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Levels of PSP toxins in bivalves exposed to natural blooms of *Alexandrium minutum* in Catalan harbours

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Abstract

The development, validation, comparison and evaluation of analytical methods for marine toxins rely on the availability of toxic material. Within the project JACUMAR PSP, our interest is mainly focused on autochthonous bivalve species with the toxic profile of *Alexandrium minutum*, since this is the principal species involved regionally in PSP outbreaks. Mussels and oysters were exposed during few days in the harbor of Vilanova i la Geltrú, to blooms reaching a maximum *A. minutum* concentration of 200,000 cells L^{-1} in 2008, and 40,000 and 800,000 cells L^{-1}, in 2009. Mussels, oysters and clams were exposed to one bloom of 22,000 cells L^{-1} in the harbor of Cambrils in 2009. In all situations higher toxic levels analyzed by HPLC-FD with postcolumn oxidation were observed in mussels (i.e. 1,200-2,500 µg eq. STX kg^{-1}) than in oysters (i.e. 60-800 µg eq. STX kg^{-1}) exposed to the same bloom. Blooms with higher concentrations of *A. minutum* did not correspond to higher levels of PSP toxins in bivalves. These differences may be explained by differences in *A. minutum* population dynamics, toxin production or in the physiological state or behaviour of shellfish. These results confirm that mussels concentrate more PSP toxins from *A. minutum* than oysters and clams.

Introduction

Marine toxins limit the development of the bivalve aquaculture industry. In order to guarantee the optimal development of the aquaculture industry and to assure the maximum safety level for consumers, monitoring programs for marine toxins need to implement robust and reliable methodologies for the quantification of toxins present in shellfish. Within the Spanish aquaculture framework, paralytic shellfish poisoning toxins, PSP (saxitoxins, STX, and derivatives) that are potentially responsible for serious food-borne intoxications are of particular relevance and are present all along the Spanish coastline.

The project "Comparison of methodologies for the evaluation of Paralytic Shellfish Poisoning (PSP) toxins in bivalves. Application for aquaculture in Spain", funded by Junta Nacional Asesora de Cultivos Marinos (JACUMAR) aims to compare five methods for detecting PSP toxins, applied to samples from Spanish coasts: mouse bioassay (MBA), chromatographic methods (HPLC-FD with pre- or post-column derivatization), Jellett™ Rapid Test kits and cell-based assays (CBA). One of the first difficulties was the obtaining of large amounts of shellfish samples naturally contaminated with PSP toxins produced by any of the two microalgal species mainly involved in toxic outbreaks in Spain, *Gymnodinium catenatum* and *Alexandrium minutum*.

The acquisition of shellfish samples contaminated with PSP toxins produced by *A. minutum* was by exposing mussels (*Mytilus galloprovincialis*), oysters (*Crassostrea gigas*) and, in one occasion,

clams (*Ruditapes* sp.) to blooms which occurred in two Catalan harbours in 2008 and 2009. This activity produced the amounts of samples required for the right performance of the comparison between techniques and participants, as well as data about the contamination of different shellfish species exposed to the same bloom in natural conditions.

Materials and methods

The exposure of shellfish to blooms of *A. minutum* in a harbour was made four times; three in the harbour of Vilanova i la Geltrú (41º12'N 1º44'E), and one in the harbour of Cambrils (41º03'N 1º03'E). In each exposure, shellfish were collected from one of the two Ebro Delta bays, Alfacs Bay or Fangar Bay. The different species of shellfish were suspended at the same point, the same depth and for the same time (Table 1). At the end of the determined time of exposure, all the shellfish were opened by cutting the adductor muscle, edible tissues were completely removed from the shell, and drained in a sieve to remove salt water before homogenization of the pooled individuals of the same species. Samples were then kept frozen (less than -15ºC) until their analysis. Aliquots of 5 ± 0.1 g of the homogenate were accurately weighed into a 50-mL falcon tube. Each aliquot was extracted with 10 mL of HCl 0.1N (double extraction, without heating); 5 mL of the extract were purified with 250 μL trichloroacetic acid 30% (TCA 30%) and the final pH was corrected to 3 with NaOH 1M. The purified extracts were filtered through 0.2 μm before their analysis by HPLC with fluorescent detection (HPLC-FD) with post-column derivatization (Franco and Fernandez Vila, 1993). A sample of seawater was taken at the same point where shellfish were suspended, at the beginning of each exposure, and fixed with buffered formaldehyde for the determination of the density of *A. minutum* by the method of Utermöhl (1958).

Results and discussion

The strategy of exposing shellfish to blooms of *Alexandrium minutum* (densities over 1,000 cells L^{-1}) occurring in small harbours succeeded in the acquisition of large amounts of contaminated shellfish matrixes (Table 2), except in the case of clams. The main PSP toxins were gonyautoxins 1, 2, 3 and 4 (GTX1, GTX2, GTX3 and GTX4); the typical toxic profile produced by *A. minutum*. The ratio of each PSP toxin varied depending on the shellfish species and the level of contamination of the sample. Mussels presented higher levels of PSP toxins than oysters and clams. Nevertheless, the content of PSP toxins did not correlate to the densities of *A. minutum*. It can be observed that the highest concentration of PSP toxins (exposure in Vilanova i la Geltrú on February 2009; the density of *A. minutum* was 39,840 cells L^{-1}) did not relate to the highest concentration of *A. minutum* (exposure in Vilanova i la Geltrú on March 2009; the toxin content in mussels was 1,256.9 μg eq. STX kg^{-1} flesh). This can be explained by the aim of this strategy. The main objective was the acquisition of large amounts of contaminated shellfish, thus, we lack of additional data for explaining the differences found between experiences. We can hypothesize explanations such as: differences in *A. minutum* population dynamics, differences in the toxin production of populations of *A. minutum* geographically separated or blooming at different seasons of the year, differences in the physiological state or filtering behavior of shellfish, differences in the hydrodynamics of the harbour, etc. It can be even a sampling bias, because we have a single estimate of *A. minutum* concentration for each exposure so we do not know to what concentration of *A. minutum* were exposed the shellfish all the time.

Conclusions

The exposure of shellfish to natural blooms of *A. minutum* in harbours is a good strategy for the production of naturally contaminated samples, useful for the development and validation of analytical methods or to be used as reference material. Mussels accumulate more PSP toxins produced by *A. minutum* than oysters and clams under the same exposure conditions.

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Table 1. Exposures of shellfish to natural blooms of *A. minutum* in two Catalan harbours during 2008 and 2009.

Date	Shellfish origin	Shellfish species	Harbour	Days of exposure
April 2008	Alfacs bay	Mussels Oysters	Vilanova i la Geltrú	4
February 2009	Alfacs bay	Mussels Oysters	Vilanova i la Geltrú	6
March 2009	Fangar bay	Mussels Oysters	Vilanova i la Geltrú	3
May 2009	Fangar bay	Mussels Oysters Clams	Cambrils	8

Table 2. Results obtained in each exposure. Note that the percentages of PSP toxins (GTX 1, 2, 3 & 4) are the proportion of each compound in the total concentration of the extract, not their contribution to the total toxicity (μg eq. STX kg^{-1} flesh).

Date	Harbour	*A. minutum* density (cells L^{-1})	Shellfish species	Toxic flesh (g)	μg eq. STX kg^{-1} flesh	%GTX1	%GTX4	%GTX2	%GTX3
April 2008	Vilanova i la Geltrú	200,000	Mussels Oysters	1,000 100	1,277.5 1,043.0	26 44	67 42	3 5	4 5
February 2009	Vilanova i la Geltrú	39, 840	Mussels Oysters	1,000 100	2,702.7 849.1	34 29	37 44	27 25	2 2
March 2009	Vilanova i la Geltrú	817, 550	Mussels Oysters	1,000 250	1,256.9 30.5	42 0	27 50	27 50	1 0
May 2009	Cambrils	21,995	Mussels Oysters Clams	1,200 600 250	45.9 1.5 n.d.	0 0 0	100 100 0	0 0 0	0 0 0