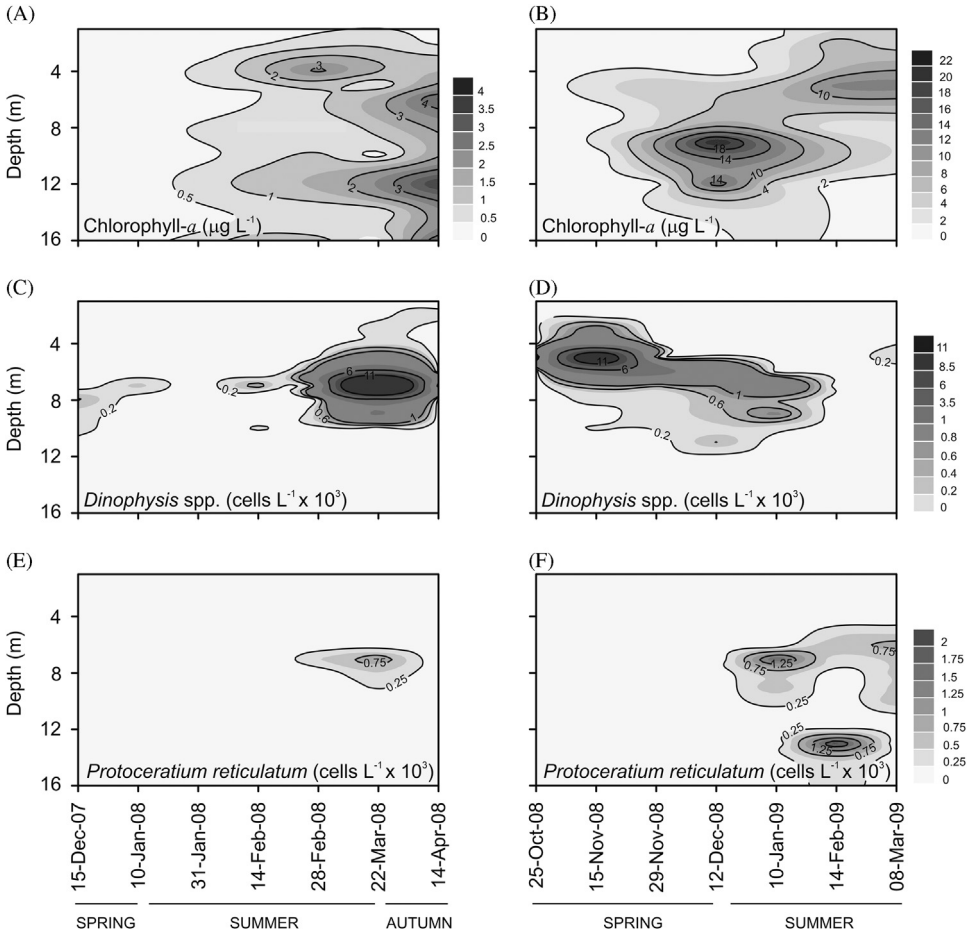

Fig. 3. Vertical distribution of (A, B) chlorophyll-*a* (μm L^{-1}) and cell density (cells $L^{-1} \times 10^3$) of (B, C) *Dinophysis* spp. and (D, E) *Protoceratium reticulatum* in Reloncaví fjord, Chile. Note that chlorophyll-*a* concentrations recorded during the 2007–2008 (A) and 2008–2009 (B) sampling periods are shown at different scales.

Table 1
Depth-integrated densities (cells $m^{-2} \times 10^3$) of *Dinophysis* spp. and *Protoceratium reticulatum* in Reloncaví fjord, Chile.

	2007–2008							2008–2009						
	15 December	10 January	31 January	14 February	28 February	22 March	14 April	25 October	15 November	29 November	12 December	10 January	14 February	8 March
Dinophysis acuminata	1.0	0.6	0.1	1.0	3.0	46.4	0.4	0.3	27.2	5.5	7.0	3.5	0.2	0.2
Dinophysis punctata	–	–	–	–	–	–	–	–	0.4	0.2	0.1	–	–	–
*Dinophysis rotundata**	–	0.1	0.0	0.5	–	1.5	0.1	0.7	0.6	0.6	0.1	0.2	–	0.5
Dinophysis subcircularis	–	–	–	–	0.2	1.3	–	–	0.1	0.5	0.3	1.3	0.3	–
Dinophysis tripos	–	0.2	0.1	–	–	0.1	–	0.0	-	–	0.1	0.1	0.1	–
Protoceratium reticulatum	–	–	–	–	0.5	1.8	–	–	–	–	–	3.9	5.5	4.1

* (=*Phalacroma rotundatum*).

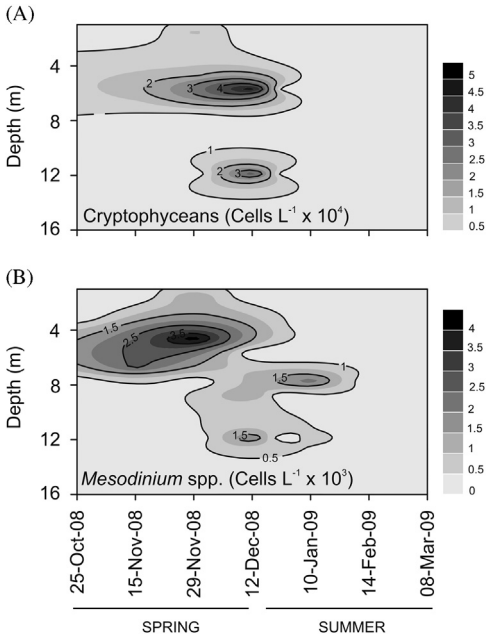

Fig. 4. Vertical distribution of cryptophyceans (cells $L^{-1} \times 10^4$) and *Mesodinium* spp. (cells $L^{-1} \times 10^3$) in Reloncaví fjord, Chile during the 2007–2008 period.

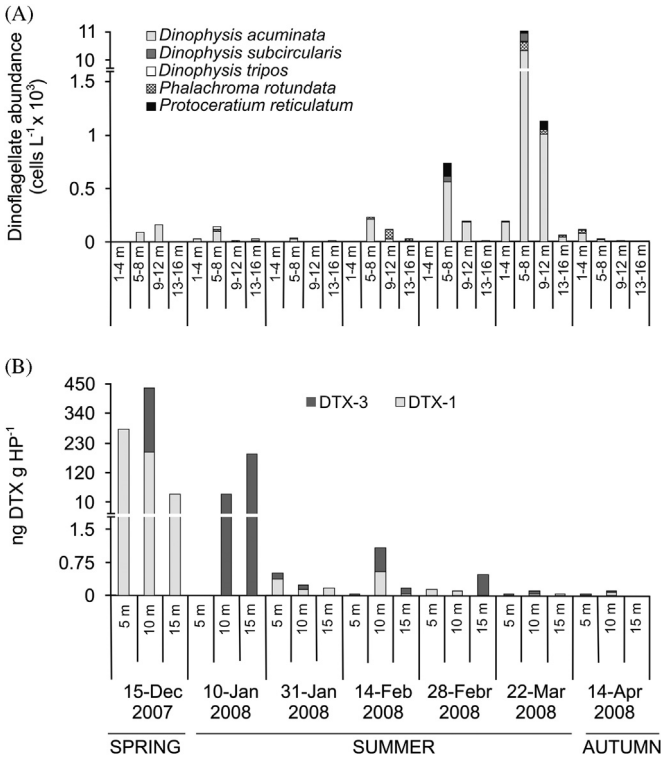

Fig. 5. (A) Occurrence of potential lipophilic shellfish toxin producing dinoflagellates and (B) average concentrations of dinophysistoxin-1 and -3 (ng g^{-1} HP) in bivalves during the 2007–2008 sampling period in Reloncaví fjord, Chile. Dinoflagellate cell densities were recorded as averages from each depth range: 1–4 m, 5–8 m, 9–12 m and 13–16 m. DTXs were determined by HPLC–FD from bivalve samples collected at 5, 10 and 15 m depths.

Dinophysis tripos, *Dinophysis rotundata* and *Dinophysis subcircularis* were the dominant species on 31 January, 25 October 2008, and on the 8 March 2009, respectively. Low densities of *Dinophysis punctata* ($< 0.1 \times 10^3$ cells L^{-1}) were observed only during the 2008–2009 period.

Similarly, a heterogeneous vertical distribution of *P. reticulatum* was observed, but it was not related to the halocline position on all sampling dates (Fig. 3e, f). During the 2007–2008 period, this species was observed only in low densities ($< 0.1 \times 10^3$ cells L^{-1}) in late summer (from 28 February to 22 March 2008), whereas during the 2008–2009 period, it had a longer growth season and was present from late spring to late summer (from 10 January to 8 March). A bloom of this species occurred on 14 February 2009 with a cell maximum (2.2×10^3 cells L^{-1}) observed at 14 m, well below the pycnocline (7–9 m).

3.3. Seasonal distribution of LSTs and its relation with their potential producers

DTXs in bivalves were detected on every sampling date during the 2007–2008 period, but there was no correlation between toxin levels and *Dinophysis* spp. densities (Fig. 5a, b). The highest concentrations of DTX-1 (280 ng g^{-1} HP) and DTX-3 (239 ng g^{-1} HP) were observed in late spring (15 December 2007). One month later (10 January 2008), only high levels of DTX-3 (184 ng g^{-1} HP) were present and DTX-1 was not detected. These levels of DTXs appeared associated with low densities (< 500 cells L^{-1}) of *Dinophysis acuminata*. After 31 January 2008, concentrations of DTX-1 and DTX-3 dropped (< 1 ng g^{-1} HP) and remained low until early autumn (14 April 2008) despite the bloom of *D. acuminata* observed on 22 March 2008.

The whole 2008–2009 period was characterized by the occurrence of trace levels (< 0.1 ng g^{-1} HP) of DTX-1 and DTX-3 in bivalves (data not shown). Although low to very high densities of

D. acuminata ($0.3–6 \times 10^3$ cells L^{-1}) were observed from the spring to early summer (from 25 October to 12 December 2008), undetectable to very low levels (< 0.16 ng L^{-1}) of PTX-2 were detected in the plankton (Fig. 6a, b). The highest levels of PTX-2 (2.2 ng L^{-1}) and YTX (3.2 ng L^{-1}) and trace levels of OA and DTX-1 (data no shown) were recorded from plankton samples at the beginning of summer (10 January 2009) and co-occured with moderate densities of *D. acuminata*, *Dinophysis subcircularis* and *Protoceratium reticulatum* ($0.2–0.6 \times 10^3$ cells L^{-1}). Moderate to high levels of YTX (51–496 ng g^{-1} HP) were detected in bivalves between 10 January and 8 March 2009 while high levels (275 ng g^{-1} HP) of 45-hydroxyyessotoxin (45-OH-YTX) and traces of PTX-2 were detected only on 14 February 2009 (Fig. 6c). A chromatogram for toxins detected in the plankton is shown in Fig. 7. Positive MBA results were obtained from bivalves collected on 14 February and 08 March 2009. In both cases, two of the three mice died within ca. 7 h after intraperitoneal injections with symptoms of YTX poisoning (including jumping, paralysis and gasping).

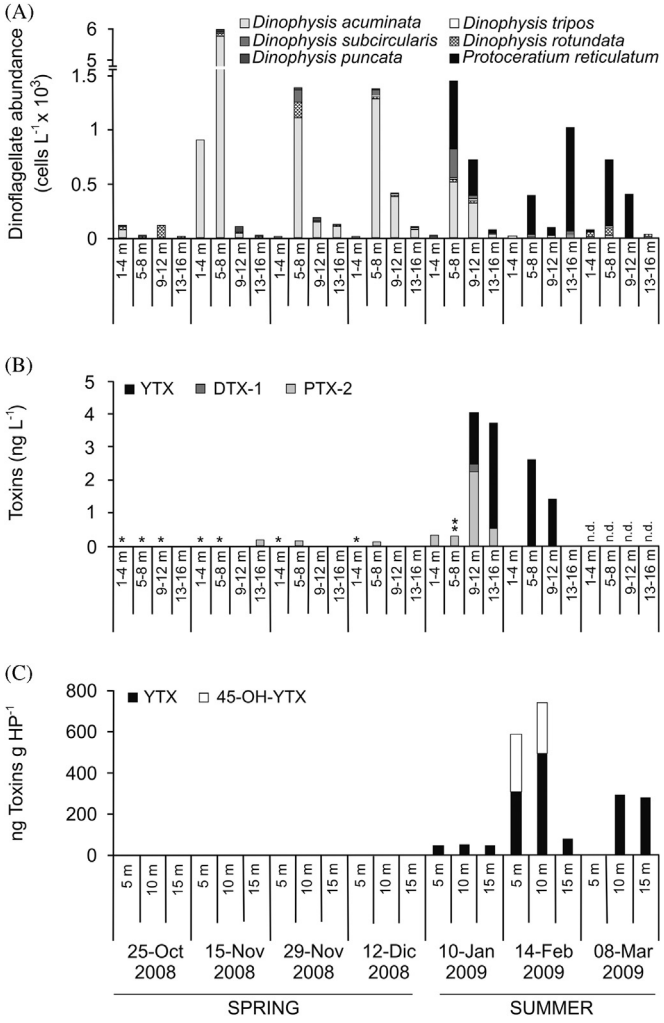

Fig. 6. (A) Occurrence of potential lipophilic shellfish toxin producing dinoflagellates; (B) and (C) concentrations of pectenotoxin-2 (PTX-2), yessotoxin (YTX) and 45-hydroxyyessotoxin (45-OH-YTX) in (B) plankton populations (20–88 μm; ng L^{-1}) and (C) bivalves (ng g^{-1} HP) during the 2008–2009 sampling period in Reloncaví fjord, Chile. Dinoflagellate cell densities were recorded as averages from each depth range: 1–4 m, 5–8 m, 9–12 m and 13–16 m. PTX-2, YTX and 45OH-YTX were determined by LC–MS from plankton samples taken from integrated depths (1–4, 5–8, 9–12 and 13–15 m) and bivalve samples collected at 5, 10 and 15 m depths. One and two asterisks indicate the presence of trace levels of PTX-2 and YTX, respectively.

A CCA was used to evaluate the relation between environmental variables and the density of *Dinophysis* spp., *P. reticulatum* the LTS profile in plankton samples during the 2008–2009 period. Together, the eigenvalues of the first two canonical axes (0.244 and 0.032, respectively) explained 87.5% of the total variance. Species and environmental variables showed correlation values of 0.88 and 0.68 on canonical axes 1 and 2, respectively. The compositional axis 1 (SPECIES AXIS 1) was correlated to PO_4^{3-} (0.72) and temperature (-0.4833), whereas $Si(OH)_4$ (0.42) and NO_3^- (-0.37) showed the highest correlations on the composition axis 2 (SPECIES AXIS 2). Both axes were statistically significant (Monte Carlo testing, $p=0.01$) and the forward stepwise model indicated that only PO_4^{3-} and temperature were statistically significant ($p < 0.05$). The ordination diagram with the obtained scores indicated that *D. tripos*, *P. reticulatum,* PTX-2 and YTX were positively related to temperature and negatively related to PO_4^{3-} (Fig. 8). Although *D. acuminata*, *Mesodinium* spp. and cryptophyceans grouped together, they were not related to any environmental variable.

4. Discussion

4.1. Distribution of Dinophysis spp. and Protoceratium reticulatum

High densities of *Dinophysis* species are known to occur in the Chilean fjords. In fact, blooms of *Dinophysis acuta* (7×10^4 cells L^{-1}) and *Dinophysis acuminata* (1.8×10^5 cells L^{-1}) reported by Lembeye et al. (1993) and Clément et al. (1994), respectively, constitute examples of the highest world records for these two species. However, with the exception of a few episodic events, *Dinophysis* species from Chilean fjords are usually recorded in low densities (< 100 cells L^{-1}) throughout the year, representing a small percentage of the microphytoplankton community (Díaz et al., 2011; Lembeye et al., 1998; Lembeye et al., 1997; Seguel et al., 2005; Uribe et al., 1995). In contrast, moderate to high densities ($> 1 \times 10^3$ cells L^{-1}) of *Dinophysis* spp. were detected throughout our study period, with the cell maxima usually located in the pycnocline region. These different outcomes were probably due to sampling design: samples were obtained in previous studies using vertical net-hauls and/or oceanographic bottles, which have low resolution over the fine scales that *Dinophysis* populations are distributed (Reguera et al., 2012). The vertical distribution for *Dinophysis* spp. throughout the fjord systems in southern Chile should be similar to those described in our study because strong salinity-driven stratification is a common feature.

Blooms of *D. acuminata* occurring at the end of summer (the 2007–2008 period) and early spring (the 2008–2009 period) suggest that temperature was not an important factor in determining the seasonal distribution of this species during this study. In fact, no relationship was detected between *D. acuminata* and temperature in the CCA performed with data obtained during the 2008–2009 period. On the other hand, *D. acuminata* grouped with cryptophyceans and the mixotrophic ciliates *Mesodinium* spp. Successful cultures of *D. acuminata* were established by feeding it the mixotrophic ciliate *Mesodinium rubrum,* which in turn preys on cryptophyceans (Park et al., 2006). In the field, close relationships between certain cryptophyceans and blooms of *Dinophysis* spp. were detected in Japanese coastal waters (Koike et al., 2007; Nishitani et al., 2005). In addition, recently fed *D. acuminata* and *Mesodinium* spp. co-occurred in the Ría de Pontevedra, NW Spain, (Gonzalez-Gil et al., 2010) and in the Northern Baltic Sea (Sjöqvist and Lindholm, 2011). Results from the CCA suggest that seasonal maxima of *D. acuminata* in Reloncavi fjord during the 2007–2008 period were conditioned by the occurrence of cryptophyceans, the base of the food chain to *D. acuminata.* However, given that

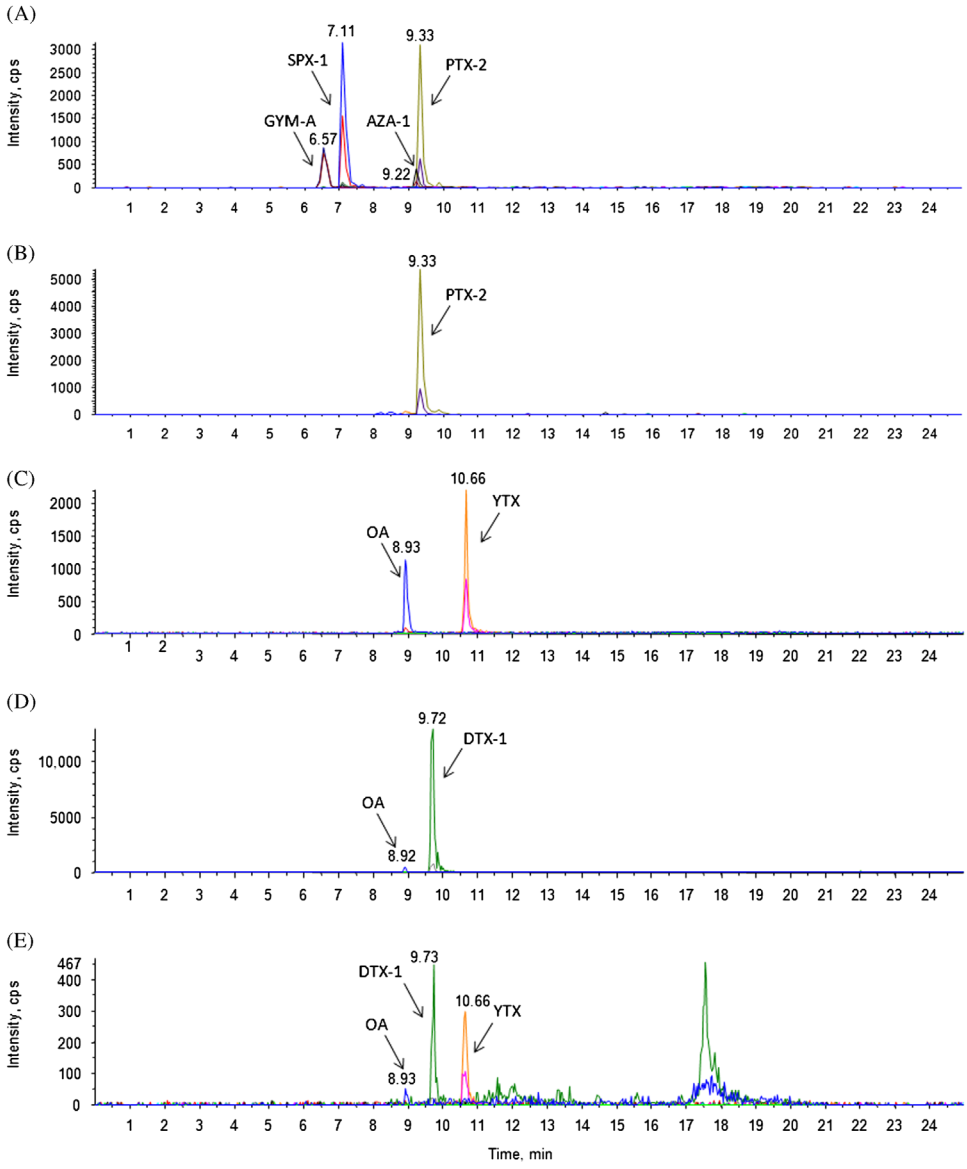

Fig. 7. LC–MS/MS chromatogram for the analysis of (A) a standard mixture under positive polarity containing GYM-A (125 ng mL^{-1}), SPX-1 (87.5 ng mL^{-1}), AZA-1 (62.5 ng mL^{-1}) and PTX-2 (107.5 ng mL^{-1}); (B) a sample under positive polarity consisting of a natural assemblage collected on 10 January 2009 at 9–12 m depth, which contained a level of 242 ± 21 ng mL^{-1} of PTX-2; (C) a standard mixture under negative polarity containing OA (100 ng mL^{-1}) and YTX (500 ng mL^{-1}); (D) a standard (qualitative) under negative polarity of DTX-1 (ca. 1 μg mL^{-1}), purchased from Wako Pure Chemical Industries, Ltd., and (E) the same sample as in (B) under negative polarity containing 171 ± 57 ng mL^{-1} of YTX, 24 ± 6 ng mL^{-1} of DTX-1, and traces of OA < 20 ng mL^{-1}. Standards other than DTX-1 were certified standards purchased from the Institute for Marine Biosciences (National Research Council, Canada). Conditions for the LC–MS/MS analysis of phytoplankton samples are described in Section 2.4.

Amoebophrya parasitoids were detected with a prevalence of 10% in *D. acuminata* populations from Reloncaví fjord in January 2009 (Alves-de-Souza et al., 2012), other biological processes such as grazing and parasitism should be considered.

In contrast to *D. acuminata*, the presence of *Protoceratium reticulatum* is associated with warm temperatures. Blooms of *P. reticulatum* have been recorded in northern Chile during the summer (Álvarez et al., 2011; Rossi and Fiorillo, 2010). In southern Chile, *P. reticulatum* occurs in low densities throughout the year (> 1 cells L^{-1}, Lembeye et al., 1997; Seguel et al., 2005), whereas the highest concentrations (~ 90 cysts cm^{-3}) of its cysts are usually detected in sediments during winter (Seguel and Sfeir, 2010; Seguel et al., 2005). Thus, the positive correlation between *P. reticulatum* and temperature observed in this study agrees with the occurrence of this species restricted to summer months. However, we cannot disregard other factors affecting its seasonal distribution. For example, the occurrence of *P. reticulatum* in Reloncaví fjord during this study was associated with maximum water transparency (estimated by Secchi depth), and physiological studies of *P. reticulatum* suggest that irradiance is the primary factor affecting its growth (Paz et al., 2006).

4.2. Seasonal variability of LST profiles

DSP toxins detected in mussels during this study were DXT-1 and DXT-3, whereas OA, associated with *Dinophysis* spp. events in other parts of Chile (Lembeye et al. 1993), was not detected. Similar DTX profiles have been recorded in shellfish from southern Chile (García et al., 2004b, 2005, 2006, 2010; Uribe et al., 2001), and seem to represent a stable pattern for this geographical area. In this study, high DTX levels in bivalves were associated with low densities of *Dinophysis* spp., whereas two dense blooms

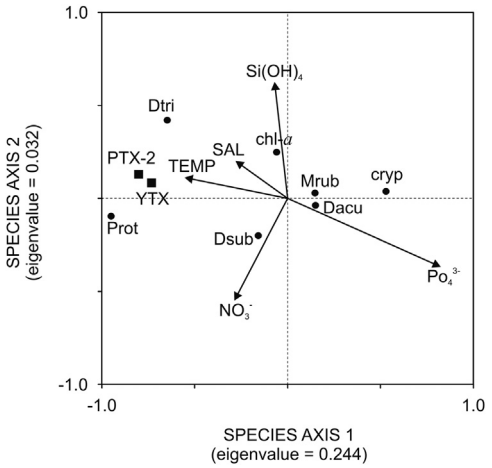

Fig. 8. Ordination diagram with the scores of species and lipophilic shellfish toxins (LSTs) obtained from the canonical correspondence analysis (CCA) related to vectors of environmental variables based on data obtained between 25 October 2008 and 8 March 2009 from Reloncaví fjord, Chile. TEMP: temperature, SAL: salinity, NO_3^-: nitrate, $Si(OH)_4$: silicic acid and PO_4^{3-}: orthophosphate. Circles and gray squares correspond to plankton variables and lipophilic toxins, respectively. Dacu: *Dinophysis acuminata*, Dtri: *Dinophysis tripos*, Dsub: *Dinophysis subcircularis*, Mrub: *Mesodinium* spp., cryp: cryptophyceans, chl-*a*: chlorophyll-*a*, YTX: yessotoxins, PTX: pectenotoxins.

of *Dinophysis acuminata* did not result in the accumulation of high levels of DTX in bivalves. Our results are in accordance with frequent reports of weak or no correlation between cell density and toxin level (Dahl and Johannessen, 2001; Holmes et al., 1999; Hoshiai et al., 1997; Rao et al., 1993; Sampayo et al., 1990; Sidari et al., 1998; Takahashi et al., 2007), although strong correlations between DSP toxicity and *Dinophysis* spp. cell densities occasionally occur (Godhe et al., 2002; Jørgensen and Andersen, 2007; Reguera et al., 1993). Correlating plankton densities and toxin concentrations in bivalves are usually difficult because bivalves can retain toxins for a long time (e.g., Moroño et al., 2003). Thus, although our data suggest that *D. acuminata* was not the source of DTXs observed in bivalves, we cannot reject the hypothesis that high levels of DTXs (Fig. 5) were produced by *D. acuminata* present before our study began. Alternatively, other *Dinophysis* species such as *Dinophysis tripos* and *Dinophysis subcircularis* that are usually present at low densities in the plankton may produce DTXs. Further studies considering a shorter temporal scale and toxin analysis of isolated cells should be performed to define the source of DTXs in Reloncaví fjord.

Unlike the DTXs and OA, PTX-2 surprisingly occurred in undetectable or trace levels in bivalves even during periods when the highest levels of this toxin were observed in the plankton. Similarly, Rodríguez et al. (2012) detected PTX-2 in plankton samples dominated by *D. tripos* but no toxicity was found in bivalves analyzed by MBA. These authors suggested that PTX-2 was likely converted by bivalve metabolism into PTX-2sa, which has no toxic effect on mice when injected intraperitoneally (Miles et al., 2004). However, that did not seem to be the case in our study. Although we did not use a PTX-2sa standard during the LC–MS analysis for bivalve samples, no chromatographic peak attributable to PTX-2sa was observed suggesting that detectable levels were not present in bivalves. YTXs were detected in the bivalves; therefore, any possible inhibition on the bivalve's clearance rates by LSTs seems unlikely. Thus, the reasons for this mismatch remain unclear.

Toxin profiles produced by LC–MS analyses of natural populations and cultures of *D. acuminata* may be dominated by PTX-2, with small contributions from OA and/or DTXs (Hackett et al., 2009; MacKenzie et al., 2005; Suzuki et al., 2009). Kamiyama and Suzuki (2008) detected DTX-1 and PTX-2 in a culture of *D. acuminata* whereas only PTX-2 was found in the natural population from which the strain was isolated, suggesting that the in situ toxin profile was affected by environmental factors. Thus far in Chile, only PTX-2 has been recorded in *D. acuminata* (Blanco et al., 2007; Fux et al., 2011), but only summer populations were characterized and OA and DTX-1 may be produced during other seasons of the year. Planktonic concentrations of PTX-2 in our study and the Baltic Sea are comparable (0.07–5.5 ng L^{-1}, Kuuppo et al., 2006), but up to 20 times higher than previously recorded for the Chilean coast (13–1031 pg L^{-1}, Trefault et al., 2011). The highest plankton concentrations of PTX-2 during the 2008–2009 period co-occurred with moderate cell densities of *D. acuminata* and *D. subcircularis*, while the toxin was undetectable during a bloom of *D. acuminata*. According to the CCA, concentrations of PTX-2 in the microplankton were more related to *D. tripos*. However, this outcome was certainly influenced by the non-toxic bloom of *D. acuminata* observed on 15 November 2008 and does not exclude the production of PTX-2 by this species and/or *D. subcircularis* during summer months. Indeed, PTX-2 was detected in a culture obtained from *D. acuminata* cells isolated from Reloncaví fjord in March 2008 (Fux et al., 2011).

PTX-2 cellular concentration in *Dinophysis* spp. during this study was extremely variable and probably affected by environmental factors. Given that the cell content of PTX-2 increases at temperatures below 10 °C in *D. acuminata* (Kamiyama et al., 2010), the CCA association between summer populations of *Dinophysis* spp. and planktonic concentrations of PTX-2 during the 2008–2009 period was most likely related to summer populations of *Dinophysis* spp. rather than a positive effect of temperature per se. Strains of a given *Dinophysis* species isolated from different geographical areas can show different toxin profiles and physiological responses (Fux et al., 2011; Reguera and Pizarro, 2008). Laboratory experiments also suggest that *Dinophysis* species need to feed on their ciliate prey to actively produce the toxins (Kamiyama and Suzuki, 2008) the PTX-2 cellular content of Japanese strains of *D. acuminata* and *Dinophysis fortii* being significantly lower in starvation conditions (Nagai et al., 2011). In this sense, the absence of PTX-2 in the plankton when the highest densities of both *D. acuminata* and *Mesodinium* spp. were observed is at least intriguing. Further laboratory experiments using strains isolated at different seasons of the year should be performed to define the role of temperature and prey availability in the production of PTX-2 by *D. acuminata* in southern Chile.

YTXs were previously reported in bivalves (Villarroel, 2004; Yasumoto and Takizawa, 1997) and plankton samples (Álvarez et al., 2011; Krock et al., 2009) from southern and northern Chile, respectively. Our study is the first to report the simultaneous presence and positive correlation of *P. reticulatum* to YTXs in bivalves and plankton populations from Chilean coastal waters. 45-OH-YTX was detected only in bivalves collected on 14 February 2008. Although Ciminiello et al. (2003) reported that small amounts of 45-OH-YTX can be produced by *P. reticulatum*, this toxin has never been detected in *P. reticulatum* cultures (Miles et al., 2005; Paz et al., 2007). Given that we did not detect 45-OH-YTX in plankton samples, it seems that the high levels of this toxin resulted from the bivalve oxidation of YTXs (Samdal, 2005). Although the concentration of YTXs in bivalves were under the European regulatory level of 1 mg K^{-1} (Tubaro et al., 2010), they gave positive results in MBAs during the summer of 2009. The time of death below 7 h associated with paralyzing symptoms were typical of YTXs (Dominguez et al., 2010). Similar symptoms have been reported from MBAs performed with extracts from bivalves collected from southern Chile (Lembeye et al., 1997; Seguel and Sfeir, 2010; Seguel et al., 2005) indicating that the occurrence of YTXs in bivalves from this geographic area is more frequent than previously thought.

4.3. Implications for monitoring

Currently, monitoring of phytoplankton aimed at the detection of HAB events in Chile is based on the deployment of 10-m tube samplers to obtain integrated water column samples (Seguel, 2008). Although tube samplers provide reliable information on the occurrence of *Dinophysis* species, they can result in the underestimation of cells densities at certain depths (Reguera et al., 2012). Indeed, a recent comparison of integrated (segmented hose) versus discrete depth sampling showed that densities estimated from segmented hose samples can be more than one order of magnitude lower than densities estimated from samples taken at specific depths under certain conditions, such as thin layer formation during marked stratification or intense physical accumulation forced by upwelling relaxation (Escalera et al., 2012). The sampling strategy used in this study, i.e. concentration of samples taken from multiple discrete depths, allowed us to face the two main features that make the accurate quantification of *Dinophysis* spp. difficult: their usual low densities and their heterogeneous vertical distribution. Our results raise the question whether dense blooms of *Dinophysis* spp. occur more frequently in southern Chile than previously thought and point out the need of further studies assessing the fine vertical distribution of these dinoflagellates in the region.

5. Conclusions

This study shows for the first time that dense populations of *Dinophysis* spp. can be observed in Chilean fjords during the entire spring–summer period, usually forming thin layers in the pycnocline region, while *Protoceratium reticulatum* seems to be restricted to summer months. Our results highlight the need for considering the strong heterogeneous vertical distribution of these dinoflagellates when monitoring their seasonal distribution in this region.

A stable pattern for the occurrence of YTXs is positively related to *P. reticulatum* densities during summer months and is described. However, different seasonal and interannual trends were observed regarding toxin profile and content of LSTs in plankton and shellfish. Although the source of DTXs in bivalves from southern Chile is largely unknown, the seasonal variability of these toxins strongly suggests a relationship to *Dinophysis* spp. populations with different LSTs profiles. Further studies on the toxicology of *Dinophysis* species present in the study area, and their accumulation and transformation in shellfish are needed.

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