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Using the radioligand-receptor binding assay for paralytic shellfish toxins: A case study on shellfish from Morocco

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ABSTRACT

Paralytic shellfish poisoning (PSP) events occur regularly along the Mediterranean and Atlantic coast of Morocco, and have been responsible for several severe cases of human intoxication. Along the southern Atlantic coast of Morocco, aquaculture and intensive artisanal fishing practices have recently been particularly heavily impacted, and toxic species have been observed in increasing intensity and frequency. In the 1990's a regulatory monitoring program was established for the coastal waters off Morocco by the National Institute of Fisheries Research (INRH), to reduce the risk of intoxication with biotoxins. The regulatory monitoring is conducted weekly and includes toxic phytoplankton enumeration and identification, as well as saxitoxin (STX) analysis in seafood using the mouse bioassay (MBA). Animal testing remains the most widely used screening method for PSP toxin detection, yet its use is being reconsidered for animal-related ethical issues, as well as for practical considerations. To be able to better evaluate alternatives to animal testing, the performance of a nuclear-based radioligand-receptor binding assay (RBA) for paralytic shellfish toxins was assessed and compared with the MBA using four commercially important shellfish matrices, including cockles *Cerastoderma edule*, razor shells *Solen marginatus*, oysters *Crassostrea gigas,* and mussels *Perna perna*.

Over 50 samples were collected and analysed as part of the regulatory monitoring framework including a suite of monthly samples from 2017 and all samples identified as toxic by MBA since 2011. Testing of reference material and evaluation of assay-critical parameters (e.g. slope of calibration curve, internal quality control QC and IC50) confirmed the robustness of the RBA methodology. With this RBA method, STX-like activity detected in shellfish samples ranged from 33 to 8500 µg STX equivalents per kg. RBA data were significantly correlated ($P < 0.0001$, Pearson $r = 0.96$) with the MBA-derived dataset. Importantly, the RBA method allowed for the detection and quantification of PSP toxins at levels not detectable by using the mouse bioassay. The limits of quantification of the RBA was calculated and found to be 10-fold lower than that of the MBA, respectively 35.24 ± 5.99 and 325 µg STX equivalents per kg of tissue. In addition, the RBA was easier to use and produced reliable results more rapidly than the MBA and without use of live animals.

Considering the increasing risks associated with harmful algal blooms, globally and in Morocco, together with the increased development of aquaculture production and seafood consumption and the difficulties of live animal testing, these findings indicate that the RBA method is a reliable and effective alternative to the MBA method.

1. Introduction

Harmful algal blooms (HABs) can have a serious impact on public health and can cause catastrophic losses to affected shellfish industries around the world (Noguchi, 2003), including in the coastal waters off Morocco. Paralytic shellfish poisoning (PSP), the most reported HAB event in Morocco, is a seafood poisoning caused by the consumption of shellfish contaminated with Saxitoxins (STXs) (Van Dolah et al., 2012) also known as paralytic shellfish toxins (PSTs). The STX family is a group of neurotoxins produced by certain species of harmful algae

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(genera) *Alexandrium, Gymnodidinium* and *Pyrodinium* (Orr et al., 2011; Pearson et al., 2010). They comprise a group of more than 50 analogues of STX (Wiese et al., 2010), but with different groups or side chains (R1-R5) (Llewellyn, 2006). At least 18 of these congeners have toxic relevancy (FAO/WHO, 2016). PSTs have been found in various marine organisms, and accumulate particularly in mussels, oysters and clams, but also in puffer fish or marine snails (Pitcher et al., 2001). PSP detection methods have been developed and validated for regulatory monitoring of seafood, including animal testing (mouse bioassay, MBA) and non-animal analytical and *in vitro* bioassays. The long-standing regulatory method for PSP toxins remains the AOAC mouse bioassay (MBA) [AOAC 959.08], with a regulatory limit of 800 μg equiv per kg of shellfish tissue. In Morocco the animal-based biotoxins surveillance program established since 1990 by the National Institute of Fisheries Research (INRH) has been very effective in protecting public health and in minimizing the number of coastal water closures for shellfish harvesting. Nonetheless, increasing resistance to whole animal testing and low sensitivity has led to the need to develop alternative methods to improve the surveillance or meet regulatory requirements.

Recently, with the technical support from the International Atomic Energy Agency (IAEA), Morocco has implemented the receptor binding assay (RBA) to estimate the concentrations of PSP toxins in fish products. This method is based on the competitive binding for a finite number of receptor sites in a rat membrane tissue homogenate, between PSTs in the shellfish tissue and a radio-labelled STX (^{3}H-STX) (VanDolah et al., 1994, 2009, 2012; Doucette et al., 1997; Dechraoui Bottein and Clausing, 2017). Since all PST analogues bind to site 1 of the voltage-gated Sodium channels and the binding affinity is proportional to potency, this makes the RBA method a very specific and accurate activity-based technique for the detection of PSTs (Ruberu et al., 2003). The assay can be performed in a 96-well plate format allowing for simultaneous testing of numerous samples. The RBA method is very sensitive, easy, simple, fast and cost-effective compared to other tests for PST analysis. In 2012, the STX-RBA was adopted as an AOAC Official Method of Analysis (OMA, 2011.27), however its applicability to shellfish species and toxin profiles from Atlantic coastline has not yet been assessed.

The present study examines the overall performance of the RBA method for PSP and its application on four shellfish species matrices collected from the southern Atlantic coast of Morocco, mussels (*Perna perna*), razor shells (*Solen marginatus*), cockels (*Cerastoderma edule*) and oysters (*Crassostrea gigas*) to quantitatively evaluate the effectiveness and reliability of this method.

2. Materials and methods

2.1. Sampling methodology

Four shellfish species were collected from January 2011 to December 2017 in two primary regions along the south Atlantic Moroccan coast as part of the seafood safety regulatory program; mussels *Perna perna* were collected from the Agadir coastal area while Razor shells *Solen marginatus*, cockels *Cerastoderma edule and* oysters *Crassostrea gigas* were collected from the Dakhla region (Fig. 1). Each sample consisted of 3 kg of shellfish, collected at low tide and kept in coolers during transport to the laboratory.

As part of the regulatory monitoring, shellfish samples analysis along Agadir coast are complemented with weekly surveillance of the algae responsible for *in situ* toxin production. Water samples are taken at high tide (± 2 h of peak) using 1 L Nalgene bottles and kept in a cooler during the transport to the laboratory. Phytoplankton identification is carried out on preserved samples using an inverted microscope following Utermöhl method (Lund et al., 1958). Cell densities, expressed on number of cells per litre for 2017 in Agadir are reported on Fig. 1.

2.2. Toxin analysis

2.2.1. Shellfish tissue extraction

Shellfish extracts were prepared following the AOAC mouse bioassay extraction protocol for the MBA and RBA methods (AOAC 959.08 and AOAC OMA, 2011.27). Shellfish samples were homogenized and an aliquote of 100 g ± 1 was extracted in 100 mL ± 1 of 0.1 M HCl. After heating and boiling for 5 min, pH was adjusted between 3 and 4. The exctract was centrifuged (3000 g for 5 min) and the supernatent directly used for toxin analysis by mouse bioassay. Remaining sample extracts tested positive by MBA between 2011 and 2016, and all extracts from monthly samples collected in 2017 were stored at −80 °C until analysis by the RBA method according to Van Dolah et al. (2009, 2012).

2.2.2. Mousse bioassay (MBA)

The MBA procedure was carried out following AOAC official method 959.08 (1995). Three mice (20 ± 2 g) were injected with 1 mL of shellfish extract. The method was standardised using a certified reference standard of STX obtained from the National Research Council Canada (IMB, NRCC, Halifax, Nova Scotia, Canada). Toxicity was correlated with death time and converted to μg STXequiv per kg of shellfish tissue.

2.2.3. Receptor binding assay (RBA)

The RBA procedure requires the use of specific reagents and a radioactive tracer (^{3}H-STX). MOst of these reagents are included in a commercially available kit (American Radiolabeled Chemicals Inc) consisting of 50 μCi of ^{3}H-STX in methanol with a specific activity 20.0 Ci mmol^{-1}, a reference standard consisting of STX dihydrochloride at 268.8 μM (100 μgmL^{-1} in 20% ethanol-water at pH 3.5) and receptors prepared from porcine brain homogenates. A buffer solution of pH 7.4 is prepared with morpholinopropanesulfonic acid (MOPS) and aqueous choline chloride, adjusted to pH 7.4 with 3N NaOH (stored at 4 °C for its use within a period not exceeding one month).

The determination of PSTs by RBA was carried out according to the protocol of Van Dolah et al. (2012), (AOAC OMA, 2011–27), with a single modification consisting of the use of porcine membrane instead of rat membrane as STX receptors.

Prior to undertaking the assay, STX standard solutions were prepared to establish a calibration curve; a set of dilutions at different concentrations of the unlabeled STX (Table 1) was prepared in 0.003 M HCl medium in a range from 10^{-6} M to 10^{-10} M.

For quality control purposes, a reference standard containing 1.8×10^{-8} M STX (3.0×10^{-9} M STX in assay) in 0.003 M HCl, aliquotted in 1 mL volumes, and stored at −80 °C, was prepared for routine use within a year. A 15 nM working stock of ^{3}H-STX was freshly prepared daily by mixing 24 μL of tritiated-STX with 100 mM MOPS/100 mM choline chloride buffer up to 3.976 mL (corresponding to 2.5 nM final in-assay concentration). Prior to running the assay, the total radioactivity counts of each working stock were checked using a low background scintillation counter (Quantullus Wallac 1220). The measured day-to-day counts per minutes (cpm) varied within less than 15% of the expected activity (Van Dolah et al., 2012).

Assay quality control was also verified using a reference material of Oyster *Crassostera gigas*and Mussels *Mytilus edulis*and *galloprovincialis* from Marine Istitute Ireland (WEPAL Quasimeme).

The receptors from porcine brain were prepared daily by thoroughly vortexing 2 mL of the preparation with 18 mL MOPS. The obtained mixture was then kept at 4 °C until use.

Sample extracts (prepared at 0.5 g tissue equivalent/mL) were first tested at three dilutions in triplicate (i.e. dilutions by 10, 50 and 200). The RBA procedure involved the initial addition of 35 μL of buffer (MOPS/choline chloride) to a 96-well MultiScreen HTS (MerkMillipore, part # MSFBN6B50), followed by the addition of 35 μL of unknown sample (or STX diHCl standard or QC check), 35 μL of ^{3}H-STX, and