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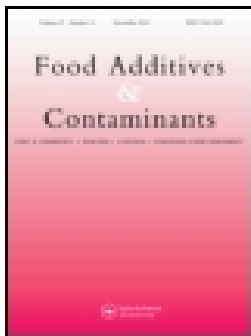Langlois, Gregg W
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Receptor Binding Assay for the Detection of Paralytic Shellfish Poisoning Toxins: Comparison to the Mouse Bioassay and Applicability under Regulatory Use

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Receptor Binding Assay for the Detection of Paralytic Shellfish Poisoning Toxins: Comparison to MBA and Applicability under Regulatory Us

The receptor-binding assay (RBA) method for the detection of paralytic shellfish poisoning (PSP) toxins was evaluated for its overall performance in comparison with the mouse bioassay (MBA). An initial study to evaluate the effects of filtering shellfish extracts prior to running the RBA indicated no significant difference between filtered and unfiltered extracts on the determined saxitoxin concentrations. Next, we tested the RBA assay on 295 naturally contaminated mussel tissue samples, ranging in concentrations from 320 µg STX equiv. kg^{-1} to 13,000 µg STX equiv. kg^{-1} by MBA. An overall trend was observed with the RBA giving higher results (256 µg STX equiv. kg^{-1} on average) than the MBA; however at low concentrations (< 500 µg STX equiv. kg^{-1}) the RBA results were marginally lower. A third study was conducted using spiked mussel tissue analysed by three independent laboratories, two of which performed the RBA and one the MBA. This multi-laboratory study again showed the RBA to give higher results than the MBA; however, it also revealed that STX determination was accurate by the RBA, unlike the MBA. To optimize the assay for efficient usage under regulatory practice, three suggestions have been made: the use of an initial screening plate to separate those samples that exceed the alert level; use of rapid PSP test kits in the field and in the laboratory for screening negative samples and for early detection of toxicity; and use of an alternate commercially-available porcine membrane in place of the laboratory-prepared rat membrane homogenate. The large number of samples analysed and the diversity of the tests conducted in this study further supports the RBA as an affordable rapid method for STX detection that is also free of the routine sacrifice of live animals.

Introduction

Saxitoxins (STX) are trialkyl tetrahydropurines with more than 30 naturally occurring congeners, and are highly potent blockers of the voltage-gated sodium channels that are present in neuronal cell membranes. Human consumption of shellfish contaminated with STX and its numerous analogs causes Paralytic Shellfish Poisoning (PSP), which has resulted in a number of illnesses and deaths in California historically. Although there is no antidote for PSP, full recovery can be achieved by placing the victim on a mechanical ventilator until the toxins are excreted. The biological half-life of STX is approximately 90 minutes, with survival chances increasing significantly after 12 hours from the initial exposure (Pearson et al. 2010). These naturally occurring and highly potent neurotoxins are produced by microscopic algae (e.g., the dinoflagellate *Alexandrium spp.*) along the California coast, which can accumulate in filter-feeding molluscan shellfish such as mussels, clams, oysters, and scallops.

To protect the public from PSP, the California Department of Public Health (CDPH) initiated a PSP monitoring program in response to a large-scale outbreak in 1927. The current CDPH Marine Biotoxin Monitoring Program (MBMP) consists of program participants from county health departments and other local, state, federal, tribal, and academic organizations, shellfish aquaculture companies, and a growing number of citizen volunteers, who collect shellfish and phytoplankton samples at numerous points along the California coastline. Shellfish samples, predominantly sea mussels (*Mytilus californianus*), are immediately frozen, and then shipped to the CDPH laboratory campus in Richmond for toxin testing. This monitoring data is used to establish commercial shellfish harvesting closures and to issue health advisories and quarantines for recreational harvesting areas.

The PSP test method currently used at the CDPH Microbial Diseases Laboratory

(MDL) is the AOAC-approved Mouse Bioassay (MBA), an *in vivo* test that has until

recently been the only approved method under the National Shellfish Sanitation

Program (NSSP). In the MBA method, 19-21 gram mice are injected with 1 mL of an

acid extract of the shellfish and the time of death (i.e. from inoculation to last gasping

breath) is recorded. CDPH-MDL has previously reported on the comparison of a

number of different promising screening assays for the PSP toxins (Inami et al. 2004).

This work led to the adoption of a commercially available qualitative lateral flow

immunoassay (Scotia® Rapid Testing Systems Ltd.) (Jellett et al. 2002) for screening

incoming shellfish samples. This qualitative test was approved by the Interstate

Shellfish Sanitation Conference (ISSC 2013) and incorporated into the NSSP as a

screening tool in 2005. Although use of this screening method has significantly reduced

the number of animals sacrificed (Oshiro et al. 2006), it is still necessary to have

positive samples tested by MBA for quantitation, which is needed for regulatory

decision-making.

The receptor binding assay (RBA) does not use live animals and is based on the

competitive binding for a finite number of receptor sites in a rat membrane tissue

homogenate between PSP toxins in the shellfish tissue and added radiolabeled STX (^{3}H-

STX), (Hartshorne & Cattrall, 1984). Since all STX congeners bind to site 1 of voltage-

gated sodium channels and binding affinity is proportional to potency, this makes the

RBA a very specific method for the detection of PSP toxins (Lipkind & Fozzard 1994).

One of several advantages of the RBA over the MBA is its lower detection limit (60 -

100 µg STX equiv. kg^{-1} compared to 400 µg STX equiv. kg^{-1}, respectively), allowing

the detection of early stages of toxic algal blooms. Early detection increases the margin

of safety by allowing public health agencies additional time to survey shellfish

resources and alert the public. RBA for PSP has undergone single and multi-lab validation studies, leading to acceptance by AOAC as an official method of analysis (OMA # 2011.27). In 2013, the RBA was approved by the ISSC (ISSC, 2013) as an Approved Limited Use Method, which is defined as a permanent method accepted for use in the NSSP but of limited scope due to a lack of data for some applications (NSSP Guide for the Control of Molluscan Shellfish, 2017). The ISSC limited the RBA to the testing of mussel and clam tissue, which were the shellfish matrices most thoroughly evaluated, with other matrices to be incorporated pending approval of matrix extension study results. Currently laboratory testing is underway at CDPH to validate the RBA for use with oyster tissue. An alternative technique for the detection of PSP toxins is a HPLC method (Lawrence et al. 2005; van de Riet et al. 2009), which has also been accepted by the ISSC and U.S. Food and Drug Administration (USFDA) as an Approved Method for unlimited use within the NSSP.

Our effort has been to eliminate the reliance on live animals while improving our analytical capacity with a method that is more sensitive and allows for some automation, thereby increasing sample throughput. We have been exploring the RBA as an alternate test method for the MBA (Ruberu et al. 2003 and Ruberu et al. 2012) in joint studies with the Center for Coastal Environmental Health and Biomolecular Research, National Oceanographic and Atmospheric Administration (NOAA) and the USFDA. Our initial work demonstrated PSP toxin concentrations measured by the RBA to be generally higher than those measured by the MBA (Ruberu et al. 2003). This difference has been attributed to salt/shellfish tissue matrix effects (LeDoux & Hall 2000, Schantz et al. 1958), to the presence of metals such as zinc and manganese (Turner et al. 2011), or to the difference in response to the various PSP toxin congeners between the *in vitro* RBA and *in vivo* MBA (Usup et al. 2004). The current work

focused on testing the accuracy, recovery, ruggedness, and comparability of the RBA

under regulatory conditions. We began with a single laboratory spiking study, looking

at shellfish extracts that were filtered through a 0.45 micron membrane filter and those

that were unfiltered, spiked with known STX concentrations and analysed for recovery.

Furthermore, with technical support from the USFDA Center for Food Safety and

Applied Nutrition, a multi-laboratory spiking study was designed to compare variability

among laboratories for the recovery of STX spiked shellfish extract by both RBA and

MBA. In addition, a thorough comparison between RBA and MBA was conducted by

analysing 295 naturally-contaminated shellfish samples collected from 92 sites along

the coast of California. Finally, we have provided suggestions for optimizing the RBA

in a regulatory laboratory environment.

Materials and methods

Chemicals and Reagents

- ^{3}H-STX diacetate in methanol (Lot# 040616, 0.1 mCi mL^{-1}, specific activity

  18.0 Ci mmol^{-1}) – American Radiolabeled Chemicals Inc. (St. Louis, MO).

- USFDA STX reference standard, STX dihydrochloride at 268.8 µM (100 ug

  mL^{-1}) (Lot #088, 100 µg mL^{-1} in 20% ethanol-water at pH 3.5) – USFDA,

  Office of Seafood (Laurel, MD).

- Rat Membrane Homogenate – The rat membrane preparation containing the

  sodium channel receptors was composed of 20 brains from 6-week old male

  Holtsman rats (Harlan Bioproducts, Indianapolis, IN) and prepared according to

  the methodology of Doucette et al. (1997). This preparation was divided into 2

  mL aliquots and frozen at -70° C. A single aliquot was thawed for each RBA

  plate and diluted with cold buffer (see RBA Buffer below) to yield a protein

  concentration of 1 mg mL^{-1} (dilution determined by conducting a protein assay).

- RBA Buffer – For the multi-laboratory study, reagents, standards, and dilutions were prepared in 100 mM MOPS/100 mM choline Cl buffer at pH 7.4. To prepare the MOPS buffer, 20.9 g of MOPS (3-morpholinopropanesulfonic acid), 13.96 g of choline chloride was dissolved in 900 mL of water, pH adjusted to 7.4 with NaOH and the final volume brought to 1 L. For all other studies, 75 mM HEPES/140 mM NaCl buffer at pH 7.5 was used. To prepare the HEPES buffer, 17.9 g of HEPES and 8.2 g of NaCl was dissolved in 900 mL of water, pH adjusted to 7.5 with NaOH and the final volume brought to 1 L.

Instrumentation

Scintillation counting was performed on a PerkinElmer TopCount plate reader with 12 detectors for all samples analysed at RBA-Lab 1, and on a PerkinElmer TopCount plate reader with 2 detectors for all samples analyzed at RBA-Lab 2. MicroScint-20 cocktail (PerkinElmer) was used as the scintillant for all RBA work.

Shellfish tissue extraction

Tissue homogenates were extracted using the AOAC mouse bioassay extraction protocol (AOAC, 1999) for the MBA, RBA and for screening using the Scotia Rapid Test (SRT) in the laboratory. For the AOAC extraction (SRT-AOAC), 100 g of shellfish tissue homogenate was combined with 100 mL of 0.1 M hydrochloric acid, pH adjusted between 1 – 4, boiled for 5 min, cooled, and final volume made to 200 mL. The mixture was centrifuged for 5 min at 3000 x *g* and the supernatant analysed.

When shellfish samples were screened in the field using the SRT, a rapid extraction method (SRT-RE) provided by the manufacturer, consisting of rubbing alcohol and vinegar, was used. For the rapid extraction 10 mL of shellfish tissue homogenate was combined with 10 mL of rapid extraction liquid (2.5:1 solution of 70% isopropyl alcohol and 5% acetic acid), shaken thoroughly to mix the contents and the

homogenate drained through a paper paint filter. The filtered extract was used for analysis.

RBA-Lab 1 Protocol

The methodology of Doucette (Doucette et al. 1997) as modified by Ruberu (Ruberu et al. 2003) was followed. The RBA procedure involved the initial addition of 35 μL of buffer (MOPS/choline Cl or HEPES/NaCl) to a 96-well microtiter filtration plate (EMD Millipore, part # MAIBN0B50) to 'wet' the wells, followed by the addition of 35 μL of unknown sample (or STX diHCl standard), 35 μL of ^{3}H -STX, and 105 μL of a 1:6 diluted rat membrane homogenate, in this order. A typical plate outline is given in Figure 1. All 8 calibration standards, quality control (QC) samples, reference samples, and shellfish sample extracts were run in triplicate on each plate. STX was used for the calibration curve in the following final in-assay molar concentrations: 1×10^{-6}, 1×10^{-7}, 3×10^{-8}, 1×10^{-8}, 3×10^{-9}, 1×10^{-9}, 1×10^{-10}, and 1×10^{-11}. Three wells per plate served as a *reference blank*, containing the material and reagents described above but omitting a source of non-radiolabeled saxitoxin. The *reference blank* establishes the maximum binding (B_{max}) for each plate. A QC sample yielding an in-assay concentration of 3.0×10^{-9} M STX standard, independently made, was used as a daily QC check. All pipetting was carried out using an 8-channel pipet. To achieve equilibrium binding, the plate was incubated for 1 hour at 4º C, then filtered using a MultiScreen vacuum manifold system (EMD Millipore, part # MSVMHTS00), and rinsed with 200 μL of 4º C buffer to remove unbound toxin. To each well, 50 μL of the scintillant (MicroScint) was added, and the top of the plate sealed with tape. The prepared plate was placed inside the TopCount scintillation counter for 30 minutes. This allowed the scintillant to dark-adapt and the contents to mix, prior to counting the receptor-bound ^{3}H-STX. Counting time was 5 minutes per well.

Criteria that must be met for *assay* acceptance are as follows: (1) the slope of the standard curve must be between 0.8 to 1.2, (2) the counts per minute (CPM) relative standard deviation (RSD) for each standard must be ≤30%, and (3) the QC check must be +/- 30% of the in-assay concentration of 3.0×10^{-9} M STX. Criteria for *sample* acceptance and quantification are; (1) $B/B_0 = 0.3$ to 0.7 and (2) RSD of triplicate wells for a sample CPM must be ≤30%.

RBA-Lab 2 Protocol

A modification of the methodology described in Van Dolah (Van Dolah et al. 2012) was followed. Briefly, the following reagents were added in sequence to each well of a Costar 3795 96-well microplate: 35 µL of unknown sample (or STX standard), 35 µL of ^{3}H-STX, and 140 µL of rat membrane homogenate (0.5 mg protein mL^{-1}). STX diHCl standard was used for the calibration curve in the following final, in-assay molar concentrations: 1×10^{-6}, 1×10^{-7}, 3×10^{-8}, 1×10^{-8}, 3×10^{-9}, 1×10^{-9}, 1×10^{-10}, and 1×10^{-11}. Three wells per plate served as reference blanks, containing the material and reagents described above but omitting a source of non-radiolabeled saxitoxin. The average reference blank established the maximum binding (B_{max}) for each plate. A QC sample yielding an in-assay concentration of 3.3×10^{-9} M STX standard was independently made and used as a daily QC check. All calibration standards, QC samples, reference blanks, and spiked shellfish sample extracts were run in triplicate on each plate. The plate was incubated for 1 hour at 4º C to achieve equilibrium binding. The contents of the microplate was transferred to a UniFilter-96 GF/B 96-well filter plate (Whatman, part # 7700-2803) using a FilterMate™ Universal Harvester (PerkinElmer). The wells of the microplate were then filled with 200 µL of 4º C buffer and drawn through the filter plate twice to ensure quantitative transfer and removal of unbound toxin. After removing the filter plate from the manifold and applying an adhesive backing to the

underside of the plate, 30 µL of Microscint-20 was added to each well, and the top of

the plate was sealed with TopSeal-A (PerkinElmer). The plate was counted for the

receptor-bound ^{3}H-STX on the TopCount 2-detector scintillation counter. Counting

time was 1 minute per well.

MBA Protocol

The AOAC procedure was followed for sample assay by the MBA method (AOAC,

1999).

Shellfish Extracts for the Single Laboratory Study

Sea mussels (*Mytilus californianus*) from Del Norte County (100 – 120 µg STX equiv.

kg[-1] measured by RBA) were used as the blank tissue matrix for the Range I study,

while bay mussels (*M. galloprovincialis*) from Agua Hedionda Lagoon in northern San

Diego County (<LOD µg STX equiv. kg^{-1} measured by RBA) were used for the Range

II study. Over many years of performing the MBA at CDPH, there has not been any

indication of differences in toxin uptake related to the species of mussel sampled (*M.

californianus* versus *M. galloprovincialis*). Mussel tissues were extracted following the

AOAC MBA extraction procedure (AOAC, 1999) and the extract was divided into two

portions, one used without any modification (unfiltered) and one filtered through a

Whatman Puradisc 0.45 micron PTFE membrane filter (filtered). Spiking ranges are

discussed in the Results and Discussion section.

Spiking Shellfish Extracts for the Multi Laboratory Study

Bay mussel tissue extracts free of PSP toxins (<LOD measured by RBA) collected from

Agua Hedionda Lagoon in San Diego was spiked with USFDA STX reference standard

to yield the final concentrations of 400, 500, 600, 700, 800, 900, 1000, 1500, 1750, and

2000 µg STX equiv. kg^{-1}. Each spiked tissue extract was divided into four separate

aliquots and labelled with a randomly assigned unique identification number. Three of

the aliquots were for the participating three laboratories while the fourth aliquot was

archived. All prepared samples and blank tissue extract were held at -80° C prior to

shipment. They were packed with dry ice in an insulated styrofoam container and

shipped via next day courier service. Samples arrived in frozen condition at each

laboratory. Aside from the coded identification number on each sample, the study was

blind to participants.

Results and Discussion

Statistical Analysis of all Data

RBA samples were run in triplicate, resulting in some variability among a set of

replicate wells. According to the AOAC protocol's acceptance criteria, any sample

with an RSD > 30% among triplicate wells is rejected. This can be problematic, as a

discarded sample must be re-assayed, resulting in a delay in obtaining critical data and

increasing the operating cost of the assay. Furthermore, if the *reference blank* is

rejected then all data from that plate will be lost, since the percent binding is determined

using its CPM value (i.e. 100% binding). One of our efforts has been focused on ways

to evaluate a set of triplicate data from a single plate for acceptability (Ruberu et al.

2012). In that study we explored two types of data treatments, Grubbs' test and

Student's *t*-test for rejecting outliers in triplicate wells, and concluded there was the

possibility of compromising accuracy with either approach. After careful consideration,

we chose to evaluate data presented in this study according to the AOAC protocol

criteria. It should be noted that NOAA criteria are part of the AOAC method as

accepted by ISSC. All statistical analyses were performed using MedCalc for Windows

Windows, version 14.10.2 (MedCalc Software, Ostend, Belgium).

Single Laboratory Spiking Study

Study Plan

Two spiking ranges were chosen; Range I samples were spiked at 400, 600, 700, 800, 1000, 2500, 5000, and 10,000 µg STX equiv. kg^{-1}, and Range II samples were spiked at 50,100, 200, 300, and 400 µg STX equiv. kg^{-1} using the USFDA STX standard. Range I concentrations are detectable by MBA while Range II concentrations fall below the MBA limit of detection, with the possible exception of the 400 µg STX equiv. kg^{-1} spike. Two tissue extract preparations were used, filtered and unfiltered (see experimental section). The comparison of filtered and unfiltered extracts revealed whether further purification of the sample extract improved the accuracy or precision of spike recovery. Each concentration range was analysed on three separate days to examine assay reproducibility from day-to-day and from plate-to-plate. Each concentration was run at 3-4 dilutions on each plate and the average result was used. If any of the dilutions failed the AOAC criteria with RSD >30%, that value was not used to calculate the average. A tissue blank was run on each plate and its CPM value was subtracted from the spiked sample CPM values.

Unfiltered and Filtered Samples

Data from the two spiking ranges for all three days were combined and analysed to determine if there was a significant difference in the results between the filtered and unfiltered samples (Table 1). Although overall variability among replicates (based on RSD) for the filtered sample data were higher (average RSD 32%) compared to that of the unfiltered (average RSD 15%), a paired-comparison *t*-test showed no significant difference (P = 0.05) in the results between the filtered and unfiltered samples (P = 0.62). The high variability of the filtered sample data was of concern and may have affected the ability to determine if a statistically significant difference existed between data sets. The unfiltered sample data was less variable and represented one less step in

processing samples, therefore all subsequent data analyses were conducted on the unfiltered sample results.

A linear regression (Figure 2) for the entire range of spike concentrations demonstrated very good agreement between the expected values and the spike recoveries (slope = 1.09; regression coefficient = 0.95). With respect to the predicted values from the line of equality, there is a greater positive bias to the RBA recoveries between the spike concentrations of 800 to 1000 µg STX equiv. kg^{-1} tissue. This increased variability at the highest concentrations can be associated with high dilution ratios and very small volumes used in the assay.

Accuracy/Trueness

Method accuracy/trueness was determined by averaging the triplicate recoveries for all of the 12 spike concentrations (n = 42), dividing this mean by the average of the spike concentrations, and then multiplying this quotient by 100. The estimated accuracy/trueness of the RBA for unfiltered samples was 115%. An alternative approach used in the AOAC inter-laboratory validation study (Van Dolah et al. 2012) was to determine accuracy based on the recovery of the 3.0 nM QC sample data from multiple plates. To use this approach, we looked at our control chart data (Ruberu et al. 2012) for the QC sample. Based on the actual mean recovery of the QC sample for 66 plates run over many years, the calculated accuracy was 101%.

Recovery

The results for the triplicate unfiltered samples results were averaged for each spike concentration. Each mean value was then subtracted from the corresponding spike concentration. A one-way analysis of variance (ANOVA) determined that there was no significant difference (P>0.05; P = 0.66) in spike recovery over the range of concentrations used (50 – 10,000 µg STX equiv. kg^{-1}). Recoveries (Table 1) were

greater than 100% for all spike concentrations, ranging from 106% to 189%, except for

the 2500 µg STX equiv. kg^{-1} which was 93%. There was no pattern of increasing or

decreasing recovery with increasing spike concentration. The AOAC single laboratory

validation study (Van Dolah et al. 2009) reported recoveries of 115 to 129% for spiked

mussel tissue, while recoveries have shown to vary from 82% to 131% in different

congeners of toxins (Usup et al. 2004).

Repeatability

Method repeatability was evaluated by comparing data from the unfiltered samples in

the spiking study over the three different days that plates were run. A one-way

ANOVA demonstrated that there was no significant difference (P > 0.05; P = 0.94) in

spike recoveries among the three days. It should be noted that the reagents used on the

different days were from the same stock solutions and standards.

Precision: The precision of the RBA was evaluated using the RSD for the triplicates of

each of the twelve spike concentrations used in the unfiltered samples. RSDs ranged

from 4% to 25% (Table 1), with an average of 15%. There was no pattern of increasing

or decreasing precision relative to the spike concentration. The 2012 collaborative

study (Van Dolah et al. 2012) had found the average RSD for routine users of the RBA

to be 17%, which is consistent with the present study.

Comparison of RBA and MBA in Testing Naturally-Contaminated Shellfish Tissue

Sampling Procedure

The CDPH MBMP generates weekly mussel samples and oyster samples as needed

from commercial shellfish growing areas, with coastal recreational sites sampled by

program participants collecting samples consisting mostly of sea mussels and

occasional bay mussels. On average over 1200 samples per year are collected state-

wide for PSP toxin analysis. Mussels are efficient at filtering out the toxin-containing

dinoflagellates, and are ubiquitous along most of the California coast, making them the best indicator species for marine toxin monitoring. Typically, samples are processed by the collectors prior to shipping to the laboratory by removing the shell, placing the drained shellfish tissue in a bottle and freezing overnight prior to shipping. Upon receipt in the laboratory, samples are extracted and screened with the Scotia Rapid Test PSP assay, with all positive samples being assayed by MBA. For the current comparison study, the remaining extracts after MBA testing were transferred to 15 mL screw cap centrifuge tubes, stored at -70°C and analysed by RBA within 1-4 weeks. The RBA testing was not 'blinded', as the MBA results were used as a guide for the initial sample dilutions.

Correlation between RBA and MBA

A total of 295 samples were used for this study, 190 of which contained detectable PSP toxins by both the MBA and RBA. STX equivalents in these naturally-contaminated samples ranged from 320 to 13,000 µg STX equiv. kg^{-1} by MBA, with 42 samples equal to or above the 800 µg STX equiv. kg^{-1} (80 µg STX equiv. 100 g^{-1}) alert level. A Deming Regression of the RBA and MBA data for 190 samples indicates a slight positive bias to the RBA, with a slope of 1.2 (Figure 3). This is consistent with our previous work (Ruberu et al. 2003) and deemed acceptable considering the inherent variability or uncertainty of each method (~ +/- 20%) (Doucette et al. 1997). Conversely, rather than indicating a positive bias in the RBA, the slope of the Deming Regression could be the result of a negative bias in the MBA. For the sake of simplifying this discussion we will refer to the RBA bias relative to the MBA rather than cite the converse relationship each time, keeping in mind that the latter is just as likely.

The relationship between the two assays is clearer in the Bland-Altman plot of the difference of the method recovery percentages versus the mean of the paired recoveries (Figure 4). There is an average positive bias for the RBA of 184 µg STX equiv. kg^{-1}, with the majority of values (n=181) within 1.96 standard deviations of the mean. At the lowest concentrations, from the MBA detection limit up to approximately 500 µg STX equiv. kg^{-1}, there is a slight negative bias to the RBA. One possible contributing factor for this observation could be the variability of the MBA near its detection limit (~320 µg STX equiv. kg^{-1} in CDPH-MDL, varying with mouse weight). Between 500 and approximately 2500 µg STX equiv. kg^{-1} there is a strong positive bias to the RBA recoveries. At increasing concentrations greater than 2500 µg STX equiv. kg^{-1} there is increasing scatter in the relationship between the assays, with an increasing number of results demonstrating a positive bias in the MBA values or a negative bias in the RBA results.   The highest point of 13,000 µg STX equiv. kg^{-1} by MBA, although included in the calculations, is not shown in Figure 4 so that more detail in the critical concentration range can be displayed.

One possibility for the observed discrepancy in the relationship between assays lies in the ability of the RBA and/or MBA to reliably quantify the different STX congeners present in mussels. Failure to do so would result in an inaccurate estimate of the total toxicity and the degree of inaccuracy could vary considerably if the subset of congeners present differed with the species or location of the samples. Studies have shown different receptor binding affinities for STX congeners by the RBA with the order of binding affinities matching the order of mouse toxicity, however the detection and quantitation of PSP toxin congeners by RBA has shown to provide a reliable integration of total toxicity present in a sample (Usup et al. 2004).   In contrast, MBA has shown to consistently underestimate PSTs with mean recoveries as low as 35% in a

collaborative study of eight MBA proficient laboratories that tested spiked shellfish samples (LeDoux et el. 2000). The RBA demonstrated acceptable linear response in the two spiking studies reported here ($r^2 = 0.95$, slope = 1.09, Figure 2; $r^2 = 0.94$, 0.99 slope = 1.01, 1.36 Figure 5), and in the AOAC single laboratory validation study (slope = 0.98, $r^2 = 0.97$; Van Dolah et al. 2009). This evidence suggests that the discrepancy of results observed between the assays in the present study is most likely due to the various sources of error in the MBA, as explained below. Matrix effects responsible for poor toxin recoveries by the MBA at lower concentrations (500 – 2000 µg STX equiv. kg^{-1}) would explain the positive bias of the RBA (Figure 4). This does not explain the negative bias to the RBA at very low concentrations (<500 µg STX equiv. kg^{-1}) relative to the MBA detection limit (320 – 400 µg STX equiv. kg^{-1}). Given the linear response of the RBA at concentrations well below this range it is more likely that the error resides with the MBA. The source of increased variability at higher concentrations also could not be determined. Possible explanations include the lack of precision in determining death times in the MBA ("last gasping breath") and error associated with high dilution ratios and very small volumes in the RBA. Regardless, the RBA performed reliably in terms of accuracy, precision, and linearity in the critical range about the 800 µg STX equiv. kg^{-1} alert level (500 – 2000 µg STX equiv. kg^{-1}) examined in both the single lab validation study and the naturally incurred samples.

Multi-Laboratory Spiking Study

Study Plan

The disparity observed in the paired RBA-MBA results was suspected to be the result of matrix effects and other sources of error within the MBA as discussed previously, however there was no direct evidence that this was the case. Therefore, a study was designed to evaluate the recovery of shellfish extracts spiked with STX at several

concentrations, and analysed by one laboratory using the MBA (data set MBA-Lab 1)

and two laboratories using the RBA (data sets RBA-Lab 1 and RBA-Lab 2). The three

participating laboratories (not in any specific order) were: CDPH Drinking Water and

Radiation Laboratory (DWRL), the Florida Fish and Wildlife Conservation

Commission, Fish and Wildlife Research Institute (FWC-FWRI), and the Maine

Department of Natural Resources (MDNR). It should be noted that the two labs

performing RBA had different instruments and slightly different protocols (see

experimental section). All three laboratories conducted triplicate analyses on each

blinded sample within the same week time frame. Results were sent back to CDPH for

decoding and evaluation.

Data Comparison

Spike recovery data from the three labs are presented in Table 2. The mean spike

recoveries and the expected recoveries were plotted against the actual spike

concentrations (Figure 5). Results show close agreement between the two RBA data sets

with respect to the expected values, however there is poor agreement between the

MBA-Lab 1 spike recoveries and the actual spike concentrations. The best fit line for

RBA-Lab 1 data closely parallels the line of equality, having a slope of 1.007 (r^2 =

0.94), with average spike recoveries ranging from 98% to 151%. Spike recoveries were

generally higher for the RBA-Lab 2 data (112 – 152%) and the regression line slope

was 1.36 (r^2 = 0.99). With increasing spike concentrations the regression lines for the

two RBAs diverges from the line of equality, indicating decreasing accuracy at higher

spike concentrations. Reproducibility of triplicate assays was best for RBA-Lab 2,

having an average RSD of 3.6% compared to 20% (excluding the blank) for RBA-Lab 1

data (Table 2). The MBA-Lab 1 data exhibited high reproducibility, with an average

RSD of 5% for triplicate spike recoveries. As noted MBA-Lab 1 accuracy for all spike

recoveries were biased low, which is consistent with previously cited studies (Le Doux et al. 2000; Van Dolah et al. 2012). MBA-Lab 1 raw data was re-evaluated by the laboratory and by two independent reviewers that had significant experience with the MBA, and no errors were found. As an additional confirmation of MBA-Lab 1 data, a second laboratory (MBA-Lab 2) that routinely performs the MBA for regulatory monitoring analysed two of the spike tissue concentrations, 750 and 1500 µg STX equiv. kg^{-1}, from the archived samples. There was good agreement of the MBA-Lab 2 results (560 and 750 µg STX equiv. kg^{-1}, respectively) with the MBA-Lab 1 data (440 and 710 µg STX equiv. kg^{-1}, respectively).

Statistical Evaluation of Data

There was a general pattern of increasing variances of the RBA triplicate spike recovery data with increasing spike concentration. This pattern was most pronounced at the two highest concentrations. One assumption of parametric statistics is that the variance is independent of the mean, which does not appear to be the case with the RBA spike recovery data. Therefore, the raw spike recovery data for all three labs was log-transformed, which corrected the inequality of error variances according to the Levene's test ($P>0.05$; $P = 0.17$). The log-transformed data was analysed by one-way ANOVA (MedCalc, version 14.10.2), which determined that there was a significant difference between laboratories for spike recoveries ($P<0.05$). Post Hoc pairwise comparisons using the Student-Neuman-Keuls method showed no significant difference between the RBA-Lab 1 and RBA-Lab 2 data sets ($P>0.05$) but showed a significant difference between the MBA-Lab 1 and each set of RBA data as well as with the expected values (i.e., spike concentrations) ($P<0.05$).

The results of this inter-laboratory study clearly demonstrate the RBA to be more accurate than the MBA for recovery of STX-spiked mussel tissue extract. The

RBA also demonstrated acceptable ruggedness, with no significant difference in spike recoveries between two different labs running this assay on different instruments. This finding is consistent with that of the AOAC collaborative study (Van Dolah, 2012) that reported no significant difference in assay performance among different instruments used by the participating labs. The considerably lower recoveries in the MBA are consistent with other MBA recovery studies, at least for the lower concentrations where no or minimal sample dilution has occurred. The AOAC collaborative study reported MBA recoveries of 68.6% and 40.5% for spike concentrations of 500 and 1200 μg STX equiv. kg^{-1}, respectively while Le Doux et al. (2000) determined in an inter-laboratory study of the MBA that this method underestimated STX concentration by as much as 60%, which was attributed to matrix or 'salt' effects described by others (Schantz et al., 1958; McFarren 1959; Ares et al. 1982; Park et al. 1986).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Using the IC_{70} values ($B/B_0 = 0.7$) from the standard curves run at CDPH and following the method given by Van Dolah (Van Dolah et al. 2012), LOD and LOQ were calculated. The IC_{70} values were determined by the software Prism ECAnything equation and assuming a minimum ten-fold dilution. The latter is the minimum dilution required to remove matrix effects when a quantification cut off of $B/B_0 < 70\%$ is used (Van Dolah et al. 2009). It should be noted that there were only four plates run for this study and hence only four IC_{70} values available for use. With this minimal data set a LOD of 105 μg STX equiv. kg^{-1} and a LOQ of 198 μg STX equiv. kg^{-1} was calculated. These values are comparable to those reported in the literature of 64 μg STX equiv. kg^{-1} and 130 μg STX equiv. kg^{-1} respectively (Van Dolah et al. 2012). The lower working range of the RBA is well below that of the MBA and the federal alert level of 800 μg STX equiv. kg^{-1} and is a factor of 3 lower than the MBA detection limit in CDPH-MDL

($\sim$ 320 µg STX equiv. kg^{-1}). Therefore, a determination of the RBA detection limit in our laboratory is of little importance relative to the working range for routine monitoring.

Optimizing RBA for Regulatory Use

Screening Plates

Under regulatory conditions, state laboratories test thousands of samples every year. Rapid throughput, with the need for results to be reported the same day that samples are received, is critical for proper management of recreational and commercial shellfish resources for public health protection. Since samples are blind to the analyst, dilution levels for positive samples are 'trial and error' until the proper range is achieved to accurately determine the concentration of STX present. Therefore, quantification of a single sample could require multiple assays (i.e., multiple plates). To use the RBA efficiently for regulatory samples, a strategy for initial sample dilution is necessary. To address this aspect of the assay we have established a 'screening plate' step, where all incoming shellfish sample extracts are assayed at two dilutions: 1:35 and 1:70.   Based on our experience with running the RBA on naturally contaminated shellfish extracts, it was determined that, if targeting 50% binding on the RBA calibration curve, 1:35 and 1:70 dilutions would approximate 400 µg STX equiv. kg^{-1} tissue and 800 µg STX equiv. kg^{-1} respectively. At the action level of 800 µg STX equiv. kg^{-1}, a 1:70 dilution will fall within the acceptable 30 – 70% binding range. Samples that fall below 30% binding can be assumed to be greater than 800 µg STX equiv. kg^{-1}.   Thus, samples that exceed the alert level can be acted upon immediately by the shellfish program manager while the laboratory prepares a subsequent plate with additional dilutions to determine the final concentration in these samples.   Samples that are above 70% binding at the 1:35 dilution could subsequently be assayed at a lower dilution if knowing the absolute

concentration (e.g., for tracking potential bloom development) was of value to the

shellfish program manager.   We believe this would be the most efficient way to

implement the RBA in a regulatory monitoring program. It is recommended that each

lab conduct an evaluation of this approach to determine the appropriate screening

dilutions to minimize the probability of 'false alarms', in which a screening result ≥ 800

µg STX equiv. kg^{-1} was subsequently quantitated and determined to be < 800 µg STX

equiv. kg^{-1}.

Rapid PSP Test Kits for Screening of Negative Samples and Early Detection

Of the qualitative test kits commercially available for PSP toxins testing, the Scotia®

Rapid Test (SRT) from Scotia Rapid Testing® (Laycock et al. 2010) was approved for

use as a screening method by the ISSC in 2005. This qualitative lateral flow

immunochromatography test was developed based on antibodies against neosaxitoxin

(NEO), with cross-reactivity evaluated for STX and several analogs (STX, GTX 2/3,

NEO, and GTX ¼) (Burk et al. 1995). The SRT-AOAC test uses the same AOAC

extraction procedure as the MBA and thus one sample extraction can be used for both

methods. As a result of its acceptance for screening, the CDPH-MDL has focused their

attention on the evaluation of this test kit. Prior to the ISSC approval of SRT-AOAC,

every shellfish sample received by CDPH-MDL was tested by MBA. Following the

ISSC acceptance of the SRT-AOAC and its incorporation into the NSSP Model

Ordinance, CDPH-MDL began using this presence/absence test to identify negative

samples, with only the positive samples requiring quantitation by MBA. Because the

majority of samples received are negative by MBA, CDPH-MDL reported a significant

savings in animals and lab resources after this screening procedure was implemented

(Oshiro et al. 2006). Although SRT-AOAC has proven to be a reliable and useful

method for this initial screening process, oftentimes samples positive by SRT-AOAC

have shown to be negative by MBA. The LOD for the SRT-AOAC PSP test can be

expected to vary regionally, depending on the subset of STX congeners present and how

these compare to the subset used to produce the antibody in the kit. We evaluated 106

samples that were tested by SRT-AOAC, MBA, and RBA. Of the 57 samples that were

positive by MBA (330 – 6320 µg STX equiv. kg^{-1}), all were positive by SRT-AOAC

(i.e., no false negatives by SRT-AOAC). RBA values for these 57 samples ranged from

240 to 3990 µg STX equiv. kg^{-1}. Of the 23 samples that were negative by MBA and

positive by SRT-AOAC, the concentrations determined by RBA ranged from LOD to

550 µg STX equiv. kg^{-1}, with an average concentration of 200 µg STX equiv. kg^{-1}. For

the 26 samples that were negative by both MBA and SRT-AOAC, the RBA results

ranged from LOD to 240 µg STX equiv. kg^{-1}. The RBA data support that the SRT-

AOAC has slightly greater sensitivity to PSP toxins in California shellfish compared to

the MBA, erroneously referred to as producing false positives, and can provide an

additional advance warning of a developing PSP event.   One potential source of error

with the SRT-AOAC test strip is the analyst-dependent interpretation of a qualitative

color change in a test line in comparison to a control line. As the level of toxin

increases, the strength of the test line decreases and is therefore easier to report as a

positive result. A concentration of PSP toxins close to the SRT-AOAC detection limit

is the most difficult to interpret, and a conservative approach of reading any

questionable result as a positive is appropriate. This potential source of error does not

represent a public health risk given the low concentrations of toxin involved. It should

be noted that there is significant overlap in the reported MBA values for negative and

low positive results due to varying mouse weights. Continuation of pre-screening

samples with SRT-AOAC will eliminate the need to analyse negative tissue and

optimize the RBA for regulatory use.

Field testing for PSP

Although not approved for regulatory use, there is a test kit for PSP toxins from Scotia

Rapid Testing® designed for field use that utilizes a simpler and faster extraction

method. This rapid extraction procedure is a mixture of 70% isopropyl alcohol (i.e.,

rubbing alcohol) and 5% acetic acid (i.e., vinegar) and uses the same test strip as the

SRT-AOAC test. As a separate part of this project, three field sites were established for

sampling of mussels and field testing with the Scotia® Rapid Extraction procedure

(SRT-RE) over a 3-4 year period of time. The sites were located at (1) Cal Poly Pier,

Avila, San Luis Obispo (35.2828^{o} N, 120.6596^{o} W), (2) Santa Cruz Wharf, Santa Cruz

(36.9741^{o} N, 122.0308^{o} W), and (3) Drakes Bay Fish Dock, Marin County (38.0834^{o} N,

122.7633^{o} W). A weekly mussel sample was collected from the sentinel mussel bag at

each location, processed in the field, tested with SRT-RE, and the homogenate shipped

to the laboratory for testing with SRT-AOAC, MBA, and RBA.

There were 356 samples tested by SRT-RE and MBA. Of the 356 paired SRT-

RE/MBA samples, 39 were also tested by SRT-AOAC and RBA, with an additional 30

samples with results for RBA but not SRT-AOAC. Of the 356 SRT-RE results, 259

were positive, corresponding to 137 positive and 122 negative MBA results. There were

no false negative SRT-RE results for positive MBA results (340 to 8790 µg STX equiv.

kg^{-1}). In summary, of the 219 negative MBA results 97 were negative and 122 were

positive by JRT-RE. Of the 122 positive JRT-RE samples, 24 were also analysed by

RBA, ranging from LOD to 550 µg STX equiv. kg^{-1}.

There were 212 samples tested by both SRT-RE and SRT-AOAC. Of these

pairs, 177 were in agreement (107 positive and 70 negative by both tests) and 35 were

in disagreement (23 +/- and 12 -/+ combinations, respectively). MBA results were

negative for those 35 samples and RBA results ranged from the LOD to 240 µg STX

equiv. kg^{-1}. The disagreement between test kits may reflect operator error in interpreting the test strip at extremely low concentrations of toxin. The absence of false negatives relative to the MBA and the agreement with the ISSC-approved SRT-AOAC for all samples that were positive by MBA demonstrates that the rapid extraction procedure coupled with the SRT-RE test strip for PSP toxins is a viable option as a field screening tool for monitoring shellfish in recreational and commercial shellfish beds.

Porcine Membrane Alternate

Early work on the binding properties of STX to voltage gated sodium channels was conducted on a variety of animal tissue models, including rabbit brain (Strichartz 1981a; Strichartz & Hansen Bay 1981b), rat skeletal muscle (Barchi et al. 1980), rat brain (Hartshorne et al. 1984), and frog sciatic nerve (Strichartz 1984). During studies with G. R. Strichartz on the pharmacology of purified saxitoxins using an RBA, Hall noted that the binding affinities correlated well with the potencies observed in the mouse assay. It was therefore evident that the receptor assay could be a useful alternative to the mouse assay. Although the RBA represented a dramatic reduction in the sacrifice of animals, it still depended on this practice for the supply of brain tissue. To explore the use of slaughterhouse byproduct instead of tissue from experimental animals, Hall prepared a suspension of bovine brain and demonstrated that it worked well (S. Hall, personal communication). In addition to the continued sacrifice of live animals, another impediment to the adoption of the RBA for regulatory use is the fact that, while the rat brains are commercially available, the prepared membrane homogenate is not. The preparation of this membrane and its standardization is a labour-intensive procedure in the laboratory. The rat membrane is also a very heterogeneous mixture and needs to be mixed (agitated) each time an aliquot is pipetted to a set of eight wells. One batch of homogenate prepared using 20 brains is adequate for 100 plates (or 700 samples using

the plate layout in Figure 1); however the ultimate goal for the RBA is to eliminate the

use of live animals completely. Hall's earlier suggestion to use brain tissue from

slaughterhouse animals was taken up by Sigma-Aldrich Corporation, which developed a

standardized porcine membrane homogenate (Cat. No. Q3639) that is now

commercially available (American Radiolabeled Chemicals, Inc., Cat. No. ARCD 1301)

for use in the RBA. We performed initial testing of this porcine membrane for its use

with the RBA (Figure 6).   The porcine membrane was very homogenous and unlike the

rat membrane didn't require continuous agitation. Overall, the porcine membrane

reference values were lower ~ 700 cpm (counts per minute) compared to that of the rat

membrane values of ~900 cpm, indicating possible lower binding capacity i.e., a lower

number of receptor sites. This can be easily overcome by finding an optimum working

dilution, by performing a protein assay of the membrane preparation (Van Dolah et al.

2013). Reproducibility between wells was better with the porcine membrane than with

the rat membrane, with RSDs of 12% and 18% respectively, probably due to the

homogeneity of the former. These initial results are very promising and demonstrate

the porcine membrane, which can be commercially obtained already standardized with

high homogeneity, would be an excellent candidate for use with RBA in regulatory

applications.

Conclusion

The receptor binding assay approved recently by ISSC for testing of mussel tissue for

PSP toxins was further evaluated for its overall performance (accuracy, recovery,

ruggedness, comparability) and for its use under regulatory conditions. The large

number of samples analysed and the diversity of the tests conducted in this study

demonstrates a robust set of quality assurance data to affirm the use of the RBA as an

affordable rapid method for STX detection that is also free of the routine sacrifice of

live animals. Three different studies were launched for testing, the first of which was to

evaluate the effects of filtering shellfish extracts prior to running the RBA. There was

no significant difference in the outcome of spike recoveries by filtering the tissue prior

to analyses, with the overall variability being lower in unfiltered tissue (15%) compare

to filtered tissue (32%).

The second study compared the MBA and the RBA recoveries for 295 naturally

contaminated shellfish tissue samples, with STX values ranging from 320 µg STX

equiv. kg^{-1} to 13,000 µg STX equiv. kg^{-1} as determined by MBA. The relationship

between the two assays (RBA and MBA) from a Bland-Altman plot indicated an

average positive bias for the RBA of 184 µg STX equiv. kg^{-1}. At concentrations

between the MBA detection limit and 500 µg STX equiv. kg^{-1} a slight negative bias to

the RBA was observed. Between 500 µg STX equiv. kg^{-1} and 2500 µg STX equiv. kg^{-1}

there was a strong positive bias to RBA, while concentrations higher than 2500 µg STX

equiv. kg^{-1} showed scatter in the relationship. Given the excellent recovery of spiked

tissue by RBA, the variability must lie in the MBA results, which suffers from matrix

effects and a lack of precision in determining death times.

The third study was conducted between three independent laboratories, two of

which performed the RBA and one the MBA. Mussel tissue extracts were spiked and

sent blind to each laboratory for analysis. Results demonstrated very close agreement

between the two RBA laboratories and those recovery values closely paralleled the

actual spike concentrations. Conversely the same samples analysed by MBA showed

recoveries significantly biased low from the true values. This inter-laboratory study

clearly demonstrated the RBA to be more accurate than the MBA for recovery of STX-

spiked mussel tissue extracts, as well as good method ruggedness. The study revealed a

general pattern of increasing variances of the triplicate spike recovery data with increasing spike concentration.

Lastly we explored ways to optimize the RBA under regulatory use, where laboratories test thousands of samples every year with rapid throughput of results. Several ways of optimizing the assay for its efficient use are discussed, such as the use of an initial screening plate to separate those samples that exceed the alert level from the rest; using rapid PSP test kits in the field and in the laboratory for screening negative samples and early detection; and using an alternate commercially available Porcine membrane replacing the laboratory prepared rat membrane homogenate.

References

Ares, JA, Dymsza HA. 1982. Development and use of a proficiency test specimen for paralytic shellfish poisoning. *J. Food Sci*. 47: 1041-1079.

Barchi RL, Cohen SA, Murphy LE. 1980. Purification from rat sarcolemma of the saxitoxin-binding component of the excitable membrane sodium channel. *Proc. Natl. Acad. Sci. USA*. 77: 1306-1310.

Burk C, Usleber E, Dietrich R, Martlbauer E. 1995. Production and Characterization of Antibodies against Neosaxitoxin Utilizing a Novel Immunogen Synthesis Procedure. *Food and Agric. Immun.* 7: 315-322.

Doucette GJ, Logan MM, Ramsdell JS, Van Dolah FM. 1997. Development and preliminary validation of a microtiter plate-based receptor binding assay for the paralytic shellfish poisoning toxins. *Toxicon.* 35(5): 625-636.

Hartshorne RP, Catterall WA. 1984. The sodium channel from rat brain. *J. Biological Chem*. 259: 1667-1675.

Inami GB, Crandall CL, Csuti D, Oshiro M, Brenden A. 2004. Feasibility of reduction in the use of the mouse bioassay: Presence/absence Screening for saxitoxin in frozen acidified mussel and oyster extracts from the coast of California with in vitro methods. *J AOAC Int*. 87: 1133-1142.

ISSC [Internet]. 2013. Interstate Shellfish Sanitation Conference: Single laboratory validation protocol for method approval (iii); [cited 2017 Mar 24]. Available from: http://www.issc.org/single-lab-validation-for-laboratory-methods.

Jellett JF, Roberts RL, Laycock MV, Quilliam MA, Barrett RE. 2002. Detection of paralytic shellfish poisoning (PSP) toxins in shellfish tissue using MIST Alert, a new rapid test, in parallel with the regulatory AOAC mouse bioassay. *Toxicon: official journal of the International Society on Toxicology* 40:1407-1425.

Lawrence JF, Niedzwiadek B, Menard C. 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. *J AOAC Int.* 88: 1714-1732.

Laycock MV, Donovan MA, Easy DJ. 2010. Sensitivity of lateral flow tests to mixtures of saxitoxins and applications to shellfish and phytoplankton monitoring. *Toxicon*. 55: 597-605.

LeDoux M, Hall S. 2000. Proficiency testing of eight French laboratories in using the
AOAC mouse bioassay for paralytic shellfish poisoning: interlaboratory
collaborative study. *J. AOAC Int.* 83: 305-310.

Lipkind GM, Fozzard HA. 1994. A structural model of the tetrodotoxin and saxitoxin
binding site of the Na+ channel. *Biophysical J.* 66: 1-13.

McFarren EF. 1959. Report on Collaborative Studies of the Bioassay for Paralytic
Shellfish Poison. *J. Assoc. Off. Anal. Chem*. 42: 263 – 271.

NSSP [Internet], National Shellfish Sanitation Program, 2017. Guide for the Control of
Molluscan Shellfish, Model Ordinance, 445pp. [cited 2017 Mar 24]. Available
from:http://www.fda.gov/Food/GuidanceRegulation/FederalStateFoodPrograms/uc
m2006754.htm

AOAC INTERNATIONAL. 1999. Official methods of analysis: method 959.08. 17th
ed. Gaithersburg: MD.

Oshiro M, Pham L, Csuti D, Dodd M, Inami GB, Brenden A. 2006. Paralytic shellfish
poisoning surveillance in California using the Jellett Rapid PSP test. *Harmful
Algae*. 5: 67-73.

Park DL, Adams WN, Graham SL, Jackson RC. 1986. Variability of mouse bioassay
for determination of paralytic shellfish poisoning toxins. *J. Assoc. Off. Anal.
Chem*. 69: 547-550.

Pearson L, Mihali T, Moffitt M, Kellmann R, Neilan B. 2010. On the chemistry,
toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin,
saxitoxin and cylindrospermopsin. *Mar. Drugs*. 8: 1650-1680.

Ruberu SR, Liu YG, Wong CT, Perera SK, Langlois GW, Doucette GJ, Powell CL.
2003. Receptor binding assay for paralytic shellfish poisoning toxins:
optimization and inter-laboratory comparison. *J AOAC Int*. 86: 737-745.

Ruberu SR, Langlois GW, Masuda LM, Perera SK, 2012. Evaluation of variability and
quality control procedures for a receptor-binding assay for paralytic shellfish
poisoning toxins. *Food Additives & Contaminants*: Part A. 1-10.

Schantz EJ, McFarren EF, Schafer ML, Lewis KH. 1958. Purified shellfish poison for
bioassay standardization. *J. Assoc. Off. Anal. Chem*. 41: 160-168.

Strichartz GR, Hansen Bay CM. 1981. Saxitoxin binding in nerves from walking legs of
the lobster Homarus americanus. *J. Gen. Physiol.* 77: 205-221.

Strichartz GR. 1981. Structure of the saxitoxin binding site at sodium channels in nerve
membranes. *Molec. Pharmacology*. 21: 343-350.

Strichartz GR. 1984. Structural determinants of the affinity of saxitoxin for neuronal sodium channels. *J. Gen. Physiol.* 84: 281-305.

Turner AD, Dhanji-Rapkova M, Algoet M, Suarez-Isla BA, Cordova M, Caceres C, Murphy CJ, Casey M, Lees DN. 2011. Investigations into matrix components affecting the performance of the official bioassay reference method for quantitation of paralytic shellfish poisoning toxins in oysters. *Toxicon* 59: 215-230.

Usup G, Leaw CP, Cheah MY, Ahmad A, Ng BK. 2004. Analysis of paralytic shellfish poisoning toxin congeners by a sodium channel receptor binding assay. *Toxicon.* 44: 37-43.

van de Riet JM, Gibbs RS, Chou FW, Muggah PM, Rourke WA, Burns G. 2009. Liquid chromatographic post-column oxidation method for analysis of paralytic shellfish toxins in mussels, clams, scallops, and oysters: single-laboratory validation. *Journal of AOAC Int.* 92, 1690-1704.

Van Dolah FM, Leighfield TA, Doucette GJ, Bean L, Niedzwiadek B, Rawn DFK. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. *Journal of AOAC Int.* 92, 1705-1713.

Van Dolah FM, Fire SE, Leighfield TA, Mikulski CM, Doucette GJ. 2012. Determination of paralytic shellfish toxins in shellfish by receptor binding assay: collaborative study. *Journal of AOAC Int.* 95, 795-812.

Table 1. Spiked mussel tissue extract, filtered and unfiltered, determined by RBA on three different days.

STX Spike Level µg STX equiv. kg⁻¹	Unfiltered Tissue					Filtered Tissue				
	µg STX equiv. kg⁻¹			RSD (%)	Average % recovery	µg STX equiv. kg⁻¹			RSD (%)	Average % recovery
	Day 1	Day 2	Day 3			Day 1	Day 2	Day 3		
50	70	70	50	18	127	50	80	60	24	127
100	100	120	140	17	120	50	150	90	52	97
200	270	250	310	11	138	110	360	240	53	118
300	420	350	470	15	138	260	560	300	44	124
400	720	540	660	14	160	310	820	630	44	147
400	500	430	340	19	106	590	350	430	27	114
600	860	660	700	14	123	920	460	670	34	114
700	980	860	690	17	120	1130	670	620	35	115
800	1330	950	1590	25	161	980	600	780	24	98
1000	2320	1770	1580	20	189	1290	1080	1000	13	112
1000	1180	1060	1040	7	109	1710	1440	1140	20	143
2500	2380	2200	2360	4	93	3150	2070	2080	26	97
5000	5550	5210	4840	7	104	7010	5390	4670	21	114
10,000	11,820	13,480	8490	23	113	18,700	9740	11,450	36	133

Table 1

Table 2. Performance of individual laboratories on blind spiked shellfish extracts in the multi-laboratory spiking study. Column values without any STX measured data represent those results that were not accepted after QC criteria (>30% RSD in triplicate wells).

STX Spike level (μg STX equiv. kg^{-1})	MBA-Lab 1 (μg STX equiv. kg^{-1})				RBA-Lab 1 (μg STX equiv. kg^{-1})				RBA-Lab 2 (μg STX equiv. kg^{-1})			
	STX measured	Mean	Recovery	RSD %	STX measured	Mean	Recovery	RSD %	STX measured	Mean	Recovery	RSD %
0	< DL				30	23		49	<DL	10		
	< DL				10				<DL			
	< DL				30				10			
400	< DL					490	123%	0.0	580	557	139%	7.3
	< DL				490				510			
	< DL				490				580			
500	370	387	77%	4.0	590	640	128%	9.8	650	653	131%	0.9
	400				710				660			
	390				620				650			
600	380	387	64%	1.5	1170	830	138%	38		805	134%	0.9
	390				540				800			
	390				780				810			
700	430	427	61%	1.4	1240	1057	151%	23		785	112%	0.9
	420				1150				780			
	430				780				790			
800	470	440	55%	6.8	990	953	119%	15	1080	1097	137%	4.3
	410				1070				1060			
	440				800				1150			
900	460	450	50%	2.2	840	1187	132%	26	1340	1330	148%	1.1
	440				1440				1320			
	450				1280							

1000	480	503	50%	6.4	1450	1263	126%	20	1340	1340	134%	0.0
	540				1370							
	490				970				1340			
1500	790	710	47%	11	1540	1467	98%	20	2050	2280	152%	11.6
	630				1720				2220			
	710				1140				2570			
1750	890	830	47%	7.9	2600	2207	126%	21	2220	2367	135%	5.8
	760				2340				2390			
	840				1680				2490			
2000	1000	1005	50%	0.7	2360	1990	100%	26	2740	2613	131%	4.2
	1010				2200				2560			
					1410				2540			

Table 2

Figure 1

1	2	3	4	5	6	7	8	9	10	11	12
$1X10^{-6}$	$1X10^{-6}$	$1X10^{-6}$	Ref Blank	Ref Blank	Ref Blank	Sample 3 1:10	Sample 3 1:10	Sample 3 1:10	Sample 5 1:200	Sample 5 1:200	Sample 5 1:200
$1X10^{-7}$	$1X10^{-7}$	$1X10^{-7}$	QC	QC	QC	Sample 3 1:50	Sample 3 1:50	Sample 3 1:50	Sample 6 1:10	Sample 6 1:10	Sample 6 1:10
$3X10^{-8}$	$1X10^{-8}$	$1X10^{-8}$	Sample 1 1:10	Sample 1 1:10	Sample 1 1:10	Sample 3 1:200	Sample 3 1:200	Sample 3 1:200	Sample 6 1:50	Sample 6 1:50	Sample 6 1:50
$1X10^{-8}$	$5X10^{-9}$	$5X10^{-9}$	Sample 1 1:50	Sample 1 1:50	Sample 1 1:50	Sample 4 1:10	Sample 4 1:10	Sample 4 1:10	Sample 6 1:200	Sample 6 1:200	Sample 6 1:200
$3X10^{-9}$	$1X10^{-9}$	$1X10^{-9}$	Sample 1 1:200	Sample 1 1:200	Sample 1 1:200	Sample 4 1:50	Sample 4 1:50	Sample 4 1:50	Sample 7 1:10	Sample 7 1:10	Sample 7 1:10
$1X10^{-9}$	$1X10^{-10}$	$1X10^{-10}$	Sample 2 1:10	Sample 2 1:10	Sample 2 1:10	Sample 4 1:200	Sample 4 1:200	Sample 4 1:200	Sample 7 1:50	Sample 7 1:50	Sample 7 1:50
$1X10^{-10}$	$1X10^{-10}$	$1X10^{-10}$	Sample 2 1:50	Sample 2 1:50	Sample 2 1:50	Sample 5 1:10	Sample 5 1:10	Sample 5 1:10	Sample 7 1:200	Sample 7 1:200	Sample 7 1:200
$1X10^{-11}$	$1X10^{-11}$	$1X10^{-11}$	Sample 2 1:200	Sample 2 1:200	Sample 2 1:200	Sample 5 1:50	Sample 5 1:50	Sample 5 1:50	QC	QC	QC

Figure 1. A typical 96-well plate layout for RBA with three dilutions per sample (1:10, 1:50 and 1:200). A 1:10 lowest dilution is required to minimize matric effects.
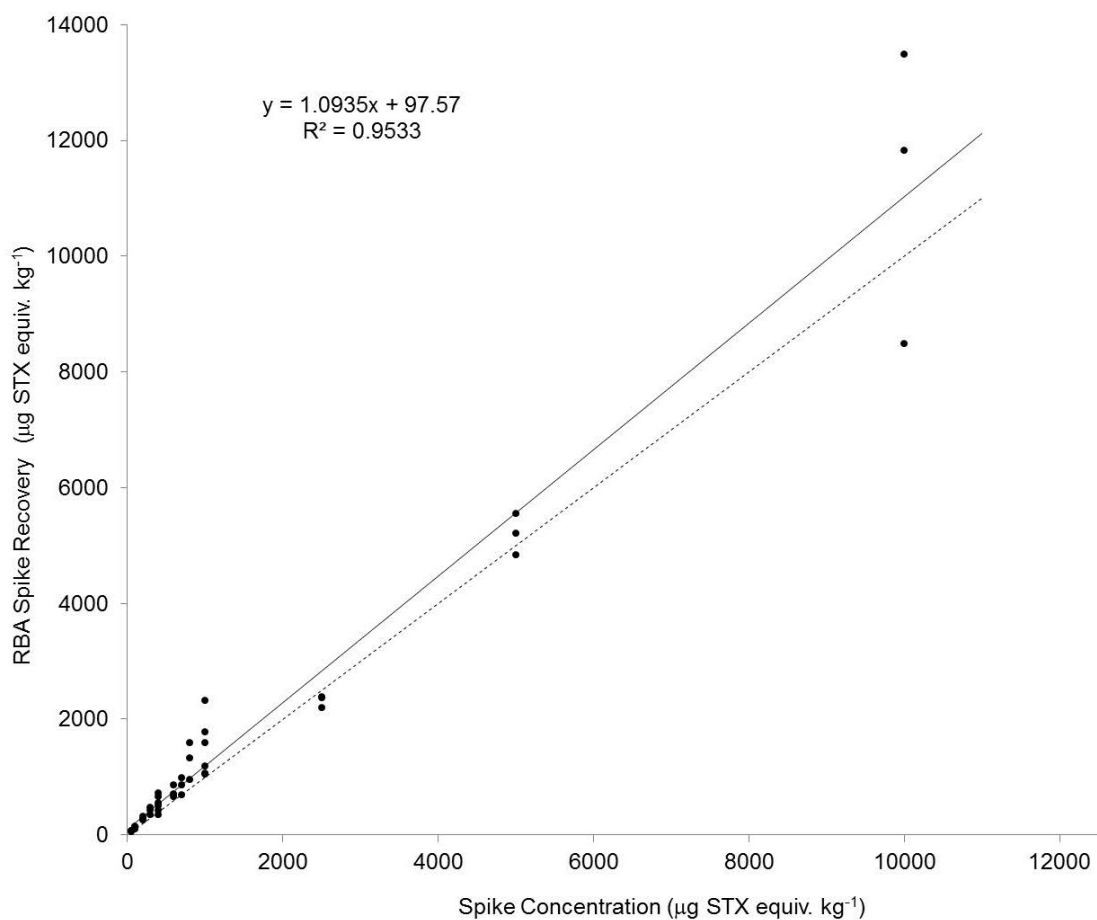
$$y = 1.0935x + 97.57$$
$$R^2 = 0.9533$$

Figure 2. RBA recoveries from the single laboratory spiking study of spiked unfiltered

shellfish tissue extracts showing the variation in the assay results between three days.

Each data point is an average of multiple measurements on a single plate. The dotted
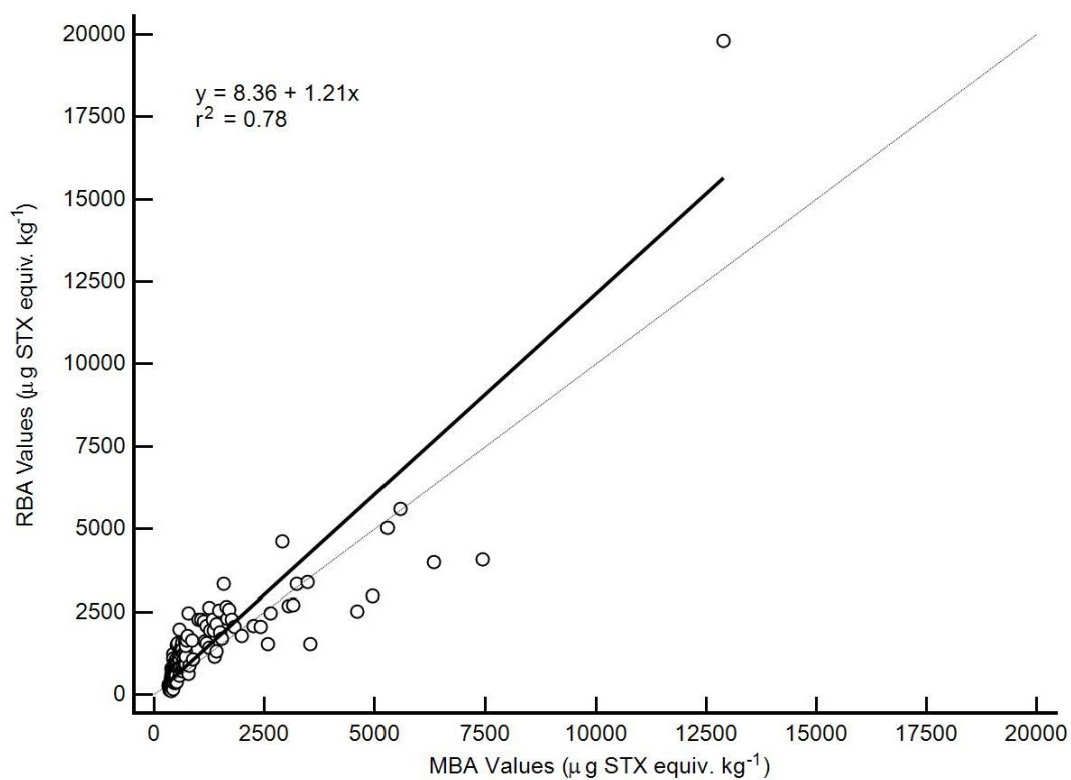
line is the Expected (1:1) line.

Figure 3. Deming regression of RBA and MBA for naturally-contaminated shellfish
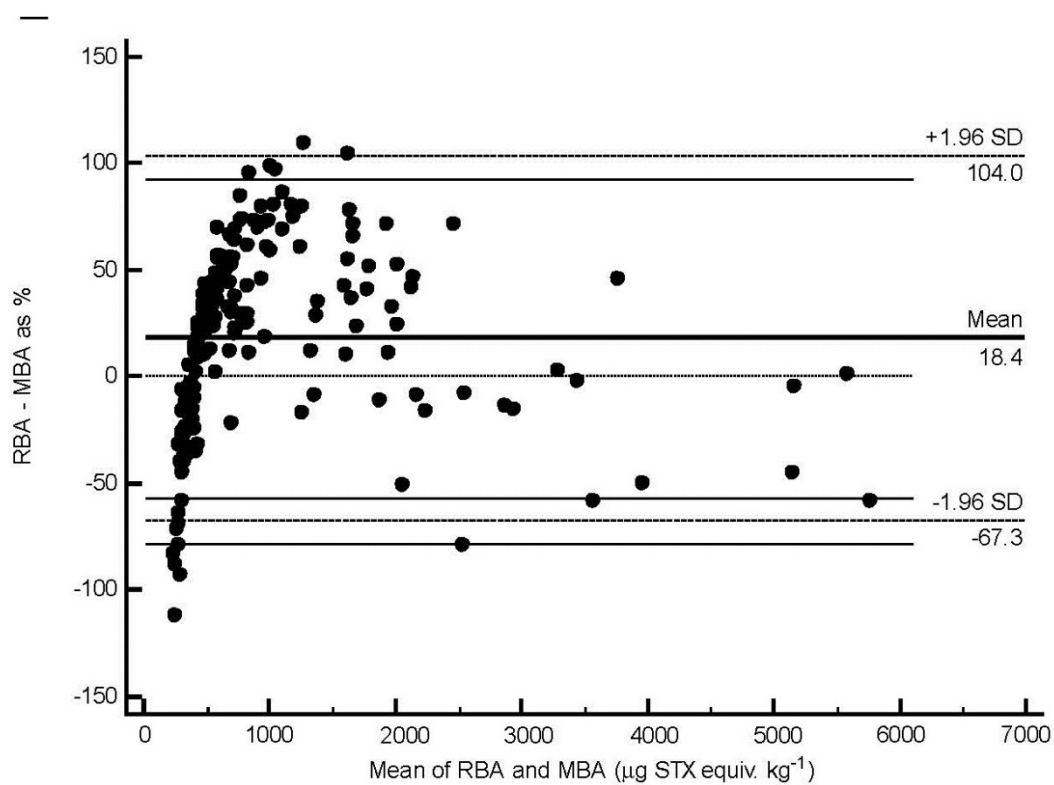
samples. The dotted line is the Expected (1:1) line

Figure 4. Bland-Altman plot of the difference of RBA and MBA percent recovery values versus the mean of the RBA and MBA recoveries, for naturally contaminated shellfish samples. The highest point (13,000 μg STX equiv. kg^{-1} by MBA) was included in the calculations but is excluded in the plot to allow more detail to be shown in the critical concentration range.
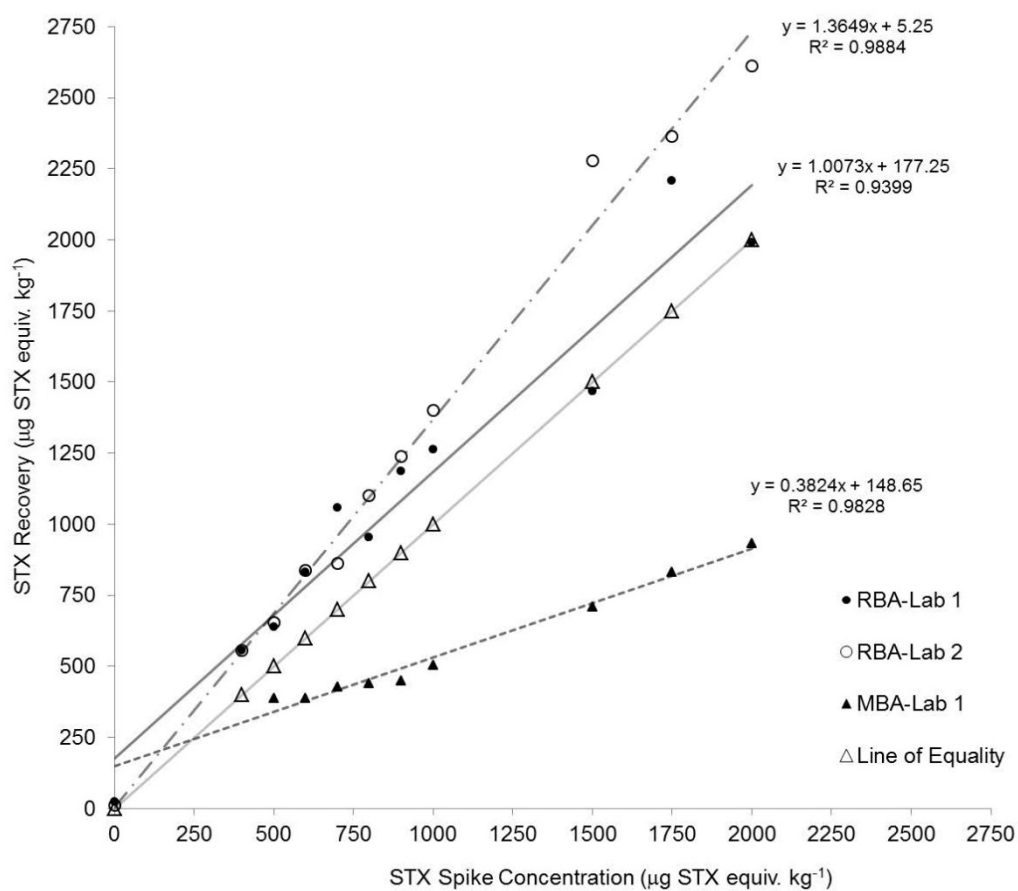
Figure 5. Plot of multi-laboratory spiking study results showing the recovery of STX-spiked mussel extracts of each laboratory compared to the spike concentrations.
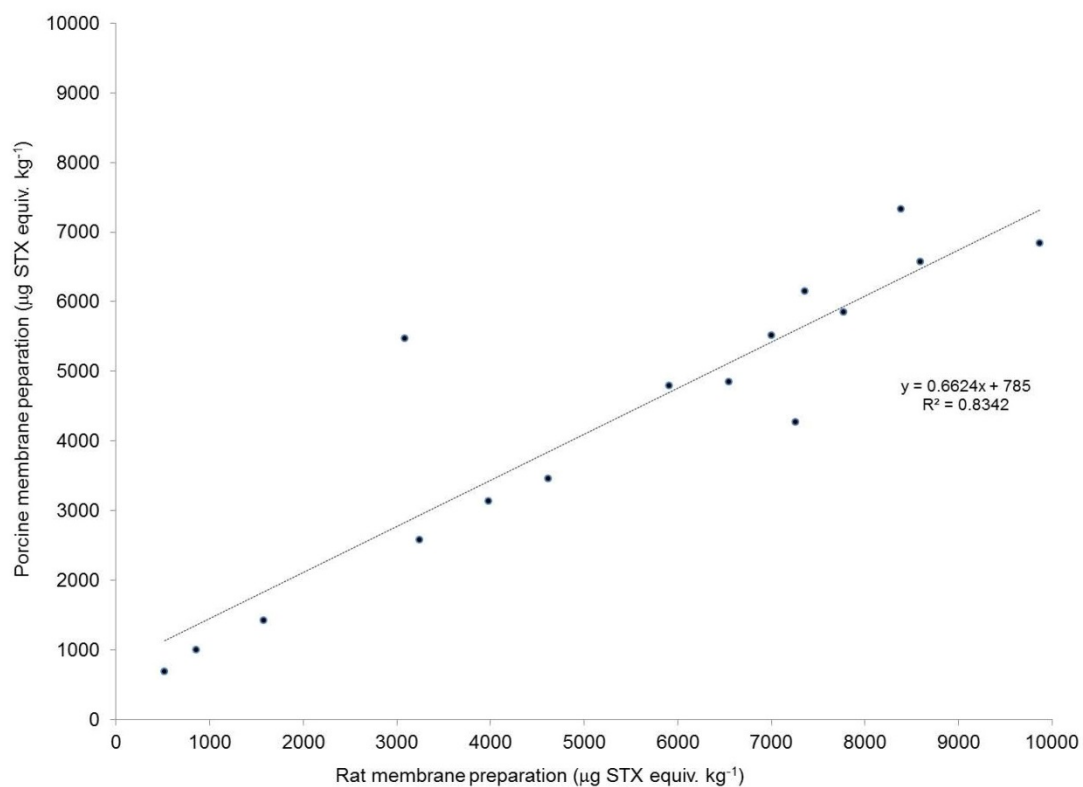
Figure 6. Comparison of rat membrane preparation to porcine membrane preparation. Naturally contaminated shellfish tissue extract was processed using the two membranes on a single plate.