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Occurrence and profiles of lipophilic toxins in shellfish harvested from Argentina

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ABSTRACT

The presence of phytoplankton responsible for the production of lipophilic marine biotoxins is well recognised throughout parts of South America. To date, the quantitation of lipophilic toxins in Argentinean shellfish has been limited to select and highly focussed geographical studies. This work reports the analysis for lipophilic marine biotoxins in shellfish harvested across five regions of Argentina between 1992 and 2012. LC-MS/MS analysis was used for the quantitation of all regulated lipophilic toxins. High concentrations of okadaic acid group toxins were quantified, with a clear dominance of the parent okadaic acid and more than 90% of the toxin present as esters. Results showed DSP toxins in shellfish from the Buenos Aires Province during 2006 and 2007, earlier than previously described. There was also strong evidence linking the presence of okadaic acid to human intoxications. Other lipophilic toxins detected were yessotoxin, pectenotoxin-2 and 13-desMeC spirolide. With evidence published recently for the presence of azaspiracid producers, this work reports the detection of low concentrations of azaspiracid-2 in shellfish. As such the data provides the first published evidence for yessotoxins and azaspiracids in Argentinean shellfish and further evidence for the continuing presence of lipophilic marine toxins in Argentinean waters.

1. Introduction

Lipophilic marine biotoxins comprise groups of naturally-occurring compounds present in a number of specific species of phytoplankton which are known to accumulate through the feeding behaviours of bivalve molluscs. Compounds of interest include the diarrhetic shellfish poisoning (DSP) toxins (okadaic acid (OA), dinophysis toxin-1 and -2 (DTX1 and DTX2), including their ester derivatives (often termed DTX3), the azaspiracids (AZAs), yessotoxins (YTXs), pectenotoxins (PTXs) and a number of cyclic amines including the spirolides (SPXs) and gymnodimine (GYM) (McNabb et al., 2005) which are not yet included in the list of regulated toxins. Human consumption of molluscs containing high enough concentrations of some of these lipophilic toxins is known to cause human sickness. The acute effects of DSP-poisoning are less severe than the effects from other marine biotoxin poisoning syndromes such as paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP), with no known fatalities resulting from intoxication following ingestion of any of the regulated lipophilic toxins (Blanco et al., 2005). However, DSP poisoning can be widespread and highly unpleasant, with symptoms including severe abdominal pain, nausea, vomiting and diarrhoea (EFSA, 2008a). Whilst the OA/DTXs and AZAs are known to result in such symptoms (Yasumoto et al., 1978, 1979, 1980; Murata et al., 1982; Satake et al., 1998), there is little evidence for the toxicity of PTXs and YTXs (Ogino et al., 1997; EFSA, 2008b, 2009a), which were originally included in the DSP class of toxins due to their activity in the DSP Mouse Bioassay (MBA) (Yasumoto et al., 1985, 1987).

Phytoplankton responsible for DSP include *Prorocentrum lima*, and a range of Dinophysis species (Yasumoto et al., 1980; Morton et al., 2009; Reguera et al., 2014). The distribution and frequency of occurrence of harmful algal blooms including this species has appeared to increase throughout the world over recent decades (Van Dolah, 2000; Reguera et al., 2012). Along the Western Atlantic coasts there is evidence for the emergence of Dinophysis blooms and DSP shellfish toxins in North America (Tango et al., 2004; Maranda et al., 2007; Hattenrath-Lehmann et al., 2013) as well as a number of South American countries such as Brazil (Proença et al., 1998; Tavares et al., 2009) and Uruguay (Méndez and Ferrari, 2002),

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although a greater number of reports have originated from Chile (e.g. García et al., 2004a, 2004b; Fux et al., 2011; Reguera et al., 2014).

In the regions of Argentina there have been reports of the occurrence of DSP-producing microalgae as well as episodes of DSP toxin accumulation in shellfish (Van Dolah, 2000; Akselman et al., 2006) including both the northern areas of Buenos Aires province (Sar et al., 2010, 2012) and the southern regions of Patagonia (Gayoso et al., 2002, 2004; Almandoz et al., 2011). Cell concentrations of *P. lima* have been reported as being similar to those levels found in the NW Mediterranean (Gayoso et al., 2004). The earliest report DSP intoxication in Argentinean shellfish consumers originated in March 1999 where over forty people were affected following consumption of two species of mussels (*Aulacomya atra* and *Mytilus edulis*) by toxins originating from *P. lima* (Gayoso et al., 2002). DTX1 was identified in the hydrolysed extracts of samples from both species, with the majority present in esterified form. No OA or DTX2 was reported in either the hydrolysed or unhydrolysed extracts (Gayoso et al., 2002). However, more recently, two species of clams (*Mesodesma mactroides* and *Donax hanleyanus*) harvested from Buenos Aires province were shown to be contaminated with OA and DTX1 together, with both present in free and ester form (Sar et al., 2012). Mussels (*Brachidontes rodriguezii*) and clams (*D. hanleyanus*) harvested from the same region, with the latter associated with human sickness, were found to result in DSP MBA positives. These samples were thought to be associated with both *Dinophysis acuminata* and *Donax caudata* (Sar et al., 2010). Whilst *D. acuminata* has been shown to be associated with other DSP toxins such as the PTX2 and PTX-11 (MacKenzie et al., 2005; Blanco et al., 2007; Kamiyama and Suzuki, 2009; Hackett et al., 2009; Fux et al., 2011; Reguera et al., 2014) these have not been reported in shellfish from Argentina to date. Other lipophilic toxin producers identified in Argentine waters include blooms of *Azadinium spinosum* identified as the producers of the AZAs responsible for Azaspiracid poisoning (AZP). Akselman and Negri (2012) reported the analysis of two blooms of *A. spinosum* collected during 1990 and 1991 respectively from the shelf waters of Northern Argentina. There is therefore the potential for AZA-contamination in Argentinean shellfish, noting the close proximity of the detected blooms to regions where Patagonian scallops are harvested commercially (Ciocco et al., 2006), although no AZA-contaminated shellfish has been reported in Argentinean shellfish to date. Similarly, there have been no reports of YTX-producing dinoflagellates, such as *Protoceratium reticulatum* and *Lingulodinium polyedrum* in Argentinean waters.

For many years the official EU reference method for the regulatory determination of DSP toxins in shellfish has been the qualitative rodent-based assay (Anon, 2004a, 2004b, 2005), typically the mouse bioassay (MBA). Monitoring for lipophilic toxins is currently conducted in Argentina following the EU Harmonised Standard Operating Procedure for detection of Lipophilic toxins by Mouse Bioassay (EURLMB, 2013). Positive results by mouse bioassays implicate the close of harvesting areas, and the ban of commercialization of shellfish from affected areas. . Since July 2011, the EU reference method became an instrumental chemical method utilising liquid chromatography with tandem mass spectrometry (LC-MS/MS). This provides a highly specific and quantitative determination of the full range of regulated lipophilic toxins (LTs), including OA, DTXs, PTXs, YTXs and AZAs, together with the additional non-regulated toxins such as the SPXs and GYM (McNabb et al., 2005). A number of single laboratory validation studies have been published and the method has been validated by the European Union Reference Laboratory for Marine Biotoxins (EURLMB) within its network of National Reference Laboratories (NRLs) and Official Control Laboratories (OCLs).

This objective of this study were to assess the presence of lipophilic toxins in a range of bivalve mollusc shellfish harvested in Argentinean marine waters using the current regulatory LC-MS/MS detection method. Currently the only method available in Argentina for routinely monitoring lipophilic toxins is the DSP MBA, so there is very little data available concerning the presence of lipophilic toxins, particularly for those not detected by the bioassay. The assessment included the analysis of a large range of shellfish species harvested over a period of 20 years with varying geographical sources. The detection of lipophilic marine toxins would provide links to toxic phytoplankton previously reported in Argentinean waters together with evidence for the potential risk to shellfish consumers from lipophilic shellfish toxins.

2. Materials and methods

2.1. Samples

69 samples of shellfish tissue were analysed during this study. These consisted of shellfish tissues which had been stored (< -15 °C) since harvesting and shellfish shucking and represent the only samples that were held in long term storage and available for shipment and testing. Species consisted of mussels (*M. edulis*, *B. rodriguezii* and *Aulacomya ater*), scallops (*Zygochlamys patagonica* and *Aequipecten tehuelchus*), wedge clams (*D. hanleyanus*), yellow clams (*M. mactroides*) and marine snails (*Zidona dufresnei*). Table 1 summarises the samples analysed, showing the specific species, date of harvest and sampling zone. Shellfish were sampled from a wide geographical area, ranging from Ushuaia in the Tierra del Fuego Province, to Buenos Aires Province in the far north (Fig. 1). Samples were harvested over a twenty-year period between 1992 and 2012, with the majority taken from the period between 2006 and 2012 (Table 1).

The wedge clam *D. hanleyanus* and the yellow clam *M. mactroides*, are intertidal species on the Atlantic coast of northern Argentina, which dominate fine to coarse sandy beach communities of the northern Argentinean coast. Whilst commercialisation of these species is prohibited, recreational harvesting is commonplace, especially with tourists during the summer months. Study samples were always harvested from beaches. *B. rodriguezii* is a small bivalve, which grows on rocks and man-made marine structures such as piers. Whilst this shellfish is not usually consumed, it is sampled and used as an indicator species for the presence of toxins in areas where it inhabits. Patagonian scallops (*Z. patagonica*) inhabit the Argentinean continental shelf on and around the shelf front and form large beds of great commercial importance. Factory trawlers catch and process the specimens in order to produce frozen adductor muscle, free from viscera and gonads. Between 7000 and 11,000 tons/year are fished and processed in this way. This fishery was certified by the Marine Stewardship Council in 2006. Samples described in this study were consequently taken from these factory trawlers. Other species include *M. edulis* (mussels), *A. ater* (or *atra*) (mussels) and *A. tehuelchus* (scallops) and were harvested from natural beds along the Argentinean coast. Marine snails were taken as incidental fishing catches, harvested by fisherman trawling for fin-fish around the Mar del Plata coastline. Shellfish samples were collected in using the same species-dependent methods over the entire time period. Frozen homogenised tissues were held in storage until they were shipped to Cefas for LC-MS/MS testing.

2.2. Shellfish extraction and mouse bioassay analysis

Between 1998 and 2002, a DSP bioassay was conducted according to the methods of Yasumoto et al. (1978) and Yasumoto