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Research Article

Winter distribution of toxic, potentially toxic phytoplankton, and shellfish toxins in fjords and channels of the Aysén region, Chile

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ABSTRACT. The Aysén region (43°-47ºS) has been prone to intensive summer blooms of toxic microalgae and complex toxin profiles in the shellfish. However, their winter distribution, toxin profile, and resting cysts in sediments are poorly known. Frequently detected toxins are: Paralytic Shellfish Toxins (PST); lipophilic toxins, including Diarrheic Shellfish Toxins (DST), Amnesic Shellfish Toxin or Domoic Acid (DA). During a cruise, carried out in winter 2012, samples were collected at 24 stations for phytoplankton analysis and analysis of toxins in mollusks, and at 5 stations, additional samples were taken for toxin analysis in plankton and resting cysts in sediments. The results confirm the presence of microalgae associated with toxins in winter, and a higher sensitivity of the relative abundance (RA) than the cellular density, as a parameter of distribution of the microalgae. The maximum RA values were level 2 (low), for *Alexandrium catenella* and *Protoceratium reticulatum*; level 1 (rare) for *Alexandrium ostenfeldii*, *Dinophysis acuminata* and *D. acuta*; levels 3 (regular) and 4 (abundant) for *Pseudo-nitzschia* spp. *seriata* complex and *Pseudo-nitzschia* spp. *delicatissima* complex, respectively. Only PST in plankton and yessotoxins in mo llusks were detected, whereas cyst densities of *A. catenella* and *P. reticulatum* in sediments were 4 and 103 cysts mL^{-1} of wet sediment, respectively. The analyzed parameters showed a bigger inter-annual variability during the winter period, as it has been reported for the summer.

Keywords: PST, DST, domoic acid, lipophilic toxins, toxic phytoplankton, Patagonian fjords.

INTRODUCTION

The austral-southern macrozone of Chile (41°-55°S) is comprised of the three southernmost regions of the country (Los Lagos, Aysén, and Magallanes). Harmful Algae Blooms (HAB), frequently affects these regions with the presence of microalgae producers of Paralytic Shellfish Toxins (PST), lipophilic toxins, and Domoic Acid (DA) during the summer period, with the consequent risk to public health, productive activities, and geographic dispersion towards sectors free of these toxic microalgae.

Regular monitoring results in this area, for the last five years, indicate Aysén as the region with the highest blooming intensity and complexity of toxin profiles in mollusks (*e.g.*, Guzmán *et al.*, 2015). Among the toxic microalgae reported for the region as producers of PST is *Alexandrium catenella* and *Alexandrium ostenfeldii*, the latter with a different toxin profile (Salgado *et al.*, 2015). *Protoceratium reticulatum* has been confirmed as a yessotoxin (YTX) producer (Pizarro *et al.*, 2012a), however for *Lingulodinium polyedrum* and *Gonyaulax spinifera*, both species producers of YTX in other parts of the world (*e.g.*, Paz *et al.*, 2004; Rhodes *et al.*, 2006), and observed in Aysén waters, the information of their toxin profiles remains unknown for southern Chile.

Diatoms of the genus *Pseudo-nitzschia* are also been observed and linked to the production of DA. This

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toxin is commonly detected at the trace level and only occasionally exceeds the limit suitable for human consumption in the Aysén region (*e.g.*, Guzmán *et al*., 2015). However, in mytilid farm areas of Los Lagos region, it is frequent to find concentrations of DA above the normative limit. The increased frequency of monitoring at these farms makes it possible to record DA events in the mollusks at weeks' lapses (*e.g.*, Resolusión Nº227/254, SEREMI-Salud 2017, www.leychile.cl).

Of the aforementioned species, *A. catenella* has been declared a plague species in the country (Resolution Ex N°177, SUBPESCA 2009), whose spatial distribution limit has been established between 43°22' and 54°55'S. For this reason, is in the interest of the authority to have a background of the spatio-temporal distribution of this species. Moreover, the Aysén region is frequently used as a route for the transport of live fish in wellboats, to be filleted at latitudes less than 43°22'S, *i.e.*, areas declared free of plague, in Los Lagos region (*e.g.*, Pizarro *et al*., 2014a, 2014b).

The Aysén region (43-47ºS) is characterized by presenting micro-basins with a north-south direction (Fig. 1), basically delimited by the Meninea Constriction (Guzmán & Silva, 2002; Sievers & Silva, 2006). Longitudinally, this heterogeneity displays a complex channels region between the ocean and the mountain range, interrupted by a great central channel that runs for the most part of the region, separating the inner channels in insular (western) and continental (oriental). The complexity of the geographical area is reflected in the local circulation of its waters (*e.g.*, González & Cáceres, 2009; Cáceres *et al*., 2010).

The objectives of the study were to know: 1) the microalgae winter distribution associated with the production of PST, DA, and lipophilic toxins, 2) the toxic profiles of phytoplankton and on mollusks collected between Boca del Guafo and Elefantes Fjord in the Aysén region, 3) the dinoflagellates resistance cysts distribution in sediments, as evidence of their presence in the study area, and 4) to analyze the results obtained regarding the geographic sector and 2012 seasons (winter *versus* summer), and comparing with previous three-year winter records.

MATERIALS AND METHODS

Working area

The study was performed during the 18th cruise of the Marine Research Cruise Program (CIMAR), coordinated by the Chilean National Oceanographic Committee (CONA) since 1995. The campaign was carried out between June 17 and July 4, 2012, aboard the R/V "Abate Molina", in the General Carlos Ibáñez del Campo, Aysén region. Of the 31 stations considered for the cruise, 23 were sampled for one or more of the objectives of this study (Fig. 1).

Qualitative phytoplankton

Phytoplankton composition was determined in 23 stations (Fig. 1), from water samples collected with a 20 μm mesh phytoplankton net (vertical trawl from 20 m depth to surface). At each station, at least 10 tows were made until a volume of 1 L was obtained. The water was then concentrated through a 25 μm mesh until a 120 mL plastic bottle was filled. Subsequently, the sample was fixed with 3% neutralized formalin, stored in the dark at room temperature until analyzed in the laboratory with an optical microscope. Relative abundance (RA) was estimated from these same samples. For this, the number of cells of *Alexandrium catenella, A. ostenfeldii, Dinophysis acuta, D. acuminata, Protoceratium reticulatum, Pseudo-nitzschia* spp. *seriata* complex and *Pseudo-nitzschia* spp. *delicatissima* complex was counted in 0.1 mL of sedimented sample, under the surface of an 18×18 mm (three replicates) coverslip. The number of cells was taken to a qualitative scale of RA categorized into ten ranks (Table 1, modified by Uribe *et al.*, 1995 *fide* Guzmán *et al*., 2015). RA is a parameter that has been more sensitive than cell density to the presence of dinoflagellates in the water column (Guzmán *et al*., 2012), which is why it is routinely used during the monitoring.

Quantitative phytoplankton

The microalgae specific abundance was obtained from water samples collected in the same 23 stations sampled for qualitative phytoplankton. The seawater samples were collected with Niskin bottles, displayed in a Rosette, on the surface, 5, 10 and 20 m. From each sample, 100 mL of seawater were fixed with Lugol solution and stored in the dark at room temperature until transport to the laboratory, where they were maintained until analysis. Quantification was performed using an inverted microscope (Utermöhl method, 1958), with sedimentation chambers whose volume (10, 50 or 100 mL) was selected according to the sample concentration.

Dinoflagellate resistance cyst and sediments samples

The sediment samples for cyst analysis were collected in 5 stations (37, 38, 41, 86, and 88; Fig. 1) by autonomous diving between 3 and 10 m depth. The sediment was obtained by surface trawling (0-3 cm), using a 40 mL plastic bottle with a double lid, which

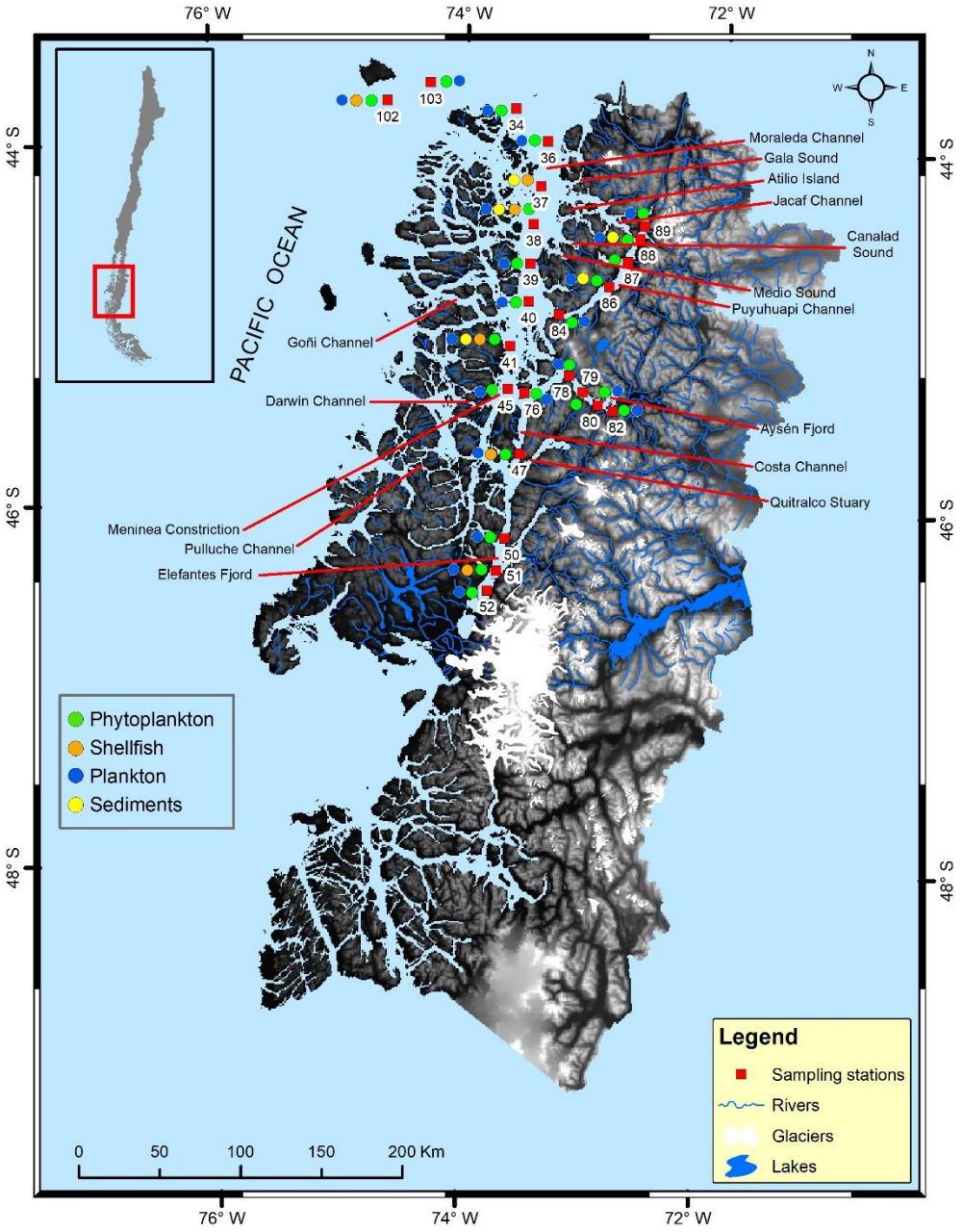

Figure 1. Sampling stations in the Aysén region, Chile.

was completely filled and closed in the same place. On the surface, the bottle was labeled, wrapped in aluminum foil, and stored at 4°C. For the identification and quantification of the cysts, a known amount of sediment was processed following the biological method of cleaning and cysts concentration proposed by Matsuoka & Fukuyo (2000). Samples were diluted in filtered seawater, sonicated for 3 min using a Sonics sonicator (model VCX130), to disaggregate the cysts from the sediment particles. Subsequently, the samples were sieved through 20 and 120 µm meshes, and the fraction retained in the 20 µm sieve was brought to a known volume. The cysts were analyzed using a 1 mL Sedgewick-Rafter counting chamber under magnification of 100x and 400x using an optical microscope equipped with a camera. The results are presented as number of cysts per mL of wet sediment (cysts mL^{-1} w.s.) corresponding to an average of three counts. The identification of cysts was determined according to their morphological characteristics (*e.g.*, shape, color, archeopyle, type of wall and processes) referred in specialized publications (*e.g.*, Nehring, 1997; McMinn *et al.*, 2010).

Table 1. Relative abundance scale of toxic and potentially toxic phytoplankton species.

Category	Level	*Dinophysis acuta* *Dinophysis acuminata* *Alexandrium ostenfeldii*	*Alexandrium catenella* *Protocoratium reticulatum*	*Pseudo-nitzschia* spp. *seriata* and *delicatissima* complexes
Absent	0	0	0	0
Rare	1	1 - 5	1 - 2	1 - 10
Scarce	2	6 - 15	3 - 10	11 - 50
Regular	3	16 - 35	11 - 42	51 - 210
Abundant	4	36 - 75	43 - 170	211 - 850
Very-abundant	5	76 - 155	171 - 682	851 - 3410
Extremely-abundant	6	156 - 315	683 - 2730	3411 - 13650
Hyper-abundant	7	316 - 635	2731 - 10922	13651 - 54610
Ultra-abundant	8	---	10923 - 43690	---
Mega-abundant	9	---	43691 - 174762	---

Toxins analyzed

The analyzed toxins were: PST, DA and lipophilic toxins. The PST included saxitoxins (STX) and its derivatives, gonyaulatoxins (GTXs) and carbamates (Cs). In the cluster of lipophilic toxins, okadaic acid (OA), dinophysistoxin-1 (DTX1), and dinophisistoxin-3 (DTX3) were considered; all of them, called okadaic, are responsible for the diarrheal syndrome. Also included in the lipophilic pectenotoxins (PTXs), yessotoxins (YTXs), spirolides (SPXs) and azaspiracids (AZPs). As certified reference material, commercial standards solutions (NRC, Halifax, Canada), were used.

Plankton samples for toxin analysis

Seawater samples for toxin analysis in plankton were taken at 23 stations (Fig. 1) using a submersible electric pump (Eingell 7835, 260 L min^{-1}) for 5-10 min (*i.e.*, the total volume between 1,300 and 2,600 L) at a depth close to the maximum fluorescence value recorded by the CTD. The pumped water was transferred to a container covered with a set of sieves of 200, 100, 25 and 10 μm of mesh size arranged in descending order to filter the water. The 10 μm sieve was used to increase the likelihood of detecting *Azadinium* spp., microalgae of small size (12-15 μm in length, 6-8 μm in diameter) and primary source of azaspiracids (Tillman *et al*., 2009). Finally, four fractions of the plankton were obtained, whose size in descending order was: >200, 100-200, 20-100 and 10-20 μm.

An aliquot of each fraction of concentrated phytoplankton was transferred separately to a graduated vessel, filtered through pre-calcined glass fiber filter (GF/F) of 25 mm diameter. Filters were stored in a 1.5 mL Eppendorf tube and deep-frozen until analysis in the laboratory. Analysis of lipophilic toxins was performed in the 10-20 μm size fraction, while the PST, DA and lipophilic toxins were analyzed in the remaining fractions, to determine their potential transfer in the food chain.

For detection of DA dissolved in water, an alternative methodology was followed, collecting 100 mL of water from each size fraction of plankton, transferred to a plastic bottle, and frozen at -20°C until analysis in the laboratory.

Analysis of toxins in shellfish

Shellfish were collected at five stations (37, 38, 41, 51, 88; Fig. 1), according to the availability of resources and maximum bathymetry. Sampling was carried out by autonomous diving between 3 and 10 m depth. The shellfish collected were: blue mussel (*Mytilus edulis chilensis* Hupé, 1854), present in all stations, ribbed mussel (*Aulacomya atra* Molina, 1782), at stations 51 and 88; clam 1 (*Retrotapes exalbidus* (Dillwyn, 1817)), at stations 38 and 88; Clam 2 (*Venus antiqua* (King & Broderip, 1832), at stations 37, 38, and 41; giant mussel (*Choromytilus chorus* Molina, 1782), at station 41; limpet (*Nacella* sp.), at station 51; Chilean abalone (*Concholepas concholepas* Bruguière, 1789), at station 38; and sea snail (*Adelomelon ancilla* (Lightfoot, 1786), at station 38. The samples were shelled, labeled in a double nylon bag and frozen until analysis in the laboratory.

PST analysis

The identification and quantification of PST were performed following the method of Franco & Fernández-Vila (1993), using a liquid chromatograph (LC) Shimadzu LC-10ADvp coupled to a fluorescence detector RF-551 (LC-FlD). For PST identification, commercial standards of Saxitoxin (STX), Neosaxitoxin (NeoSTX), Decarbamoylsaxitoxin (dcSTX); Gonyaulatoxin (GTX) -1-4, 2-3 and 5; Decarbamoyl gonyau-

latoxin (dcGTX) -2-3; N-sulfocarbamoyl gonyaulatoxin (C 1-2) were used.

The PST and lipophilic toxins were analyzed in the plankton and mollusk samples according to protocols described in detail in Pizarro *et al*. (2015). Briefly, in the case of plankton, the filters were thawed and immersed in 300 µL of 0.05 M acetic acid, vortexed for 1 min and centrifuged at 20160 g for 10 min. The supernatant was transferred to a 1.5 mL vial. The sedimented material was re-suspended, in another 300 µL of 0.05 M acetic acid, mixed and centrifuged again. The supernatants were mixed, and 5 µL of this extract were injected into the LC-FlD after filtering using 0.45 µm (Nylon 13 mm) filters (Franco & Fernández-Vila, 1993).

In the case of shellfish, once in the laboratory, these were thawed and macerated with a food processor. 10 g of shellfish meat from this macerate were immersed in 5 mL of 0.1N HCl; vortexed, sonicated, and centrifuged at 2656 g for 10 min. After the supernatant was recovered, the sedimented material was re-extracted a second time with 5 mL of 0.1N HCl, following the same procedure described. The supernatants were mixed and their pH adjusted (between 3 and 4), with 0.1N NaOH. 10 µL of this extract, previously filtered by 0.45 µm (Nylon 13 mm), was injected into the LC-FlD. Excess maceration was stored for extraction of DA and lipophilic toxins.

Analysis of lipophilic toxins

The detection of lipophilic toxins was performed by MALDI-TOF (Matrix-Assisted Laser Desorption/ Ionization Time-Of-Flight) mass spectrometry at the Center for Scientific and Technological Support for Research (CACTI), University of Vigo in Spain, following the methodology described in Paz *et al*. (2011). For the identification of these toxins, commercial standards of okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2), pectenotoxin-2 (PTX2), spirolide-1 (SPX1), azaspiracids (AZP1, 2,3) and yessotoxins (YTX) were used.

The extraction protocols in plankton and shellfish are described in detail in Pizarro *et al*. (2015). Briefly, the filters containing the different plankton size fractions were thawed and immersed in 500 µL of 100% methanol, vortexed for 1 min and centrifuged at 20160 g for 10 min. The supernatant was transferred to an amber vial of 1.5 mL, being previously filtered by 0.45 µm (13 mm PTFE filters). The Eppendorf sediment residues were resuspended, with 500 mL of 100% methanol, and according to the previous procedure, extracted again. The supernatants were and evaporated with nitrogen gas at 38°C. The vials were stored at 4°C until sent for analysis by MALDI-TOF.

In the case of the shellfish, 2 g of macerated meat was extracted two times with 1 mL of 100% methanol. Each extract was centrifuged at 2656 g for 20 min. The supernatants were recovered, emptied into an amber vial, mixed and filtered by 0.45 µm (13 mm PTFE filters). The final extract was evaporated with nitrogen gas at 38°C and stored at 4°C until sent for analysis.

DA analysis by HPLC

The detection of DA was performed in water samples (dissolved DA), plankton (filters, particulate DA) and shellfish, with an SDP-M10Avp (DAD) diode array detector (LC-DAD). The conditions of the LC-DAD were as follows, according to the protocol described by Quilliam *et al*. (1995): Mobile phase Acetonitrile 10% (CH_3CN: H_2O): 1% Trifluoroacetic acid (TFA); flow rate: 0.8 mL min^{-1}; column: LiChroCART 125-4 Merck with LiChrospher 100 RP-18 filler (5 µm); column temperature 27°C; lambda absorbance: 242 nm.

For plankton samples, 500 µL of methanol was added to each filter stored in the 1.5 mL Eppendorf tube, vortexed for 1 min and centrifuged at 10285 g for 10 min. The supernatant was filtered by 0.45 µm (Millex PVDF, 13 mm in diameter), and collected in an amber vial of 1.5 mL. The pellet was re-suspended in another 500 µL of methanol following the same procedure as above. The supernatants were vortexed for 1 min and 5 µL of this extract were injected into the LC-DAD.

Quilliam *et al*. (1995) methodology was used for the analysis of DA in shellfish. 2 g of macerated shellfish meat were immersed in a 1 mL volume of 50% methanol in 15 mL centrifuge plastic tubes, vortexed and centrifuged at 2656 g for 10 min. The supernatant was recovered and the pelleted material was re-suspended in 1 mL of 50% methanol, following the same procedure described above. The supernatants were mixed in a 2 mL vial. Finally, 5 µL of this extract previously filtered by filters (Millex PVDF 0.45 µm, 13 mm) were injected into the LC-DAD.

The Vera-Ávila *et al*. (2011) modified (Pizarro & Frangópulos, 2012) methodology was used for the detection of DA dissolved in water. For this purpose, a 50 mL elution plastic syringe was prepared at the outlet of which a silicone hose plus a stopcock was inserted. The syringe was filled with 20 mL of Diaion resin (HP-20, Supelco) activated according to MacKenzie *et al*. (2004). The resin was pretreated with 5 mL of methanol followed by 5 mL of 0.01N HCL and allowed to drain.

Once the water sample (100 mL) was thawed, it was acidified with 0.1 N HCl to a pH of 3.5 ± 3.7. Octane-1-sulfonic acid was then added as the sodium salt (OSA) to a final concentration of 0.005 M and eluted (5-6 mL min^{-1}) through the syringe with pre-treated

resin. Once drained, the resin was washed with 5 mL HCl 0.01 N. Both elutions were discarded. Finally, the toxin bound to the resin was washed with 4 mL of 30:70 (v/v) ammonium acetate in methanol (0.01 M, pH 7). The extract was collected in a 15 mL plastic tube. 4 mL of this extract was evaporated with nitrogen gas under the hood, and re-suspended in 300 µL of 50% MeOH, vortexed for 1 min and filtered through 0.45 µm (Millex PVDF, 13 mm diameter). A 10 µL aliquot of this extract was injected into the LC-DAD.

Database analysis

The results obtained were analyzed and discussed with reference to the information reported by the "Red Tides Management and Monitoring Program in the regions of Los Lagos, Aysén and Magallanes", stages III-VI, executed between 2009 and 2012 (Guzmán *et al.*, 2010, 2011, 2012, 2013). For this purpose, the historical database of relative abundance (RA) of microalgae and dinoflagellate cysts, both variables estimated according to the methodology described in this study, was considered. In the case of PST and lipophilic toxins, the methodology used was the mouse bioassay, an analysis performed by the Environmental Laboratory of the Ministerial Health Secretariat of Aysén.

RESULTS

Relative abundance of toxic and potentially toxic species

The RA index showed the presence of these species in a greater number of stations with respect to the number of stations in which it was possible to estimate cell density (Figs. 2-3). The toxic species *Alexandrium catenella* was present in eleven stations, with levels of 1 (rare) and 2 (low), while *Alexandrium ostenfeldii* was present in eight stations with RA level 1 (rare) (Fig. 2a). The RA of *Dinophysis* species was estimated at level 1 (rare), in eleven stations for *D. acuminata* and thirteen stations for *D. acuta* (Fig. 2b). *Protoceratium reticulatum* was detected with levels 1 (rare) and 2 (poor) in ten stations, being those localized in Puyuhuapi Channel (84, 88, 89) and Moraleda Channel (40), where they recorded abundances of level 2 (scarce) (Fig. 2b).

The relative abundance of potentially toxic diatoms, *Pseudo-nitzschia* spp. *seriata* complex and *Pseudo-nitzschia* spp. *delicatissima* complex was similar. The maximum relative abundance of the first complex was level 3 (regular) at stations 39 and in Puyuhuapi Channel while the second complex showed a maximum abundance of level 4 (abundant), but only at stations 39 and 40. However, both diatom complexes were present throughout the study area (Fig. 2c).

Density of toxic and potentially toxic species

From a total of 23 stations sampled, *A. catenella* was present at stations 36, 40 and 88 with cell densities of 200, 1,700 and 800 cells L^{-1} at depths of 10, 5 m and surface, respectively (Fig. 3a). *A. ostenfeldii* was not observed in any of the quantitative samples from the study area. Within the *Dinophysis* species, densities of *D. acuminata* were 100 cells L^{-1} in the 5 m surface at stations 38 and 89, while *D. acuta* presented this same density at the surface of station 86. *P. reticulatum* was detected only at stations 47 and 52, with densities of 100 and 200 cells L^{-1} at 10 and 25 m depth, respectively (Fig. 3a). The distribution and cell density of diatoms *Pseudo-nitzschia* spp. *seriata* complex and *Pseudo-nitzschia* spp. *delicatissima* complex was similar in the study area (Fig. 3b). For the first complex, the density range was between 100 and 15400 cells L^{-1}, with the maximum value observed at station 84. The density range for the second complex was between 100 and 13100 cells L^{-1} with the maximum density observed at station 40.

Dinoflagellates cysts

The five sediment samples collected during the cruise presented dinoflagellate cysts. Common in all of them was the presence of *P. reticulatum* cysts (Fig. 3c, Table 2), with a maximum of 126 cysts mL^{-1} w.s. in station 88, of which 18% corresponded to cysts. The latter were corroborated by the absence of the archeopile, a structure that allows the germination of the cyst. Station 86 was the second station to present the highest number of *P. reticulatum* cysts, with densities of 13 cysts mL^{-1} w.s. Stations 37, 38 and 41, located in the central channel of the region, presented a number of 11, 4 and 3 cysts mL^{-1} w.s., respectively. Station 38 only had empty *P. reticulatum* cysts. Station 37 was the only station that presented cysts of *A. catenella*, although in a low number, 4 cysts mL^{-1} w.s. It was also possible to observe cysts of other non-toxic dinoflagellates at stations 37, 41, 86 and 88. These were cysts of six species of the genus *Protoperidinium* and one species of the genus *Scripsiella* (Table 2).

PST and DA toxins in plankton, shellfish, and waters matrices

Two of the three toxin cluster was detected in the different matrices analyzed. The PST was detected only in the plankton (Table 3) and the YTXs only in the shellfish (Figs. 4a-4b).

Only N-sulfocarbamoylgonyaulatoxin (C1-2) was detected at a quantifiable level in concentrations of 0.03-0.70 pg μL^{-1} in the different plankton fractions collected at six stations (34, 36, 38, 80, 40 and 52) of a total of 22 stations sampled (Fig. 5).

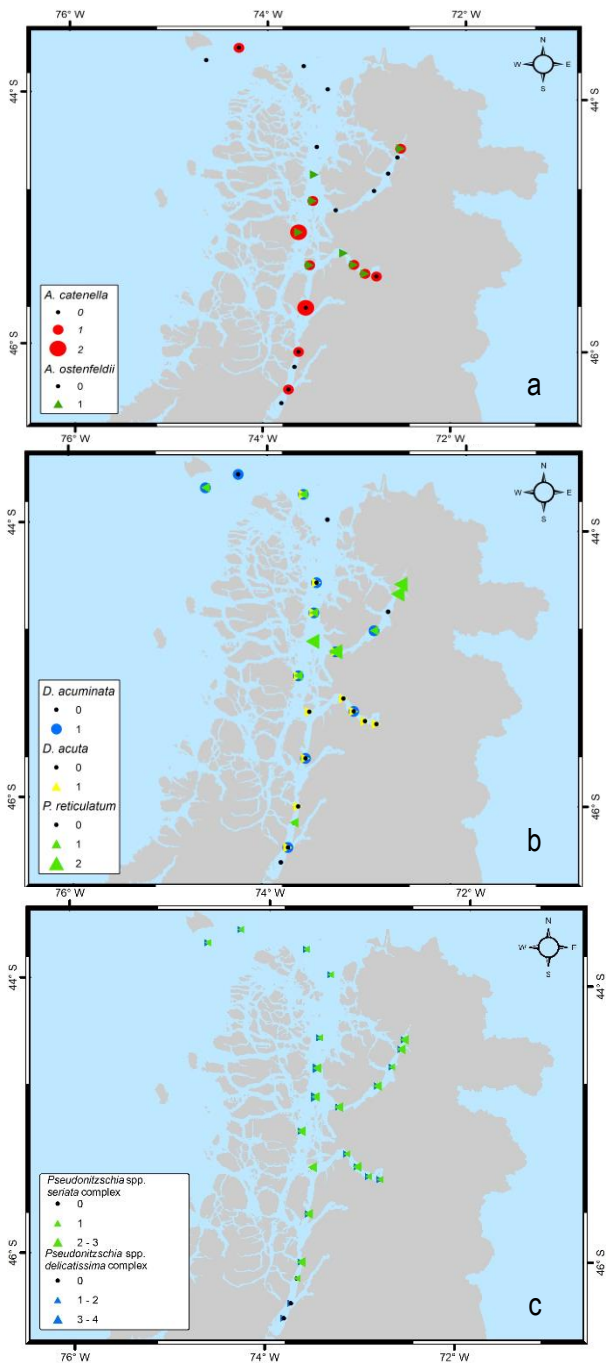

Figure 2. Relative abundance observed during the study period. a) *A. catenella*, *A. ostenfeldii*, b) *D. acuminata*, *D. acuta* and *P. reticulatum*, c) *Pseudo-nitzschia* spp. *seriata* and *delicatissima* complexes.

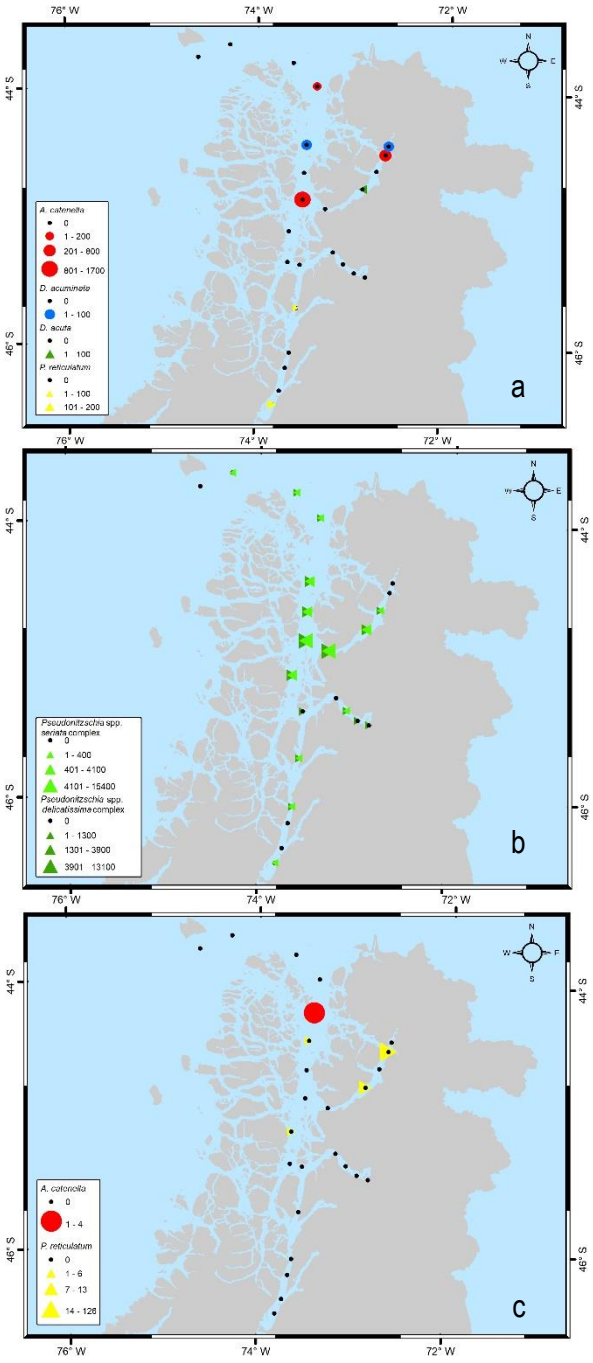

Figure 3. Cell density (cells L^{-1}) detected during the study period. a) *A. catenella*, *D. acuminata*, *D. acuta* and *P. reticulatum*, b) *Pseudo-nitzschia* spp. *seriata* and *delicatissima* complexes, c) Cyst number (cysts mL^{-1}) of *A. catenella* and *P. reticulatum*.

Of these stations, the toxin was detected mainly in the fraction >100 µm (33%), corresponding to the microzooplankton, and in 4% in the fraction with predominance of phytoplankton (20-100 µm) detected only in station 40. The size fraction <20 µm only showed toxicity at station 38 and represented 4% of plankton fractions with quantifiable toxin. However, it is noteworthy to mention that the C1-2 toxin was detected at trace level in one or more of the size fractions analyzed for the remaining 16 stations. In percentage terms, this is fraction of size >100 µm in 34%; fractions 20-100 µm and <20 µm in 19%. The DA was not detected in any of the analyzed matrices.

Table 2. Dinoflagellate cysts detected in the sediments of the study area.

Sampling station	Species	N° of cysts mL^{-1}
37	*Alexandrium catenella*	4
	Protoceratium reticulatum	11
	Protoceratium conicum	8
	Pentapharsodinium dalei	3
	Protoperidinium minutum	3
	Scrippsiella lachrymose	7
	Protoperidinium spp.	27
38	*Protoceratium reticulatum* (empty)	4
41	*Protoceratium reticulatum*	3
	Protoperidinium excentricum	3
	Protoperidinium avellana	3
	Protoperidinium conicum	8
86	*Protoceratium reticulatum*	
	Protoperidinium conicum	
	Protoperidinium spp.	
88	*Protoceratium reticulatum*	103
	Protoceratium reticulatum (empty)	23
	Protoperidinium conicum	17
	Protoperidinium spp.	7

Lipophilic toxins

Of the five lipophilic toxins SPXs, YTXs, AZPs, PTXs and okadaic, searched in plankton and shellfish samples by MALDI mass spectrometry, YTXs were the only detected in some shellfish extracts: mussels (stations 38, 41 and 51), Magellan mussels (station 88) and clams (station 41) (Fig. 5). The different ionic forms of YTX detected were 45-hydroxy-yesotoxin, 45-hydroxy-carboxy-yotoxin (commonly found in shellfish as a biotransformation product), and an unknown ionic derivative, like to 41-keto-andotoxin, confirmed by MALDI-TOF (Table 4).

Analysis of historical records

In this section, we perform a brief analysis of the variables RA, PST, lipophilic toxins and cysts recorded in the historical database between 2009 and 2012 (Figs. 6, 9) generated by regular monitoring in the macro zone, to enrich the discussion of the results obtained during the CIMAR-18 cruise. The months considered for comparison were June or July for the winter period and March 2012 for the summer period.

Relative winter abundance of toxic and potentially toxic species

The relative historical abundance of *A. catenella* (Fig. 6a) shows that in June 2012 it reached a maximum of level 3 (regular) in the northwestern sector of the region. These levels were observed again in June 2009 but in the south-central eastern sector of Aysén. The geographic coverage of the species was also different between these two years. The stations in which it was present in June 2009 (90%) were located in the sectors bordering the main channel, *i.e*., latitudinal distribution. Particularly in 2009, level 1 of the species was concentrated on the western side of the main channel (Aysén insular), while levels 2 and 3 on the eastern side (continental Aysén). In 2012, the presence of the microalgae was concentrated in the longitudinal sense, in the northeastern and western sector of the region. In this case, 80% of the stations (12 in total) with the presence of the microalgae were located in the northern sector, and only 25% of the total stations located in the north-western sector presented level 2-3. However, it should be noted that in both years the relative abundance gradient was longitudinal, in E-W direction in 2012, and E-W in 2009. In 2010 and 2011 levels of relative abundance only reached the level 1 (rare) and with different geographic coverage. Regionally dispersed in 2010, with 70% of the stations located in the interior channels of insular and continental Aysén, while in 2011 the species was only detected in a station south of insular Aysén.

For *A. ostenfeldii* (Fig. 6b), the maximum relative abundance reached only level 1 (rare) in June 2012 and in previous years. In 2012, it was observed in the northern sector of Aysén, while in 2010 and 2011 it was observed more frequently in Jacaf Channel, and in 2009 in the central sector of the region. In none of these years, the microalgae was observed in a number greater than three stations, and only in July 2012 was detected in the Elefantes Fjord.

Table 3. Toxins C1-2 detected in the size-fractionated plankton (µm). Dark gray indicates the toxin concentration (ng μL^{-1}), tr: indicates the trace level.

Sampling station	C1-2	10-20	20-100	100-200	> 200
102	C1	0	tr	0	0
	C2	0	tr	0	0
103	C1	tr	tr	tr	tr
	C2	tr	0	tr	0
34	C1	0	0	0,69	0,70
	C2	0	0	tr	tr
36	C1	tr	tr	0,69	0
	C2	tr	tr	tr	0
38	C1	0,69	0	tr	0,69
	C2	tr	0	0	tr
40	C1	tr	0,69	0,69	0,69
	C2	tr	tr	tr	tr
39	C1	tr	tr	tr	tr
	C2	tr	tr	tr	tr
41	C1	tr	tr	tr	tr
	C2	tr	tr	tr	tr
45	C1	tr	tr	tr	tr
	C2	0	0	tr	tr
47	C1	tr	0	tr	0
	C2	tr	0	tr	0
50	C1	tr	0	0	0
	C2	0	0	0	0
51	C1	0	0	tr	0
	C2	0	0	tr	0
52	C1	tr	tr	tr	0,69
	C2	tr	tr	tr	0,03
76	C1	0	tr	tr	tr
	C2	0	tr	tr	0
78	C1	0	0	tr	0
	C2	0	0	0	0
80	C1	tr	tr	0,70	0
	C2	tr	tr	0,03	0
82	C1	0	tr	0	tr
	C2	0	0	0	0
84	C1	tr	0	0	0
	C2	0	0	0	0
86	C1	0	tr	tr	tr
	C2	0	0	tr	tr
87	C1	0	0	0	tr
	C2	0	0	0	0
88	C1	0	0	tr	0
	C2	0	0	0	0
89	C1	tr	tr	tr	tr
	C2	0	0	0	0

The historical distribution of *D. acuminata*, *D. acuta* and *P. reticulatum* is shown in Fig. 8. The maximum level of relative abundance reached by *D. acuminata* in the analyzed period was 3 (regular) in June of 2012 (Figs. 7a-7b), however, the distribution of both species never reached the southern part of the Meninea Constriction sector, observing a rather longitudinal distribution pattern. In June 2009 and July 2010, the distribution pattern of these species was latitudinal. *D. acuminata* was detected between Moraleda Channel and Elefantes Fjord, while *D. acuta* was distributed from the central sector to the south. *P. reticulatum* (Fig. 7c), was detected mostly in the northern sector of the region in June 2012, with a longitudinal distribution similar to *D. acuminata* and *D. acuta*. The relative abundance of this species reached maximum levels of 2 (low) and 3 (regular) in Puyuhuapi Channel and in the channels of the northwestern sector of the region.

During 2009 and 2010, the distribution of this species was rather latitudinal like the *Dinophysis* species, with a common distribution area as the Puyuhuapi Channel. As well as the *Dinophysis* species, *P. reticulatum* was not detected in June 2011.

The relative abundance of *Pseudo-nitzschia spp. seriata* complex and *Pseudo-nitzschia* spp. *delicatissima* complex reached levels of up to 6 and 8 in June 2012 (Fig. 8a-b), respectively, inside Puyuhuapi Channel. In both diatoms species, it was possible to observe a latitudinal and longitudinal distribution, the latter in the northern sector of the region. Latitudinally, relative abundance levels were lower, with levels ≤4 for the *seriata* complex and ≤5 for the *delicatissima* complex, however this distribution pattern was different over the years. In June 2009, both species had a higher frequency of occurrence in the southern sector with maximum abundance levels of 4 (abundant) for the *seriata* complex and 5 (very abundant) for the *delicatissima* complex. In June 2011, they had a central-northern distribution of Aysén continental where *Pseudo-nitzschia* spp. *delicatissima* complex reached a maximum level of 6 (extremely abundant) and *Pseudo-nitzschia* spp. *seriata* complex reached level 3 (regular). In June 2010, both species only reached the minimum level of relative abundance (1, rare) with totally different distributions: Latitudinal in the case of the *delicatissima* complex, while the *seriata* complex was detected only in a station located at the entrance of the Jacaf Channel (Fig. 8a).

Relative summer abundance of toxic and potentially toxic species

The distribution of species in March 2012, a seasonal period prior to that recorded during the CIMAR-18 cruise in winter season, is shown in Fig. 9. The relative abundance levels of 5 (very abundant) for *A. catenella*, *D. acuminata* and *D. acuta* (Fig. 9a) were relatively high. In the case of *A. catenella*, level 3 (regular) is considered the limit value on which the odds of observing increases in the toxicity of shellfish by PST that exceeds the limit of human consumption. *A. ostenfeldii* and *P. reticulatum reached* a maximum re-

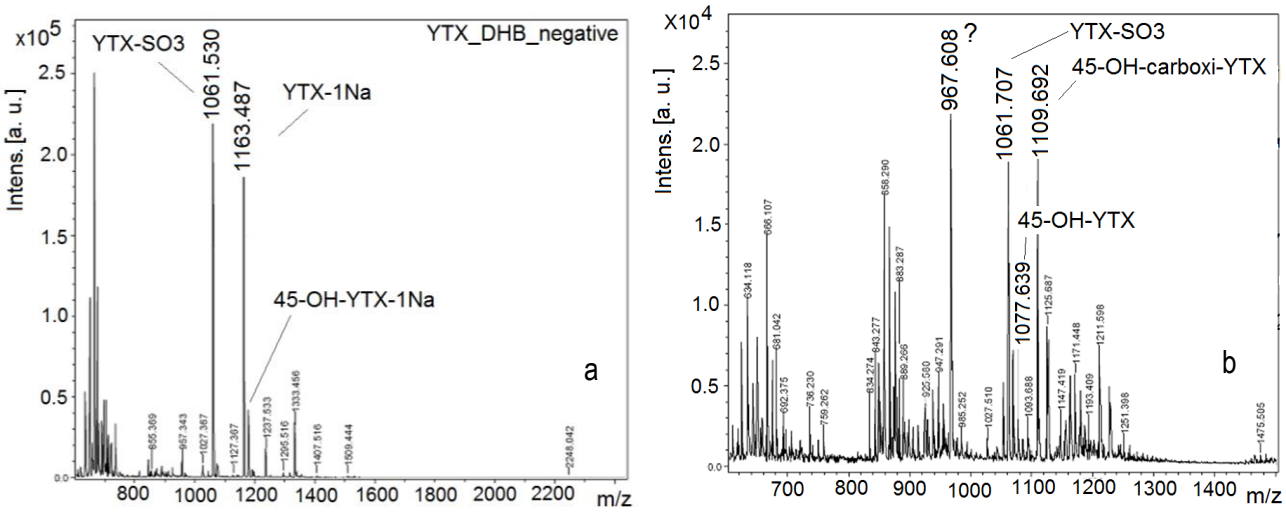

Figure 4. Mass spectrum of YTX in negative mode. a) Standard, at *m/z* 1061, 1163 and 1179 for the ions $[M-2Na-SO3]^-$, $[M-Na]^-$ and $[M+Na]^-$, respectively, b) example of mass spectrum of a mussel sample, station 88. Ion at *m/z* 1061 of YTX is highlighted.

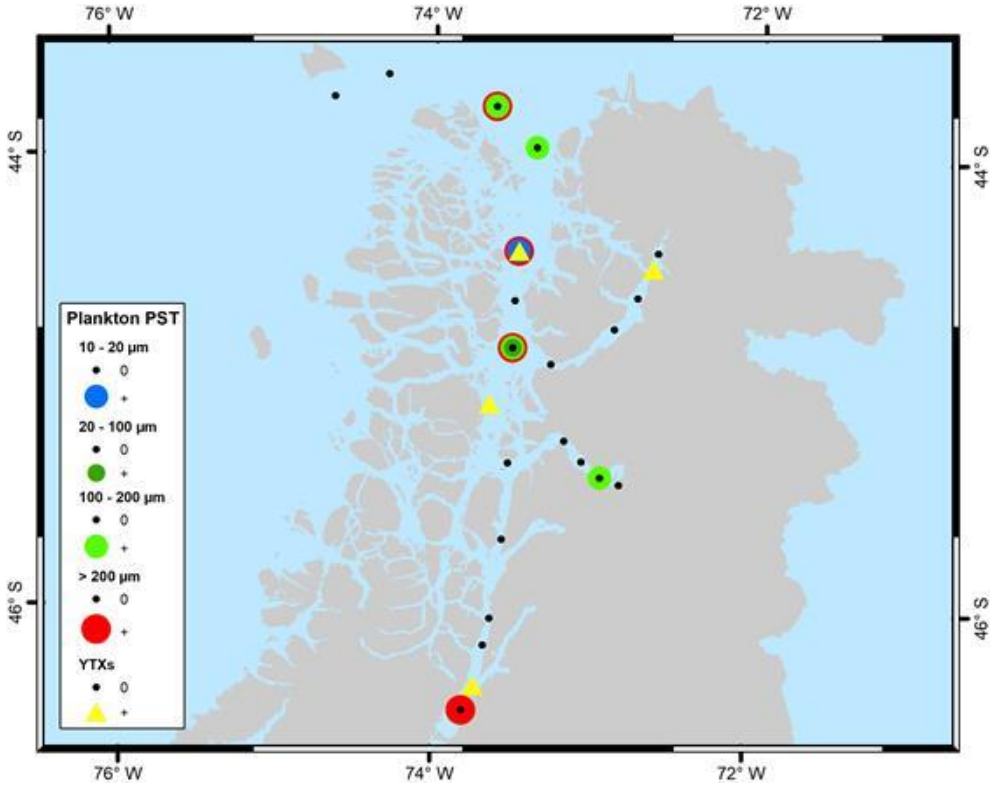

Figure 5. Analytical detection of PST (C1-2) in the size-fractionated plankton, and YTXs in shellfish.

lative abundance level of 3 (regular), a high value in the first case considering that the maximum value historically reached in the same month of previous years was 1 (rare). For *P. reticulatum*, the levels reached during March of the previous years were between 4 and 7 (abundant and hyper-abundant, respectively) (Guzmán *et al*., 2015). For diatoms, the levels of 6 (extremely abundant) and 8 (ultra-abundant) reached in March 2012 by *Pseudo-nitzschia* spp. *seriata* and *delicatissima* complexes, respectively, were similar to those found during the same month of previous years (Guzman *et al*., 2015), except for the *P.* spp. *seriata* complex that reached level 3 (regular) at most in March 2009.

Table 4. Mass (*m/z*) of the standard YTX ions (top) and in the mollusk extracts (lower). *Signal too low to be confirmed by MS/MS.

		Standard YTX *m/z*	YTX [M-2Na-SO_3] 1061	YTX [M-Na] 1163	45-OH-YTX [M+Na] 1179		
		Sample *m/z*	YTX [M-2Na-SO_3]	YTX [M-Na]	45-OH-YTX [M-SO_3]	45-OH-carboxi YTX [M-SO_3]	41-keto-YTX ? [M-SO3]
			1061	1163	1077	1109	967
Sampling station	Date	Transvector					
38	20-jun-12	blue mussel	-		-	+	-
41	02-jul-12	blue mussel	-	+*	-	+	-
41	02-jul-12	Clam 2	-		-		+*
88	04-jul-12	ribbed mussel	+		+	+	+
51	23-jun-12	blue mussel		+*			

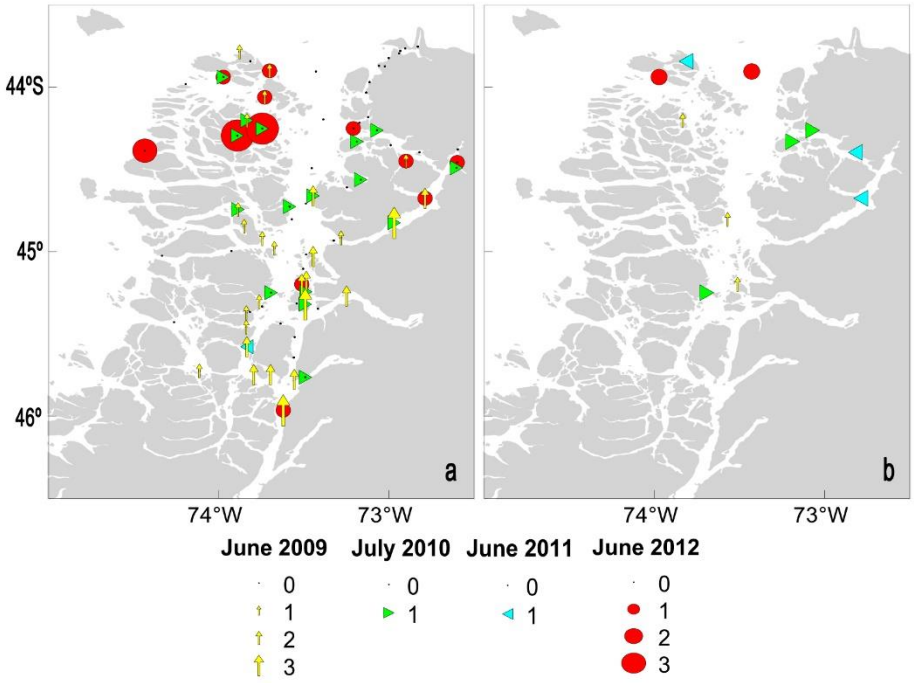

Figure 6. Historic relative abundance during June or July between 2009 and 2012 for a) *A. catenella*, b) *A. ostenfeldii*.

Winter and summer historical record of PST and lipophilic toxins

The maximum levels of winter toxicity reached by PST and lipophilic toxins were recorded in 2009 (Fig. 10), with values up to 1359 µg STX eq. 100 g^{-1} in the first case and a percentage of geographic coverage (80%, n = 55 stations) of samples positive to the mouse bioassay in the second (Guzmán *et al.*, 2015). The highest historical PST values (16052 µg STX eq. 100 g^{-1}, Guzmán *et al.*, 2015) occurred as well during March 2009, and coverage percentage of positive samples to mouse bioassay by lipophilic toxins (88%, n = 82 stations). For both variables, the lowest toxicity levels in shellfish occurred in 2012 for both, winter and summer periods. In June 2012, the PST reached 143 µg STX eq. 100 g^{-1} and lipophilic toxins reached 5% (n = 56 stations) of geographic coverage, while in March of the same year the values reached 151 µg STX eq. 100 g^{-1} and 24% (n = 71 stations) coverage, respectively.

DISCUSSION

Knowledge about the distribution of PST toxins and lipophilic toxins in transvectors as well as their primary microalgal source in the estuarine ecosystems of southern Chile channels and fjords remains low. The

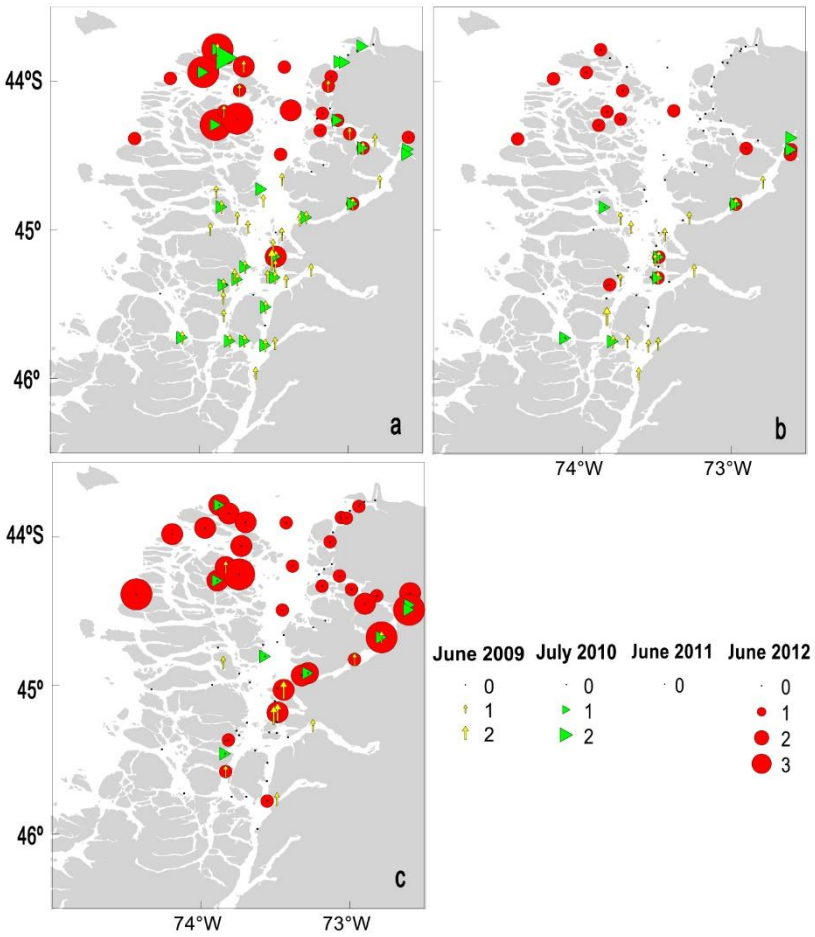

Figure 7. Historic relative abundance during June or July between 2009 and 2012. a) *Dinophysis acuminata*, b) *D. acuta*, c) *Protoceratium reticulatum*.

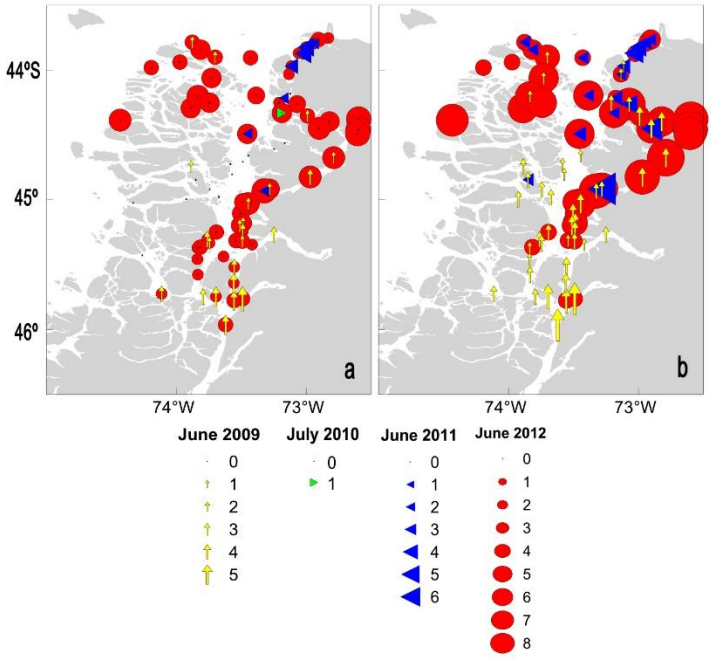

Figure 8. Historic relative abundance a during June or July between 2009 and 2012. a) *Pseudo-nitzschia* spp. *seriata* complex, b) *Pseudo-nitzschia* spp. *delicatissima* complex.

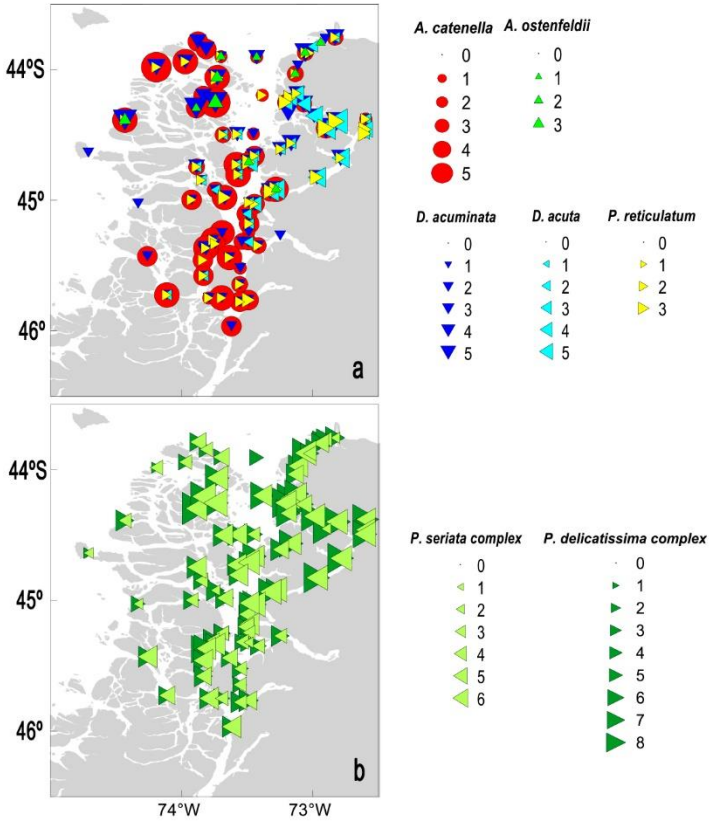

Figure 9. Relative abundance during the regular monitoring of March 2012 of the dinoflagellates. a) *A. catenella*, *A. ostenfeldii*, *D. acuminata*, *D. acuta*, *P. reticulatum*, b) diatoms *Pseudo-nitzschia* spp. *seriata* complex and *Pseudo-nitzschia* spp. *delicatissima* complex.

Aysén region is characterized by intense events of harmful microalgae blooms and high levels of toxicity in shellfish during spring-summer, a period in which efforts to prevent human intoxications are concentrated (Guzmán *et al*., 2015) and increases the risk of dispersal of microalgal plague into free sectors (*e.g.*, Pizarro *et al*., 2012a, 2012b, 2014a). However, the CIMAR-18 cruise results indicate that the microalgae source of PST and lipophilic toxins persist in the studied area during the winter period, albeit at low concentrations.

It is also confirmed that the relative abundance (Figs. 2a-2b) is a more sensitive estimator than the cell density to verify the presence of dinoflagellate species (Fig. 3a). This has been previously reported in other studies (*e.g.*, Guzmán *et al*., 2012; Pizarro *et al*., 2011, 2015), indicating that is still a good tool of dinoflagellates presence during the winter period. In the case of diatoms, both estimators show a similar distribution for the taxa considered in this study (Figs. 2c, 3b). The discussion on the distribution of toxin-producing dinoflagellates species, for this reason, is based on relative abundance results. For comparative purposes, the same analysis of the results between these two large groups of microalgae is performed for diatoms species.

Distribution of *Alexandrium* spp.

A. catenella was present in much of the region during the winter period. Relative abundance levels of up to 2 (sparse) (station 41), in the northern section of Meninea Island (45°14'S, 73°37°W) and south of the Costa Channel (station 47) (Fig. 2a) were not as expected (absence), for a winter month as June. This pattern of inter-annual variability becomes recurrent if we compare the results obtained in this study, with those recorded historically by the Regular Monitoring Program in June 2009 and 2012. The maximum relative abundance reached for this month in these two years attained level 3 (regular) (Fig. 6a) (Guzmán *et al*., 2015).

Along latitudes, the results of this study were also coincident with those recorded during the regular historical monitoring, considering the same sampling sectors, *i.e.*, Moraleda Channel, Puyuhuapi Channel,

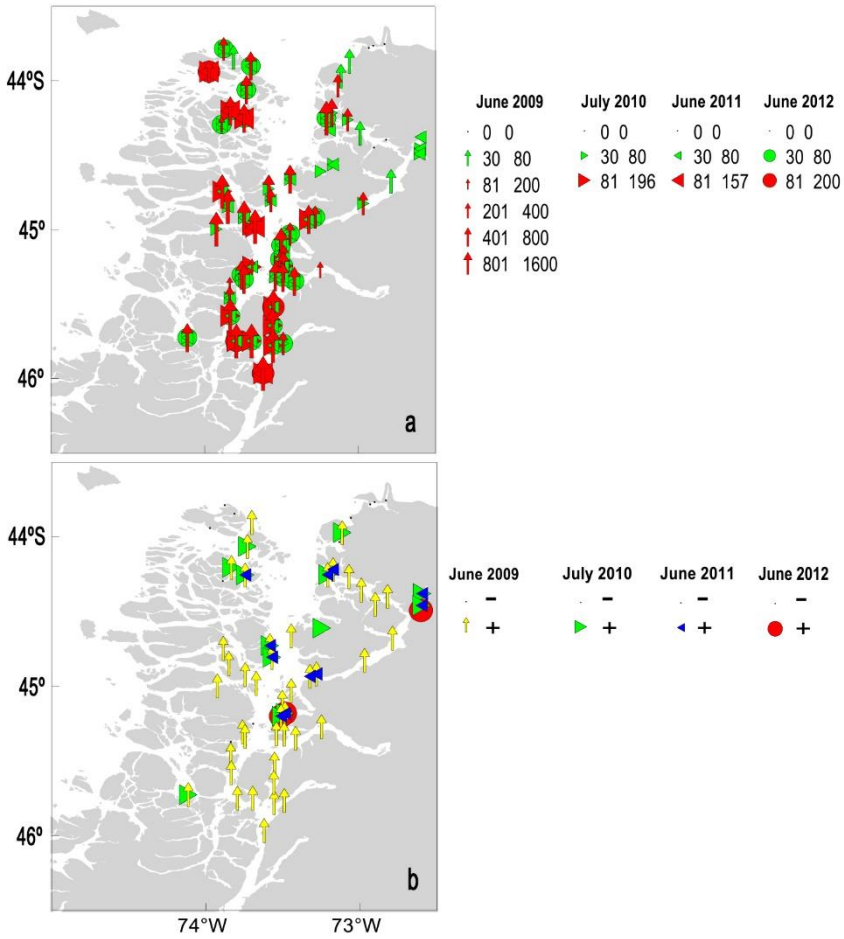

Figure 10. Historical toxicity detected by mouse bioassay in the sentinel shellfish during the regular monitoring on June or July between 2009 and 2012. a) PST, µg STX eq. 100 g^{-1}, b) lipophilic toxins expressed as presence (+) or absence (-).

Meninea constriction, western sector of the Aysén Fjord, Costa Channel and Elefantes Fjord. On the contrary, *A. ostenfeldii* always presented a relative abundance level of 1 (rare) during the CIMAR-18 cruise, as well as in regular historical monitoring, however, the frequency of occurrence was different, as were the sectors of distribution. During CIMAR-18, considering the same sampling sites, the microalgae were observed in at least 6 stations, all located in Meninea, Aysén and Puyuhuapi, while in 2012 (10 days before) it was observed in the northern Aysén sector and in the Elefantes Fjord, in 2009 was detected in the central sector of the region. In 2010 and 2011 were observed more frequently in the Jacaf Channel. The results indicate an inter-annual variability in the winter distribution as well as on a daily scale for these microalgae.

Distribution of *Dinophysis* spp. and *P. reticulatum*

During the regular historical monitoring in the winter months, the distribution of the species of *Dinophysis* and *P. reticulatum* was rather latitudinal with a common distribution area, the Puyuhuapi Channel, except for the year 2011, when these species were not detected. However, the results also suggest a spatial specific-distribution variability in the Aysén Fjord. In this study, the species of *Dinophysis* were detected in this place, not so *P. reticulatum* species. Historically, records from previous years corroborate this differential distribution during June. Although *P. reticulatum* was detected in the interior of this fjord, in June 2006 and 2009 (Guzmán *et al*., 2015) with level 1 (rare) relative abundance, it was with a lower frequency than *D. acuta*.

Our results indicate that during the winter, these dinoflagellates persist in the region of Aysén, with a spatially variable distribution, confirmed by historical data recorded in the same sampling areas: Channels Moraleda, Puyuhuapi, Costa, and fjords Aysén and Elefantes. However, in the same historical context, records also indicate a meso-spatial distribution (latitudinal and longitudinal) of short-term (days) and

inter-annual (*e.g.*, June-July, 2009-2012). These conditions are not evident only with the results of the CIMAR-18 cruise as it was carried out on a latitudinal oriented route, following the central channel, with sampling only on two inland continental channels. However, the winter distribution of the species tends to cover mainly the large central channels of the region between the Moraleda Channel and Elefantes Fjord, with very low-frequency detection of *P. reticulatum* (maximum level 1, rare) south of the Costa Channel, according to the available information analyzed in this study (*e.g.*, Guzmán *et al*., 2015). However, the species has also been recorded on the west coast of Aysén insular, Pulluche Channel sector (45°50'S, 74°32'W) in July 2002, with maximum relative abundance level 1 (rare) (Seguel & Sfeir, 2010).

Distribution of *Pseudo-nitzschia* spp. *seriata* complex and *Pseudo-nitzschia* spp. *delicatissima* complex

The distribution pattern of both diatom complexes was very similar, with no spatial or temporal differences as in the case of dinoflagellates. The variability in distribution occurs rather on a year-on-year basis. In June 2011, the highest frequency of detection was restricted to the western central-north sector. In 2010, the distribution was latitudinal and during 2009, the distribution was restricted to the Puyuhuapi Channel and southern sector of the region. Distribution records for these species indicate them with a maximum level of relative abundance 4 (abundant), in July of the year 2002 in the sector of Darwin Channel (45°24'S, 74°00'W) (Seguel & Sfeir, 2010), level of abundance and longitudinal distribution that is within the variability ranges found in this study.

Space-temporal variability of dinoflagellates and diatom distribution

Our results suggest a winter distribution pattern of interannual variability, and daily in dinoflagellates but not so in diatoms. Within the dinoflagellates, there are also differences in winter spatial distribution, such as with *P. reticulatum*, a species that were not detected in the Aysén Fjord during CIMAR-18 cruise, when its winter detection has historically been of relative abundance level 2 (rare) inside the fjord.

During the summer period, the studied microalgae remarkably change their distribution patterns, as well as their relative abundances (*i.e*., Figs. 9a-9b). Even for the western coast of insular Aysén, there are records of the relative abundance of *A. catenella* and *D. acuminata* reaching levels of up to 3 (regular) and 4 (abundant), respectively, during the summer (Seguel & Sfeir, 2010). A sector not monitored by smaller vessels due to the difficult oceanographic conditions of the sector.

The factors involved in species distribution are complex because they appear to be beyond (or over) the seasonality of the year and other classical drive forces (*e.g.*, oceanography, hydrodynamics, wind, etc.) considered so far. For example: a) during the winter period, specific environmental conditions variability required by different microalgae to persist, with rapid conditioning responses reflected, in a matter of days, in the different distribution patterns found in dinoflagellates, but not for diatoms, b) the influence of summer distribution patterns seems to be a good indicator of what will happen during the winter period. This is suggested by results reported for sectors of the Magellanes region, the southernmost region of Chile (*e.g.*, Pizarro *et al*., 2011, 2015). This is an aspect, not yet been evaluated, that needs to be incorporated in the studies carried out since they have to do with the inter-annual variability observed in microalgae distribution, c) the effects of freshwater fronts, produced by existing river and glacier discharges acting as walls or barriers to containment and physical accumulation, probably affecting important biological aspects, such as physiology, and microalgae life cycles, especially for dinoflagellates, another aspect not been evaluated at the spatial mesoscale level, d) mixotrophic capacity of dinoflagellates, unlike diatoms, in environments with high levels of dissolved organic matter (autochthonous and allochthonous), especially during winter (Pizarro *et al*., 2005), characteristic of southern fjords, channels and embayments ecosystems of Chile, an aspect that has not been evaluated as a factor favoring dinoflagellates persistence during the winter period, as well as their influence on the proliferation of others dinoflagellates species that follow diatom summer blooms or events of high seasonal primary production (Aracena *et al*., 2011, 2015; Paredes *et al*., 2014).

PST in plankton and shellfish fractions

The detectable levels of C1-2 toxins in the different plankton size fractions considered in this study, with a predominance of micro (>100 μm) and mesozooplankton (>200 μm), suggest the transfer of PST to higher trophic levels. However, these toxins do not appear to have been transformed into GTXs or dcGTXs by zooplankton as occurs with some shellfish (*e.g.*, Fast *et al.*, 2006; Krock *et al.*, 2007; Pizarro *et al.*, 2015) or as observed in the micro and mesozooplankton reported for the Magellanes region (Pizarro *et al.*, 2011). However, experimental studies are needed to determine the fate of PST toxins ingested by zooplankton, their role as eventual regulators, and a container of noxious species blooms in the inner seas of the southern macro-

region. There is proven evidence of toxic species consumption by copepods (Frangópulos *et al.*, 2011, 2014), and is necessary to elucidate important aspects regarding how much toxicity they can ingest, accumulate and transfer to higher trophic levels.

The quantifiable detection of C1-2 in the fraction 20-100 µm, in which phytoplankton predominates, occurred only in one sampling station (40), a result that is consistent with the higher cell density of *A. catenella* found in that station. These toxins were also detected in the fraction less than 20 µm at station 38, although intriguing, is consistent with the absence of *A. catenella* and with the finding of Cs in the larger size fraction (mesozooplankton) at the same station. This result suggests the presence of zooplankton remains and/or fecal pellet fragments in the fraction smaller than 20 µm. A higher percentage of quantifiable toxin in the fractions >100 µm than in the fraction with phytoplankton predominance is consistent with the transfer of PST to zooplankton, which would also act as a toxin concentrator.

Detection of C1-2 at trace level in one or more fractions per plankton size in all of the stations sampled (Table 3), suggest that the microalgae were present in the water column and that its Cs toxins could be an indicator mostly sensitive to the presence of *A. catenella* than relative abundance. The stations at which trace C1-2 were detected were all stations in which *A. catenella* and/or PST were detected in the plankton or stations adjacent to the latter (Figs. 3, 4). However, it is necessary to validate this hypothesis by contrasting the analyzes with fluorescence detector with other more sensitive ones such as mass spectrometry. In the case of shellfish, the abundance of *A. catenella* was not sufficient to detect PST in them.

Lipophilic toxins in mollusks

It is highly probable that the microalgal source of YTXs detected in bivalves is *P. reticulatum* (Pizarro *et al.*, 2012a), species reported with greater frequency and abundance in our country than *Gonyaulax spinifera* and *Lingoludinium polyedrum*, which are the other two species associated with the production of yessotoxins in other parts of the world. Both have been reported for southern Chile (*e.g.*, Salgado *et al.*, 2011; Guzmán *et al.*, 2015). Recently, *Gonyaulax taylorii* has been reported for Mejillones Bay in northern Chile (23º03'S, 70º23'W) as a fourth dinoflagellate producer of YTXs (Álvarez *et al.*, 2016), although the species has not been identified in the cold waters of the southern macrozone. *P. reticulatum* has been confirmed as the primary source of YTXs in Otway Sound (53º13'S, 72º12'W) and Adalberto Channel (48º42'S, 74º28'W) in Magallanes region (Pizarro *et al.*, 2014b) and in Mejillones Bay in northern Chile (Álvarez *et al.*, 2011). It has also been associated with YTXs toxicity in Reloncaví sea snails in Los Lagos region (41º30'S, 72º18'W) (Vivanco *et al.*, 2012; Alves de Souza *et al.*, 2014).

Yessotoxins were the only lipophilic toxins detected with the technique applied in this study, in shellfish from four stations (Fig. 5) in which *P. reticulatum* and/or its cysts were recorded (Figs. 2b, 3c) in adjacent sectors during CIMAR-18 cruise. The pattern of distribution was also consistent with that observed during regular monitoring (Fig. 7c) for common sampling areas (Moraleda and Puyuhuapi channels, Meninea sector), as well as consistent with reports of this toxin on shellfish from the Aysén region dating back to 1997 (Yasumoto & Takizawa, 1997; Goto *et al.*, 2001).

Dinoflagellates cysts

Despite the high levels of relative abundance (5, very abundant) of the vegetative phase of *A. catenella* observed in March 2012 (Fig. 9a), the number of cysts observed during the study (4 cysts mL^{-1} w.s.) was low, when contrasted with the maximum values reported for Aysén in previous years. As an example, values of 14627 and 2729 cysts mL^{-1} w.s. have been reported for Medio Sound (44º36'S, 73º14'W) and Canalad Sound areas (44º32'S, 73º9'W) respectively, in July (winter) 2010 (Guzmán *et al.*, 2011). For the Quitralco Estuary (45º46'S, 73º32'W), values of 76 cysts mL^{-1} were reported in the late summer of 1999 (Lembeye, 2004) and 17 cysts mL^{-1} in November 2001 (Seguel *et al.*, 2005). For the northern sector of Melinka (43º50'S, 73º55'W), maximums of 50 and 128 cysts mL^{-1} have been reported in June and July 2009, respectively (Díaz *et al.*, 2014), year that was characterized by an intense blooming of *A. catenella* in comparison to periods before and after that year (Guzmán *et al.*, 2015). Conversely, the cell density of *P. reticulatum* cysts (103 cysts mL^{-1} w.s.) was similar in magnitude to the maximum values for the region commonly reported in previous years, despite having regular relative abundance levels (3) in March 2012 (Fig. 9a). For Quitralco Estuary, 85 cysts mL^{-1} have been reported in July 2001 (Seguel *et al.*, 2005), 77 cysts mL^{-1} in Goñi Channel (44º50'S, 74º05'W) in July of 2002 (Seguel & Sfeir, 2010), and 50 cysts mL^{-1} in August of 2009 and February of 2010 for the northern sector of Melinka (Díaz *et al.*, 2014). Nevertheless, results are low when confronted with those obtained in July (winter) 2010, where *P. reticulatum* cysts reached values as high as 25971 cysts mL^{-1} w.s. in Medio Sound, 14643 cysts mL^{-1} w.s. in Atilio Island (44º22'S, 73º17'W) and 10773 cysts mL^{-1} w.s. in the interior of Gala Sound (44º11'S, 73º7'W) (Guzmán *et al.*, 2011).

These antecedents indicate the great variability of the density, distribution, and seasonality of *A. catenella* and *P. reticulatum* cysts. Although an increased abundance of cysts tends to be associated with the intensity of vegetative cell blooms during the summer period (*e.g.*, Díaz *et al.*, 2014), the survival of these cysts in the sediments also seems to be very variable.

CONCLUSIONS

In the winter period, RA levels of *A. catenella*, *D. acuminata* and *P. reticulatum* can reach level 3 (regular), while *D. acuta* and *A. ostenfeldii* reach level 1 (rare), but in no case, these dinoflagellates as a whole, are absent in the study sector. Results obtained during winter showed that the absence of harmful microalgae would be an abnormal condition. Dinoflagellates species may persist during winter up to levels of relative abundance 3 (regular) according to these results and the analysis of historical data performed in this study.

During the winter period, the short-term variability (days) in the dinoflagellates distribution patterns is most evident, however, they are still a reflection of the summer blooms distribution that precede them, a relation that is not linear. In the case of diatoms, a lower variability of the distribution pattern observed during the study period compared to its summer pattern.

The increase in the relative abundance of dinoflagellates from levels ≤3 during winter to levels >5 in the preceding summer period (*e.g.*, Figs. 6a, 7, 9a) reflect the complexity of time scales in which factors enhance microalgae growth, mainly short-term in dinoflagellates, and inter-annual in diatoms (Figs. 8, 9b).

In dinoflagellates species, the specific biological factor in their winter space-time distribution is most evident. The summer distribution pattern of this group varies during the winter, unlike diatoms, suggesting that physical (*e.g.*, hydrodynamic, oceanographic, bathymetric, wind) conditions alone do not explain the distribution patterns of dinoflagellates during the coldest seasonal period of the year.

There is a great variability of density, distribution and seasonality of *A. catenella* and *P. reticulatum* cysts, associated with their persistence in sediments, a condition that seems to be associated with environmental factors influencing their formation, viability, biological transport (consumers) and/or physical (advective) factors.

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