Bull Environ Contam Toxicol (2009) 82:660–664
DOI 10.1007/s00128-009-9703-y

First Evidence of Okadaic Acid in *Mytilus galloprovincialis* Mussels, Collected in a Mediterranean Lagoon, Tunisia

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Received: 30 December 2008 / Accepted: 26 February 2009 / Published online: 17 March 2009

Abstract Evidence of the presence of okadaic acid (OA) and its monthly fluctuations are reported for the first time in *Mytilus galloprovincialis* samples collected from June 2005 to May 2006 in the Bizerte Lagoon. All of the samples with the exception of those taken in August 2005 were found to be contaminated. The level of OA in mussels exceeded the regulatory limit of DSP toxins (16 μg OA/100 g mussel meat) within the European Union (2002) in only two cases: in January and February, 2006 with 31.85 ± 3.06, and 18.86 ± 1.88 μg OA/100 g mussel meat respectively. During the investigative period, potentially toxic dinoflagellates were observed. The results reported here, although based on limited sampling, demonstrate for the first time the existence of a risk to public health from diarrheic shellfish poisoning in cultivated bivalve mollusc in Tunisia.

Keywords DSP · *Mytilus galloprovincialis* · Okadaic acid · Bizerte Lagoon · Tunisia

Diarrheic shellfish poisoning (DSP) is a serious and globally widespread toxic syndrome caused by the consumption of shellfish contaminated with algal toxins produced by marine dinoflagellates. Although rarely life-threatening, DSP causes incapacitating diarrhea and vomiting with no known medical treatments. DSP is associated with the polycyclic ether toxins okadaic acid, dinophysistoxins, and pectenotoxins (Bowden 2006). The most important toxins involved in DSP are represented by the okadaic acid (OA) and dinophysistoxins (DTX1 and DTX2) (Yasumoto and Murata 1993). They are produced mainly by dinoflagellates such as *Dinophysis* spp. and *Prorocentrum* spp. (Lee et al. 1989). Chemical methods for direct DSP toxin detection such as high-performance liquid chromatography (HPLC) or liquid chromatography coupled with mass spectrometry (LC/MS) (Quilliam 1995) have been shown to be valuable methods of analysis. The ability of OA-type toxins to inhibit certain serine/threonine protein phosphatases (Honkanen et al. 1994) led to the development of colorimetric assays using *p*-nitrophenyl phosphate (*p*-NPP) as substrate (Tubaro et al. 1996). The main purpose of this study is to show the first evidence of okadaic acid presence and the monthly fluctuations of OA-like toxins in mussels collected in the Bizerte Lagoon, Tunisia. The PP2A inhibition assay using *p*-NPP as substrate, and HPLC methods using ADAM (9-anthryldiazomethane) were used to quantify the diarrheic OA-like toxins and the OA in mussels, respectively.

Materials and Methods

The Bizerte Lagoon (37°8′–37°14′N, 9°48′–9°56′E) is a semi enclosed area located on the northern coast of Tunisia. It has a surface area of 150 km^2. Exchanges with the Mediterranean Sea occur through the 7-km long channel. During the period of study (June 2005 to May 2006), phytoplankton samples were harvested from the water surface of the Lagoon and were fixed with formaldehyde at 1% (v/v) final concentration until analysis. The

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dinoflagellate species were identified using universally accepted taxonomic keys based specifically on the morphology and the valve description of the cells (Sournia 1986). During the same period, samples of 3 kg of mussels (*Mytilus galloprovincialis*) were collected monthly at the same site at 3 m depth and stored at −20°C prior to extraction.

For the extraction of phycotoxins, an aliquot of 100 g of flesh mussel tissue from each sample was homogenized and extracted in triplicate with 100 mL of aqueous methanol. Each extract was then clarified by filtration through a glass, microfiber filter. The process of homogenisation, extraction and filtration was repeated two additional times with fresh extraction solvent. For each sample, the three filtrates generated were pooled and partitioned successively using n-hexane and dichloromethane. Each dichloromethane extract was then evaporated and the residue reconstituted in methanol. All crude methanolic extracts were then analyzed with PP2A inhibition assays, HPLC-fluroescence detection and LC/MS techniques.

PP2A was used according to Tubaro et al. (1996) with minor modifications. Briefly, the PP2A activity was determined by measuring coloration associated with the formation at 37°C of *p*-NP from the substrate *p*-NPP using a microtiter plate reader. The *p*-NPP (80 mM) was dissolved in a buffer containing 40 mM Tris–HCl, 34 mM $MgCl_2$, 4 mM EDTA and 4 mM Dithiothreitol, pH 8.3. The PP2A was diluted in the same buffer, and supplemented with 0.5 mg mL^{-1} bovine serum albumin (BSA). The assay was then performed by adding 100 µL of the sample solution to 50 µL of the enzyme solution in a 96-well microtiter plate. After incubating at 37°C for 5 min, 50 µL of substrate were added. The rate of *p*-NP production was measured after 1 h at 405 nm. All assays were conducted in triplicate. The 'toxin equivalent' concentration in each extract (OA equivalent/100 g fresh mussel weight) was determined from a standard graph of the inhibition of PP2A by pure OA (Sigma) with an IC_{50} of about 320 pg mL^{-1}.

The derivatization of okadaic acid standard and mussel sample was carried out according to Lee et al. (1987). Briefly, an aliquot of dichloromethane mussel extract residues or okadaic acid standard (0–80 ng) was treated with 100 µL of a freshly prepared solution of 0.1% 9-anthryldiazomethane (ADAM) (in methanol). After 1 h at 25°C in the dark, the sample was evaporated and the residue was diluted in 500 µL of n-hexane/chloroform, 1:1 (v/v) and then transferred into 500 mg Silica gel Sep Pak cartridge. The system was washed successively with 5 mL of n-hexane/chloroform, 1:1 (v/v) and 5 mL of chloroform. Finally, the toxin derivatives were eluted with 5 mL of chloroform/methanol, 9.5:0.5 (v/v). The last fraction was evaporated, dissolved in 200 µL of methanol and then 10 µL was injected and analyzed by HPLC with fluorescent on-line detection. The separation of toxin derivatives was carried out on a reversed-phase C18 column (150 × 4.6 mm; 3.5 µm; Kromasil) using a mobile phase consisting of acetonitrile/methanol/water (70/20/10, v/v) at a flow rate of 1.2 mL min^{-1}. The excitation and emission wavelengths were set at 365 and 415 nm respectively. Peaks in the resulting chromatograms were identified by comparison with the retention time of okadaic acid analytical standard. The okadaic acid concentration in each flesh mussel tissue was estimated from a calibration curve of okadaic acid standard (0–80 ng).

To confirm the presence of okadaic acid in mussel extracts, LC/MS techniques was carried out on an Ion Trap LC/MS system equipped with a binary solvent pump coupled to an analytical work station. The dichloromethane extract of mussel collected in January 2006 was then analyzed on a reversed-phase C18 column (150 × 4.60 mm; 3.5; Kromasil) using acetonitrile/methanol/water (70:20:10, v/v/v) containing 0.05% acetic acid, at a flow rate of 0.5 mL min^{-1}. The ES/MS interface in negative mode operated at 350°C gas temperature, 8 L min^{-1} drying gas flow, 40,000 Pa nebuliser gas pressure and capillary voltage of 4,000 V. Mass spectrum were obtained by scanning from m/z 500 to 1,000 with a scan time of 0.75 s. For quantification of OA in mussel sample, data acquisition was performed in selected-ion-monitoring (SIM) mode in negative ion by selecting the $[M–H]^-$ ion, at m/z 803.7.

Results and Discussion

The microscopic examination of the phytoplankton samples during the period of investigation (June 2005 to May 2006) showed the presence of five potentially toxic dinoflagellate species such as *Dinophysis caudata, D. sacculus* and *D. acuminate, P. minimum* and *Prorocentrum lima*, in the Bizerte Lagoon. The benthic species, *P. lima* was observed at higher cell density (80; 100 cells L^{-1}) only in August and October 2005 (Fig. 1a). The species *P. minimum* was the predominant species in the seawater of the Bizerte Lagoon with cell densities intensifying in December 2005 (1×10^5 cells L^{-1}). Alternately, the planktonic species *Dinophysis caudata* was observed only in October 2005 and January 2006 at low cell density (<50 cells L^{-1}). The species *D. acuminata* was observed during the study period only at low cell density (<50 cells L^{-1}). However, the species *D. sacculus* was most frequently observed at low cell density (<50 cells L^{-1}) in June, July, September, October 2005, as well as March and April 2006; at middle cell density (80–120 cells L^{-1}) in August and December 2005; and reaching a high cell density (780 cells L^{-1}) in

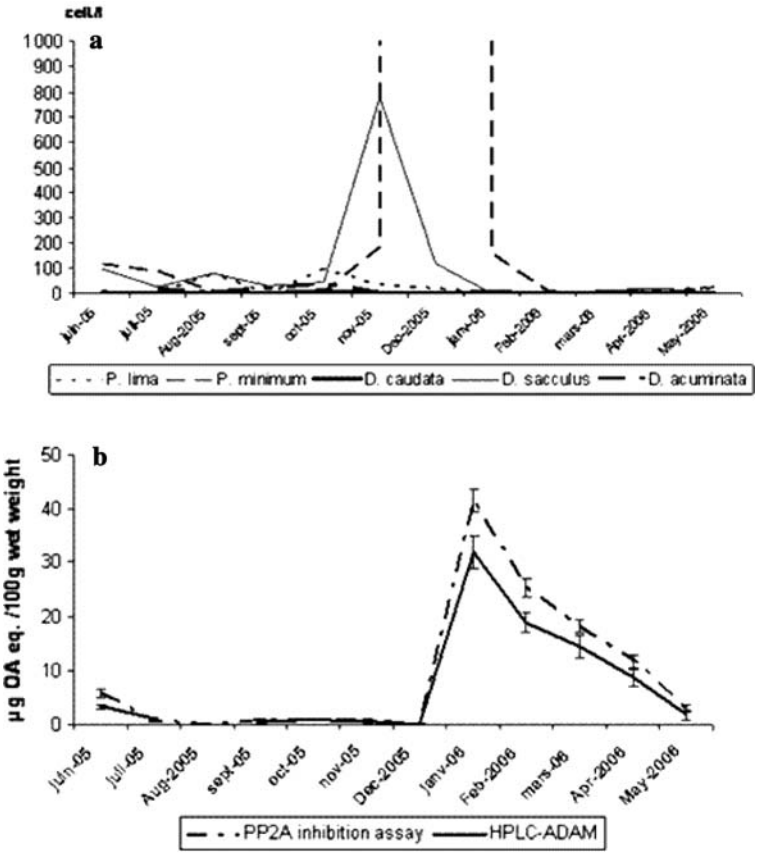

Fig. 1 Presentation of monthly fluctuations data of potentially toxic dinoflagellate collected from Bizerte's Lagoon (**a**) and monthly fluctuations of okadaic acid and okadaic acid-like toxins contents in mussel (*M. galloprovincialis*) samples collected in the Bizerte Lagoon from June 2005 to May 2006 and determined by the HPLC-ADAM method and the PP2A assay, respectively. Each value is the mean ± SD of three replicates (**b**)

November 2005. The first occurrence of dinoflagellate phycotoxins in Tunisian shellfish was reported by Biré et al. (2002), who detected the neurotoxic gymnodimine in clams harvested in the south of Tunisia. In this study, we detected for the first time OA in mussels originating from the Bizerte Lagoon, where Tunisian shellfish production farms are located. Although shellfish toxicity, with its associated human impact, has been attributed to *P. minimum* blooms from a variety of coastal environments, only clones isolated from the Mediterranean coast of France and shellfish exposed to *P. minimum* blooms in this area have been shown to contain a water soluble neurotoxic component which killed mice (Denardou-Queneherve et al. 1999). Therefore, the OA detected in the mussel samples from this Lagoon was produced by *D. sacculus* that occurred in the seawater of the Lagoon in November 2005 and which is known in the literature as DSP toxins producers (Camacho et al. 2007). However, it should be noted that the species *P. lima*, *D. caudata*, and *D. acuminata* occurred between October and December 2005 with low cell density (10–100 cells L^{-1}); only *D. sacculus* occurred at high cell densities (780 cells L^{-1}) in November and it may therefore be the origin of mussel DSP-toxicity.

The OA-like toxins and the OA concentrations in mussel samples were analyzed by the PP2A inhibition assay and by the HPLC respectively. The seasonal fluctuations of the OA-like toxins and the OA concentrations in the mussel samples are shown in Fig. 1b. Measurable toxin levels were detected in all the samples examined except in August 2005. During the 1 year monitoring program from June 2005 to May 2006, the OA equivalent concentrations calculated using the PP2A assay ranged from 0.11 to 41.70 µg/100 g wet weight mussel meat (Fig. 1b). The peak value was observed in January 2006, with a concentration of 41.70 ± 2.07 µg OA equivalents/100 g wet weight. Moreover, the OA concentrations calculated using the HPLC-ADAM method were in the same range, and varying from 0.12 to 31.85 µg/100 g wet weight (Fig. 1b) with a peak observed during the same period in January 2006. Generally a good correlation was obtained between OA determined by the PP2A method and the HPLC-ADAM method (Mountfort et al. 2001). However, in this study the concentrations of OA for some mussel samples were underestimated by the HPLC-ADAM method compared to the PP2A assay as indicated in Fig. 1b. It is likely that the PP2A method overestimated the toxin concentration due to the ability of this assay to detect a broad range of OA homologues (dinophysistoxins) present at low concentrations in the mussel samples.

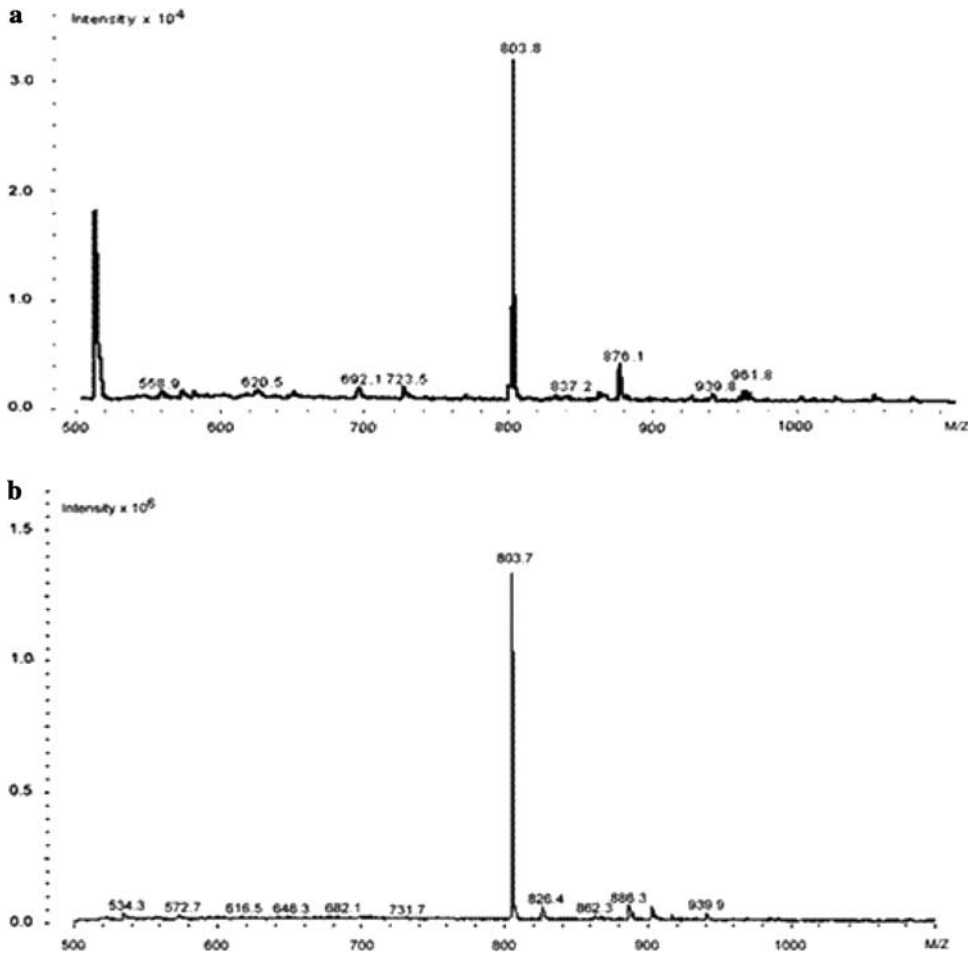

Fig. 2 LC–MS analyzes in negative selected ion monitoring (*SIM*) mode of **a** *Mytilus galloprovincialis* samples extract (January 2006) and **b** okadaic acid (*OA*) standard (50 μg). The mussel sample extract (**a**) exhibit one peak at retention time (7.50 min) and parent ion ($[M–H]^-$: 803.8) similar to those of okadaic acid standard (7.53 min; $[M–H]^-$: 803.7). The mass spectra range from m/z 500 to 1,000

The LC/MS analysis of the mussel extract harvested in January 2006, in which the highest OA equivalent concentration was observed using the PP2A assay and the HPLC-ADAM method, is shown in Fig. 2a. The mass spectrum of this sample revealed the presence of one peak assigned to OA on the basis of the presence of the parent fragment ion $[M–H]^-$ m/z 803.8 that is known to be indicative of this toxin in the standard mass spectrum (Fig. 2b). The concentration of OA in this mussel sample quantified using the LC/MS technique (33.45 ± 2.76 μg OA/100 g wet weight) and the HPLC-ADAM method (31.85 ± 3.06 μg OA/100 g mussel meat) consistently indicated that OA was the predominant toxin contained in the samples studied. In this study, the peak of OA in mussel samples from the Bizerte Lagoon followed shortly after the occurrence of *D. sacculus* in November 2005 (Fig. 1), implying their involvement as the source of okadaic acid contamination of mussels in this Lagoon. Indeed, Giacobbe et al. (2000) demonstrated the toxicity of the dinoflagellate *Dinophysis sacculus.* We observed also that after January 2006 OA levels of mussel samples in the Bizerte Lagoon began a steady decline, and by May 2006 concentration decreased to 5% of initial value. This can be explained by the slow depuration of toxins from shellfish. Although the concentrations of OA in the mussel samples (except in January and February) are under the European safe limit, vigilance is recommended as the damage caused to humans by repeated consumption of low doses of OA-like toxins probably occurs more frequently and poses a more serious threat to human health than acute intoxication since these toxins are considered as tumor promoters (Suganuma et al. 1988).

No human shellfish-related intoxications were reported during the occurrence of *D. sacculus* (November 2005) and the bloom event (December 2005), during which harvesting of bivalve shellfish was banned for 2 months. In this instance the national monitoring program, which has been in effect since 1995 in the areas of bivalve mollusc production in Tunisia, provided an early warning system to protect public health. However, the results reported here, although based on limited sampling, demonstrate for the first time that shellfish, particularly mussels, collected 2 months after the occurrence of *D. sacculus* in the Bizerte Lagoon may present a public health risk due to the presence in January 2006 of okadaic acid at a concentration (31.85 ± 3.06 μg OA/100 g shellfish meat) 2-fold the

European regulatory limit (16 μg OA/100 g shellfish meat) within the European Union (2002).

Acknowledgments We thank Audrey Solgadi (IFR141, SAMM service, University Paris-Sud 11, UFR Pharmacy) for LC/MS analysis. We are grateful to the critical comments of the referees and the help of Mrs S. Ghouti (University of Paris-Sud 11, UFR Pharmacy, the Language Department,) for critical reading of the manuscript, and to Mr. Nathanael Massey for final reading and editing. This study was supported by a fellowship given to Imène Kacem by the Ministry of higher education and scientific research, Tunisia.

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