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Paralytic shellfish toxins in *Argopecten purpuratus* and *Semimytilus algosus* from northern Chile

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Abstract – Within the framework of the Chilean molluscan shellfish safety program, blooms of *Alexandrium* were detected in several aquaculture sites in northern Chile. An outbreak of *Alexandrium* was detected in May 2006 in Mejillones Bay. Wild and cultured phytoplankton and shellfish samples from this bloom were analyzed by high performance liquid chromatography with fluorescent detection (HPLC-FD). Neither phytoplankton net tows samples nor an *Alexandrium* culture started from the bloom were found to contain PSP toxins at detectable levels. The shellfish, however, showed the presence of C2, GTX2, GTX3 and dcGTX2. Two new PSP episodes were recorded in June 2006 in Guanaqueros and Tongoy Bays. In these instances, shellfish samples from the two bays were analyzed by mouse bioassay and HPLC-FD, showing PSP toxicities that ranged from 27 to 34 μg STX eq 100 g^{-1} and a toxin profile mainly characterized by the presence of STX, GTX2 and GTX3. Differences between toxin profiles in Mejillones Bay and the two other locations suggest that, in the area, this kind of toxicity is probably produced by different regional populations or strains of the genus *Alexandrium* other than *Alexandrium catenella*, since the toxin profile and optimal environmental conditions for this species are noticeably different from those recorded in the proliferations from northern Chile. This paper therefore presents the first report of this kind of toxicity from an area between parallels 13°55'S and 33°5'S, comprising the subtropical zone of the southeastern Pacific Ocean. Results suggest that, as in the northern Pacific coastal area of America, PSP toxicity may be widely distributed on the southern Pacific coast.

Key words: Paralytic shellfish poisoning, toxicity / *Argopecten purpuratus* / *Semimytilus algosus* / *Alexandrium* sp. / Pacific Ocean

1 Introduction

Paralytic shellfish poisoning (PSP) is a neurotoxic syndrome caused by the ingestion of shellfish contaminated by saxitoxin and its analogues (reviewed in Bricelj and Shumway 1998). These toxins are known to be biosynthesized by various species of marine dinoflagellates of the genera *Alexandrium*, *Gymnodinium* and *Pyrodinium* (reviewed in Burkholder 1998).

The PSP family of toxins includes more than 20 structurally-related compounds from the marine environment. These substances can be grouped into five groups that differ substantially in toxicity. The most toxic group is that of the carbamate toxins: saxitoxin (STX), neosaxitoxin (NeoSTX) and the gonyautoxins (GTX1 to 4). The decarbamoyl (dcGTX1 to 4, dcSTX, dcNeo) and deoxy-decarbamoyl toxins (doSTX, doGTX2-3) have an intermediate toxicity. The least toxic are the N-sulfocarbamate toxins (GTX5 and 6, C1 to C4) (Oshima et al. 1990; Oshima 1993) and the hydroxybenzoate toxins (GC1 to GC3) (Negri et al. 2003b). Filter-feeders acquire these toxins by ingesting toxic dinoflagellates and accumulating them in their tissues (Bricelj and Shumway 1998).

Originally detected on the Pacific coast of the United States in 1937 by Sommer and Meyer (1937) and described by Schantz and Magnussen (1964), PSP has substantially broadened its geographical distribution since then. This is partially due to the progressive implementation of monitoring programs (Fernández et al. 2003), which have grown in parallel with the development of shellfish aquaculture, but it is also perhaps due to an true increase in the frequency of toxic outbreaks (Hallegraeff 1993).

PSP has only been reported from two biogeographic zones in South America, separated by more than 3000 km: Paracas Bay, Peru (Tropical zone) (Antinori et al. 2002) and

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Southern Chile (Subpolar Transitional zone). In the latter location, the toxins are produced by *Alexandrium catenella* (Guzmán and Campodónico 1975; Guzmán and Campodónico 1978; Compagnon et al. 1998; García et al. 2004), which has a toxin profile dominated by C1/C2, GTX1/4, GTX2/3 and GTX5 (Lagos et al. 1996; Krock et al. 2007) in phytoplankton. Bivalves that have ingested this species were found to contain large proportions of GTX2 and GTX1, as well as GTX3, GTX4, GTX5 and lesser amounts of neoSTX, STX, dcSTX and C toxins (Compagnon et al. 1998). In the former location, it has not yet been possible to identify the species responsible for PSP or the toxin profile.

Since 1989, the Chilean administration has been monitoring molluscan shellfish safety and toxic, or possibly toxic, phytoplankton by means of a specific program (Sanidad de Moluscos Bivalvos) run by Sernapesca. Within the framework of this program, although some presumed PSP producers belonging to the genus *Alexandrium* have been found in scallop aquaculture sites in the north of Chile (Uribe unpublished data), it has not been possible to associate them with PSP.

In this work, we report the first known paralytic shellfish toxin (PST) events between parallels 13°55' S and 43°5' S, which occurred in three aquaculture sites in the north of Chile (Subtropical zone) and affected the scallop *Argopecten purpuratus* and the mussel *Semimytilus algosus*. The toxin profiles were analyzed and some possible causative agents suggested in view of the results obtained.

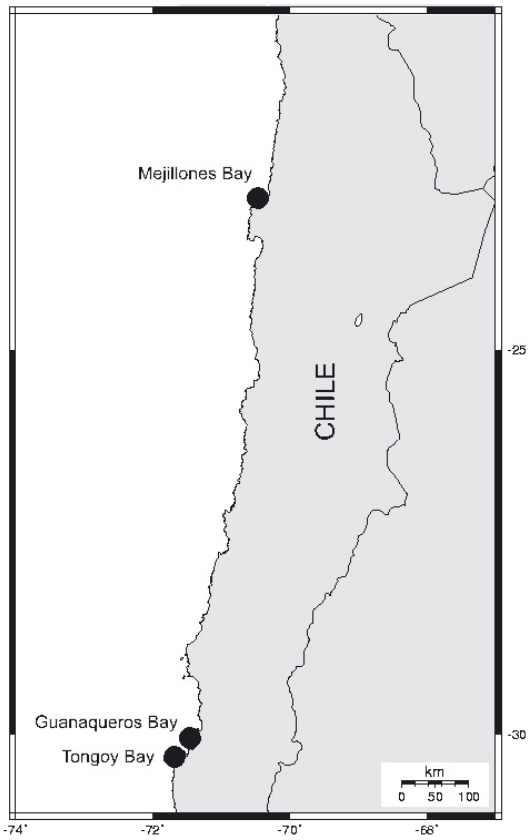

Fig. 1. Sample locations in bays along the northern Chilean coast.

2 Materials and methods

2.1 Phytoplankton samples and *Alexandrium* culture

Phytoplankton samples were obtained periodically from Mejillones Bay (23°3'S, 70°27'W), Guanaqueros Bay (30°11'S, 71°25'W) and Tongoy Bay (30°15'S, 71°20'W) (Fig. 1) from November 2005 until January 2007 by means of vertical net hauls (20 μm mesh) and a 15 m hose. Hose samples were preserved with lugol's solution, the aliquots of the net haul samples used for quantification where also preserved in 4% formalin, while other aliquots used to isolate cells that would be used to start the cultures, were maintained unpreserved.

Phytoplankton species were identified from net haul samples and their abundance quantified from the hose samples with the Utermöhl method (Hasle 1978) using an Olympus inverted light microscope.

For the taxonomic identification of *Alexandrium* from Mejillones Bay, some cells were placed on microscope slides with a drop of sodium hypochlorite to separate the thecal plates. Samples were observed and photographed under an Olympus light microscope with an Image Pro Plus image processor.

2.2 Biological material and toxicity

Scallop (*Argopecten purpuratus*) and mussel (*Semimytilus algosus*) samples were collected from Mejillones Bay (23°3'S, 70°27'W) during the *Alexandrium* bloom of May 2006. Scallop samples were obtained from Guanaqueros Bay (30°11'S, 71°25'W) and Tongoy Bay (30°15'S, 71°20'W) under the Chilean molluscan shellfish safety program.

PSP toxicity was quantified according to the AOAC mouse bioassay (AOAC 1995) in the Laboratorio del Ambiente (Secretaría Regional de Salud, Región de Coquimbo).

2.3 Sample preparation and chromatographic analysis

Net tow samples (30 ml) from Mejillones Bay and *Alexandrium* sp cultures started from cells of the bloom in the same area were filtered through 12 μm nucleopore filters and extracted following the method of Franco and Fernández-Vila (1993).

The soft tissues of each bivalve species studied (100 g) were homogenized in HCl (0.1 N) at a ratio of 1:1 (weight:volume). Extracts were clarified by centrifugation (10 000 × g, 8 min). An aliquot of the resulting extract was purified using a C18 Solid Phase Extraction (SPE) Bond Elut cartridge (3 ml, 500 mg) (Varian) following the method of Oshima (1993). A subsample of each purified sample was concentrated (15 fold) by evaporation in a Speed Vac (Thermo Fisher Scientific) and was redissolved in ultrapure water (MilliQ Gradient, Millipore). A 50 μl aliquot was hydrolyzed to transform the sulfocarbamate toxins (if present) to the corresponding carbamate toxins (HCl 0.1 N, 100 °C, 15 min). The hydrolyzed

Table 1. Limit of detection of PSP toxins analyzed by high performance liquid chromatography (HPLC) with post column reaction and fluorimetric detection.

Toxin		pmol	μg STX eq kg^{-1}
Gonyautoxin-1	GTX1	0.4	12
Gonyautoxin-2	GTX2	0.15	1.6
Gonyautoxin-3	GTX3	0.05	1
Gonyautoxin-4	GTX4	0.35	7.6
Gonyautoxin-5	GTX5	0.28	0.5
Decarbamoylgonyautoxin-2	dcGTX2	0.06	0.3
Decarbamoylgonyautoxin-3	dcGTX3	0.03	0.3
Saxitoxin	STX	0.15	4.6
Decarbamoylsaxitoxin	dcSTX	0.19	3
Neosaxitoxin	neoSTX	0.16	4.4
N-sulfocarbamoylgonyautoxin-2	C1	0.08	0.01
N-sulfocarbamoylgonyautoxin-3	C2	0.05	0.1

extract was filtered through 0.45 μm nylon Tracer syringe filters (13 mm diameter) (Teknokroma).

The presence of most of the known PST toxins was verified by different high performance liquid chromatography (HPLC) methods. NeoSTX, dcSTX and STX were analyzed following the method of Oshima (1995) using a Prodigy C8 column (250 mm × 4.6 mm, 5 μm particle diameter) (Phenomenex). GTX 1-6 and dcGTX 1-4 were examined by the method of Franco and Fernández-Vila (1993) using an Ascentis RP-Amide column (150 mm × 4.6 mm, 3 μm) (Sigma-Aldrich). In cases where GTX1 or GTX4 could have been masked by the presence of an interfering compound in the shellfish samples (even after SPE treatment), these samples were analyzed using the same technique but with a mobile phase pH of 6.7. C-toxins were analyzed following the method of Oshima (1995) using a Prodigy C8 column (250 mm × 4.6 mm, 5 μm) (Phenomenex). Finally, GCs were analyzed by the method of Negri et al. (2003b) using a Luna C18 (150 mm × 4.6 mm, 3 μm) (Phenomenex).

Toxin quantification was carried out using the techniques of Franco and Fernández-Vila (1993) (GTX1-4) and Oshima (1995) (STX, neoSTX and dcSTX). Sulfocarbamoyl toxins were quantified as their carbamate toxin equivalents by means of the two previous methods.

All HPLC analyses involving fluorescent detection were carried out with a Beckman System Gold/Nouveau workstation equipped with a post column reaction system composed of two Jasco pumps (PU1580 and PU2080) and a 10 m Teflon coil (internal diameter 0.5 mm) in a water bath at 65 °C. Detection was carried out with a Jasco FP1520 fluorescence detector (signal/noise >350 μm).

A correspondence between the chromatographic peaks observed when fluorescence detection was used and PSP toxins was confirmed by comparing the retention times with those of the reference materials (from IMB-NRC, Canada) and by suppressing the signal when the oxidant of the post column reaction system was replaced with water. The limits of detection of this technique were estimated for a signal/noise ratio of 3 (Table 1).

The presence of STX in extracts of *A. purpuratus* from Tongoy Bay was additionally confirmed by HPLC-MS/MS (high-performance liquid chromatography tandem mass spectrometer) using a Surveyor MS HPLC system, coupled to a Deca XP plus ion trap mass spectrometer (Thermo Fisher Scientific) with an electrospray interface. The chromatographic separation was carried out by hydrophilic interaction liquid chromatography (HILIC), following the method of Dell'Aversano et al. (2005). The mass spectrometer was operated in positive mode, with a spray voltage of 4.5 kV, using a stability indicating method (SIM) and MS/MS mode, for monitoring and confirmation, respectively, of the STX ions. The toxin concentration of GTX2, GTX3, GTX5, dcGTX2 and dcGTX3 in the available samples was too low to be detected by the HPLC-MS/MS method.

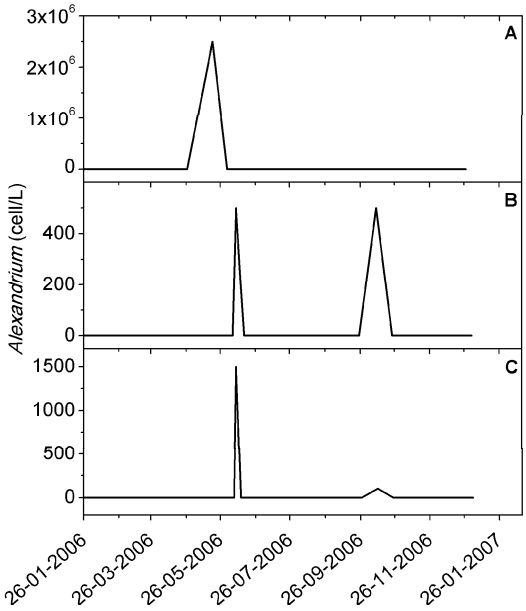

Fig. 2. *Alexandrium* sp. (cell L^{-1}) in the seawater of Mejillones Bay (A); Guanaqueros Bay (B) and Tongoy Bay (C), between January 2006 and January 2007.

3 Results

3.1 *Mejillones Bay*

The analyses of phytoplankton samples from Mejillones Bay revealed the occurrence of an *Alexandrium* bloom from May 5–24, 2006 (autumn in the southern hemisphere) with the highest concentrations of this dinoflagellate (2 500 000 cell L^{-1}) recorded on May 19 (Fig. 2A). Water temperature was 16 °C at 5 m and 14 °C at 10 m. The average water column salinity was 34 psu.

No chains were observed during the *Alexandrium* bloom and only occasional couplets were detected, with single cells being observed most frequently. Cells ranged in size from 35 to 37 μm long and from 36 to 38 μm wide, usually being slightly wider than long. The epitheca had a small ventral pore in the right margin of the first apical plate (1'). The first apical plate was directly attached to the pore plate (po) which had a small comma (cp). The anterior sulcal plate (sa) was generally

Table 2. Comparison of toxin compositions (% mol) of bivalves collected from the sample locations in north Chile (nd: not detected).

Toxin	Mejillones Bay		Guanaqueros Bay	Tongoy Bay
	S. algosus	*A. purpuratus*	*A. purpuratus*	*A.purpuratus*
C1	nd	nd	Trace	Trace
C2	Trace	Trace	Trace	Trace
GTX1	nd	nd	7.1	nd
GTX4	nd	nd	nd	nd
GTX2	Trace	Trace	3.5	3.3
GTX3	Trace	Trace	0.9	1.1
GTX5	nd	nd	Trace	nd
dcGTX2	nd	Trace	0.6	0.5
dcGTX3	nd	nd	0.5	nd
STX	nd	nd	87.4	95.1

greater in length than in width. These characteristics resemble those of *Alexandrium tamarense,* but a more detailed analysis would be required to unequivocally assign the Mejillones Bay bloom to this species.

When analyses were made by HPLC, neither phytoplankton net tows nor the *Alexandrium* culture were shown to contain PSP toxins. However, low levels of PSP toxins were found in *Semimytilus algosus* and *Argopecten purpuratus*. In both cases, the toxin profile was characterized by the presence of C2, GTX2 and GTX3 (Table 2); dcGTX2 was also present in *A. purpuratus*. The hydrolysis of the samples did not produce any noticeable increase in the concentration of GTXs, STX or neoSTX, indicating that no other sulfocarbamoyl toxins (C1, C3, C4, GTX5 and GTX6) were present in significant amounts. GC toxins were not found in any of the shellfish samples.

3.2 *Guanaqueros and Tongoy bays*

Analyses of phytoplankton samples from Guanaqueros (Fig. 2B) and Tongoy (Fig. 2C) Bays revealed the presence of *Alexandrium* sp. from 6 to 13 June 2006 (winter in the southern hemisphere) with maximum concentrations of 500 and 1500 cell L^{-1}, respectively. Two kinds of *Alexandrium* were detected in both locations. The first type, which was most abundant as single cells, had a cell size of 35.44 ± 0.22 μm long and 35.74 ± 0.27 μm wide. The second type formed short chains of 2 to 4 cells, measuring 30.71 ± 0.48 μm in length and 32.52 ± 0.29 μm in width. Both kinds of cells were usually slightly wider than long.

Argopecten purpuratus samples obtained in the same period from both locations were shown to present PSP toxicity by mouse bioassay from 6 to 22 June 2006. The toxicities observed ranged from 27 to 34 μg STX eq 100 g^{-1} (Table 3).

HPLC analyses of scallops from Guanaqueros Bay indicated the presence of nine PSP toxins (Fig. 3). The toxin profile was dominated by STX (more than 85%) followed by GTX1, GTX2, GTX3, dcGTX2, dcGTX3 and trace levels of GTX5, C1 and C2 (Table 2). Hydrolysis of the samples did not produce any noticeable increase in the concentration of GTXs, STX or neoSTX, indicating that no other sulfocarbamoyl toxins (C3, C4 and GTX6) were present in significant relevant amount. GC toxins were not found in any of the shellfish samples.

Table 3. PSP toxicity (μg STX eq 100 g^{-1} of shellfish) from Guanaqueros Bay and Tongoy Bay as determined by mouse bioassay.

	Guanaqueros Bay	Tongoy Bay
	A. purpuratus	*A. purpuratus*
6 June 2006	31.9	-
7 June 2006	-	34
9 June 2006	31	34.4
16 June 2006	30.4	27.5
22 June 2006	-	31

The toxin profile found in scallops from Tongoy Bay was similar but less complex than the one from Guanaqueros Bay, and it was dominated by STX (more than 95%) followed by GTX2, GTX3, dcGTX2 and trace levels of C1 and C2 (Table 2). It differed from the Guanaqueros Bay profile in that it lacked GTX1, GTX5 and dcGTX3. Again, the hydrolysis of the samples did not produce any noticeable increase in the concentration of GTXs, STX or neoSTX, indicating that no sulfocarbamoyl toxins were present in relevant t amounts. GC toxins were not found in any of the samples.

The presence of STX in scallops from Tongoy Bay was confirmed by HPLC-MS/MS which showed the most characteristic fragments of the m/z 300 molecular ion (m/z 282, m/z 221 and m/z 204) (Fig. 4). Unfortunately, the toxin concentration of GTX and dcGTX detected by HPLC-FD was too low to be detected by the HPLC-MS/MS method.

4 Discussion

4.1 *PSP distribution along the Pacific coast of South America*

The Pacific coast of South America seemed to be free of PSP toxicity at latitudes to the north of 43°S, with the only exception to date being a report of this type of toxicity from Paracas Bay (13° 50' S, 76° 17' W) in Peru (Antinori et al. 2002). Even considering this result from Paracas Bay, there is an area covering the entire subtropical zone from 20 °S to 40 °S (Saavedra-Pellitero et al. 2007) comprising nearly 3000 km, in which no PSP event has been reported. In this work we have unequivocally identified three events of this kind of toxicity from two areas located near the centre of the subtropical zone cited above, which are separated by

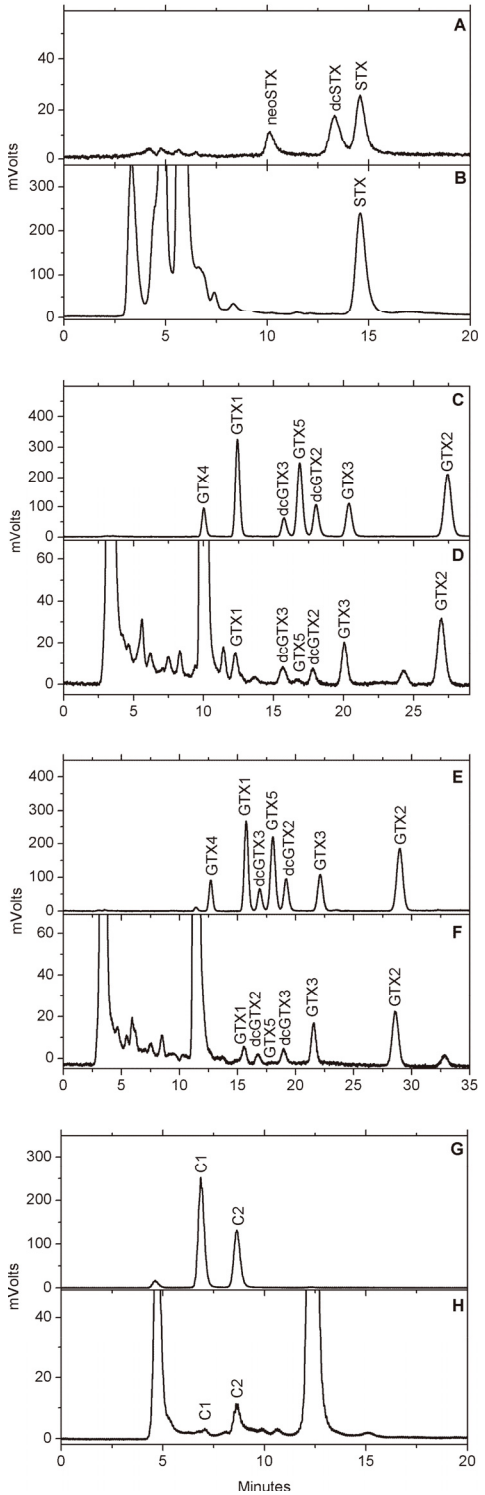

Fig. 3. PSP toxins in *Argopecten purpuratus* from Guanaqueros Bay. (A) Analytical standard for saxitoxin group. (B) *A. purpuratus*, showing only STX. (C) Analytical standard of gonyautoxins group eluted with mobile phase at pH 7. (D) *A. purpuratus* eluted with mobile phase at pH 7, showing GTX2 and GTX3 as major components. (E) Analytical standard for gonyautoxins group eluted with mobile phase at modified pH of 6.7. (F) *A. purpuratus* eluted with mobile phase at modified pH to confirm the absence of GTX4. (G) Analytical standard for C1 and C2 toxins. (H) *A. purpuratus* extract shown trace of C1 and C2 toxins.

about 1000 km. *Semimytilus algosus* and *Argopecten purpuratus* from Mejillones Bay and *Argopecten purpuratus* samples from Guanaqueros and Tongoy Bays have been shown to contain PSP toxins. This suggests that, as along the Pacific coast of North America, PSP toxicity may be widely distributed along the coast of South America.

The toxicities recorded were below the regulatory limit for most countries (80 μg STX eq 100 g^{-1}), and are substantially lower than those from the nearest locations in which this kind of toxicity has been recorded to the north and south. Toxicities of 129 and 221 μg STX eq 100 g^{-1} have been recorded in Peru (Antinori et al. 2002).In southern Chile the levels are certainly much higher as more than 400 people were intoxicated in some outbreaks (Guzmán et al. 2002), suggesting levels above the LOAEL of 800 μg STX-eq kg^{-1}.

4.2 *Toxin profiles and possible causative organisms*

The three observed outbreaks coincide with the timing of *Alexandrium* blooms. However, no phytoplankton samples for toxin analysis were available from Guanaqueros and Tongoy Bays, and no PSP toxins were found at detectable levels in those collected from Mejillones Bay, including the culture started from that population.

The toxin profiles of *A. purpuratus* found in the two southern bays (Guanaqueros and Tongoy) had more than six toxins in common (STX, GTX2, GTX3, dcGTX2, C1 and C2) and were probably caused by the same agent, but from different populations or strains because the differences observed (mainly the presence of GTX1 in one location) cannot be explained by biotransformation or the other toxins present.

The toxin profile in the same bivalve sampled in Mejillones Bay differs fundamentally from the profiles in other bays owing to the absence of STX. Even when STX can be derived from GTX2 by enzymatic desulphonation, this transformation did not seem to take place in *A. purpuratus* as the species had GTX2 but not STX in Mejillones Bay, which would have been the case if the scallops were desulphonating GTX2. Therefore the STX found in the scallops of Tongoy and Guanaqueros Bays must have been acquired from phytoplankton. It would, therefore, seem clear that the species or strain that caused the northern episode was different from that responsible for the southern one.

As far as we know, there are only three records with profiles similar to those found in Mejillones Bay. The first was described by Percy et al. (2002) in *Alexandrium minutum* from the southern coast of the UK; the second case was reported by (Furey et al. 1997) in *Mytilus edulis* and *Crassostrea gigas* following the ingestion of *Alexandrium tamarense* in Cork Harbour, Ireland (although the C2 toxin was consistently absent); and the third case was also related to *Alexandrium tamarens*, in Daya Bay, China (Chan et al. 2006).

No phytoplankton or other shellfish species are known to replicate the unusual toxin profile found in the scallops from Guanaqueros and Tongoy Bays. Nevertheless, the presence of the three main toxins in Tongoy Bay (STX, GTX2 and GTX3) has been associated with several *Alexandrium* species, such as *A. minutum* in the northern Adriatic sea (Honsell et al. 1996), *A. minutum* in the UK (Nascimento et al. 2005), *A. minutum*

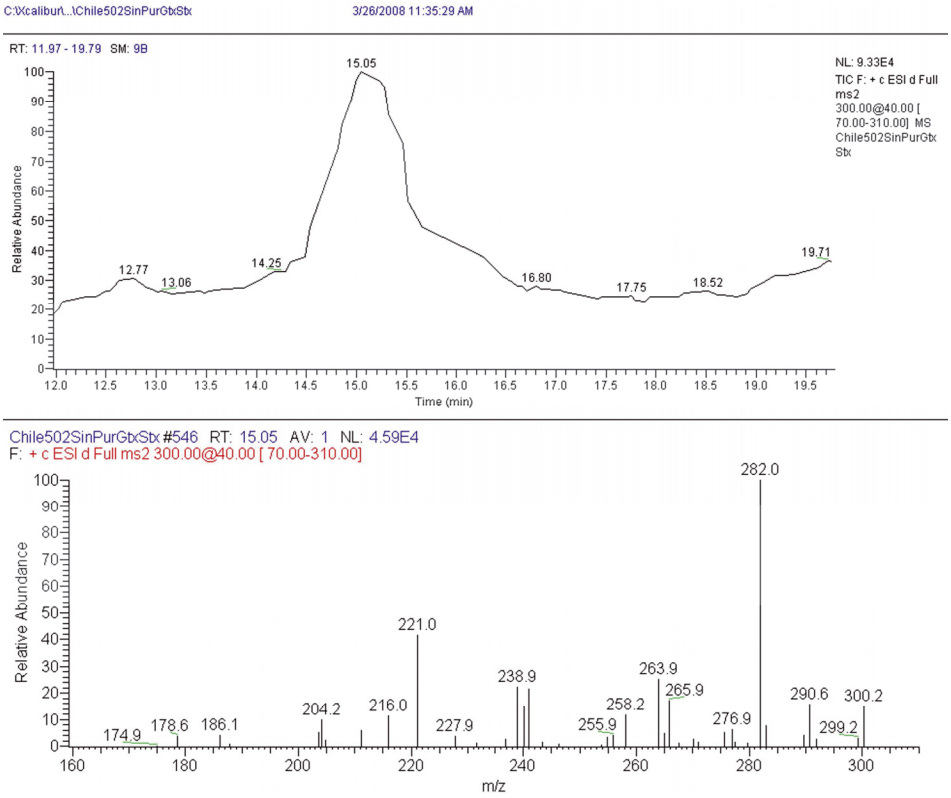

Fig. 4. MS/MS chromatograms and spectra obtained by the method of Dell'Aversano et al. (2005) of *Argopecten purpuratus* from Tongoy Bay showing the characteristic fragments of STX (m/z 282, 221 and 204).

in Denmark (Hansen et al. 2003) and *A. tamarense* in Australia (de Salas et al. 2000; Negri et al. 2003a). Similar profiles were reported in the digestive gland of the mussel *Perna perna* following the ingestion of *A. minutum* in Morocco (Abouabdellah et al. 2008). However, in no case was STX found to be the predominant toxin.

It would therefore seem that PSP toxicity in the area is likely to be produced by different regional populations or strains of the genus *Alexandrium*. In another geographical area of Chile (from 43 to 55 °S) with substantially different oceanographic and biogeographic characteristics (subpolar transitional), PSP toxicity is closely related to *A. catenella* blooms. The toxin profiles found in this study are considerably different from the toxin profiles of species characterized by the presence of high amounts of N-sulfocarbamoyl toxins (C1, C2 and GTX5) and the presence of high proportions of GTX1 and GTX4 (Lagos et al. 1996; Krock et al. 2007). Moreover, the optimal temperatures and salinities for the proliferation of *A. catenella* in south Chile – below 14 °C (Molinet et al. 2003; Navarro et al. 2006) and between 22 to 26 psu, respectively (reviewed in Villanueva 2005) – are not attained in northern Chile. Owing to a combination of the different toxin profiles observed and the unfavorable environmental conditions in northern Chile, it would be unlikely that *A. catenella* was the species responsible for the PSP events.

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