Detection of Harmful Algal Toxins Using the Radioligand Receptor Binding Assay

A Manual of Methods
DETECTION OF HARMFUL ALGAL TOXINS USING THE RADIOLIGAND RECEPTOR BINDING ASSAY
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DETECTION OF HARMFUL ALGAL TOXINS USING THE RADIOLIGAND RECEPTOR BINDING ASSAY
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FOREWORD

Marine ecosystems and their resources play major roles in sustaining human population and economic growth in coastal developing countries. These ecosystems are subjected to various natural and human-made threats. Among these are harmful algal blooms (HABs), which are natural phenomena that are increasingly being reported around the globe and responsible for human poisoning through the accumulation of potent toxins in marine food products.

The impact of HABs may be aggravated by a limited knowledge of the microalgal species that cause toxic outbreaks, their biology, their diversity, their life cycles, and by poor capabilities for predicting the outbreaks and assessing the degree of HAB toxicity. Other negative factors are the lack of recognition of the disease, the lack of epidemiological data, the lack of adequate and specific treatment and low public awareness.

Owing to the profound public health and socioeconomic impact of HABs, many countries have developed and implemented HAB related monitoring programmes and regulatory frameworks.

Following a request made by the Philippines during the IAEA General Conference in 1997 to identify possible measures to address the impacts of HABs, the IAEA initiated related Technical Cooperation projects to assist Member States in strengthening their capacities for prevention, management and mitigation of health and socioeconomic impacts of HABs.

Since 1998, the IAEA and the National Oceanic and Atmospheric Administration (NOAA) have undertaken concerted actions to develop and to validate a radioligand based method, the receptor binding assay (RBA). The RBA is now recognized by the AOAC International as an official method for the detection of paralytic shellfish poisoning toxins.

Within the IAEA Technical Cooperation programme, the RBA methodology was transferred to over 23 Member States in Africa, Asia, the Pacific region and Latin America. Transfer of knowledge and relevant equipment has enabled the development and strengthening of significant national, regional and interregional capabilities in algal toxin detection and monitoring, and management and mitigation of HABs associated with paralytic shellfish poisoning, diarrheic shellfish poisoning and ciguatera poisoning.

In order to support and to facilitate the continuing effort of Member States to acquire, develop and sustain capabilities to address the impacts of HABs, the IAEA has taken the initiative to conceive and produce this manual of radioligand receptor binding assay methods. This is the first publication produced in collaboration with the NOAA and the Intergovernmental Oceanographic Commission of UNESCO (IOC UNESCO), the Philippine Nuclear Research Institute (an IAEA collaborating centre) and the Institut Louis Malardé in French Polynesia. It complements the Manual on Phytoplankton Sampling Techniques produced under the regional project RLA7014 and published by the IOC UNESCO.

The series of well established nuclear techniques for early identification and quantification of HAB related toxins that are presented in this publication are intended to reduce the health and socioeconomic impacts of HAB events. The end users include national phytoplankton monitoring programmes with benefits to fisheries, research scientists, public health, communities, etc.

The IAEA officers responsible for this publication were J. Gerardo-Abaya of the Division for Asia and the Pacific, F. Descroix-Comanducci and M.-Y. Dechaoui Bottein of the IAEA Environment Laboratories.
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CONTENTS

1. INTRODUCTION ............................................................................................................... 1
   1.1. THE SAXITOXIN GROUP OR PARALYTIC SHELLFISH POISONING TOXINS .... 2
       1.1.1. Organisms — ecology .................................................................................... 2
       1.1.2. Toxins ............................................................................................................ 2
       1.1.3. Toxico-pharmacology .................................................................................... 3
       1.1.4. Health effects on humans and wildlife .......................................................... 3
       1.1.5. Detection methods ....................................................................................... 4
   1.2. CIGUATOXINS OR CIGUATERA TOXINS ............................................................ 5
       1.2.1. Organisms — ecology .................................................................................... 5
       1.2.2. Toxins ............................................................................................................ 6
       1.2.3. Toxico-pharmacology .................................................................................... 6
       1.2.4. Health effects on humans and wildlife .......................................................... 6
       1.2.5. Detection methods ....................................................................................... 6
   1.3. DOMOIC ACID OR AMNESIC SHELLFISH POISONING TOXINS .................... 8
       1.3.1. Organism — ecology .................................................................................... 8
       1.3.2. Toxins ............................................................................................................ 8
       1.3.3. Toxico-pharmacology .................................................................................... 8
       1.3.4. Health effects on humans and wildlife .......................................................... 9
       1.3.5. Detection methods ....................................................................................... 9
   2. THE RADIOLIGAND RECEPTOR BINDING ASSAY FOR ALGAL TOXINS .......... 11
      2.1. OVERVIEW ....................................................................................................... 11
      2.2. PRINCIPLE ....................................................................................................... 12
         2.2.1. Receptor-ligand interaction ......................................................................... 12
         2.2.2. Radioligand measurement .......................................................................... 14
         2.2.3. Analysis of data ......................................................................................... 15
   3. MEMBRANE PREPARATION ...................................................................................... 17
      3.1. RAT BRAIN MEMBRANE PREPARATION FOR CTX AND STX RBA ........... 17
      3.2. SF9 MEMBRANE PREPARATION FOR DA RBA ............................................. 19
   4. RBA FOR PARALYTIC SHELLFISH POISONING TOXINS: SAXITOXINS ........... 20
      4.1. APPARATUS, SUPPLIES AND REAGENTS ..................................................... 20
      4.2. SAMPLE PREPARATION ................................................................................... 21
         4.2.1. STX extraction from shellfish .................................................................... 21
         4.2.2. STX extraction from phytoplankton ......................................................... 22
      4.3. RECEPTOR BINDING ASSAY PROCEDURE ..................................................... 23
         4.3.1. Preparation of stock solutions and standards for assay .............................. 23
         4.3.2. Performing the assay ............................................................................... 24
         4.3.3. Analysis of data ....................................................................................... 26
   5. RBA FOR CIGUATERA TOXINS: CIGUATOXINS ............................................... 27
      5.1. APPARATUS, SUPPLIES AND REAGENT ...................................................... 27
      5.2. SAMPLE PREPARATION ................................................................................... 27
         5.2.1. CTX extraction from 1–3 g fish samples ..................................................... 28
         5.2.2. CTX extraction from large sample size ....................................................... 29
         5.2.3. Silica solid phase extraction cleanup ......................................................... 30
      5.3. RECEPTOR BINDING ASSAY PROCEDURE ..................................................... 31
         5.3.1. Preparation of stock solutions and standards for assay .............................. 31
         5.3.2. Performing the assay ............................................................................... 32
         5.3.3. Analysis of data ....................................................................................... 34
   6. RBA FOR AMNESIC SHELLFISH POISONING: DOMOIC ACID ......................... 35
      6.1. OVERVIEW ....................................................................................................... 35
      6.2. SAMPLE PREPARATION ................................................................................... 35
1. INTRODUCTION

Harmful algal blooms (HABs) are proliferations of certain algal species that cause harm to humans, wildlife, and ecosystems through the production of toxins or by causing physico–chemical stress due to features such as cell morphology (e.g. spines), exudates (e.g. surfactants), and high biomass levels (e.g. hypoxia). HABs and their potentially devastating, wide ranging impacts are now a well established threat to marine and freshwater ecosystems and the services they provide, and these events have continued to increase in frequency, duration, and distribution over the past several decades (see reviews by Glibert et al. [1] and Anderson [2]). In the case of toxic HABs, which are the primary focus of the present IAEA sponsored effort, the causative organisms are predominantly dinoflagellates, but also include diatoms, raphidophytes, and haptophytes. These classes of microalgae are abundant in coastal marine ecosystems throughout the world and although toxigenic species represent only a small percentage of the total number described (~ 2%; [3]), new toxin producing species continue to be discovered [4] and the distribution of known taxa continues to expand [5–8]. Included among the many and varied explanations for this trend are factors such as anthropogenic nutrient enrichment, ballast water introductions, enhanced aquaculture activities, and, more recently, global climate change/variability [2, 9].

Coastal communities and economic interests (e.g. commercial/subsistence/recreational fisheries, aquaculture, and tourism) worldwide depend on fishery and aquatic resources adversely affected by HABs. Management and mitigation strategies are being developed to reduce the extent and severity of HAB impacts, with an emphasis on minimizing the risk of human exposure to contaminated seafood (see recent reviews by Anderson [2, 10]). Key among the management tools employed to protect public health are monitoring programs aimed at detecting HAB toxins as they occur in both the algae that produce them and the fishery resources contaminated by these toxins. In turn, there is a considerable effort to develop and validate reliable, low cost, high throughput methods for HAB toxin detection for use in both monitoring and regulatory applications.

Within the context of the Intergovernmental Oceanographic Commission (IOC) of UNESCO, a Harmful Algal Bloom Programme (http://ioc-unesco.org/hab/) has been developed with the aim “to foster the effective management of, and scientific research on, harmful algal blooms in order to understand their causes, predict their occurrences, and mitigate their effects.” Many resources are available through the IOC HAB Programme, including the Harmful Algal Information System (http://www.iode.org/haedat/), which when fully established will “consist of access to information on harmful algal events, harmful algae monitoring and management systems worldwide, current use of taxonomic names of harmful algae, and information on biogeography of harmful algal species.” The IAEA Technical Cooperation Programme is working to integrate the HAB management and monitoring activities of its Member States through technology transfer and capacity building in partnership with the United States National Oceanic and Atmospheric Administration (NOAA), the US Food and Drug Administration (US FDA) and the Intergovernmental Oceanic Commission (IOC) Programme in order to support the need for up to date HAB event information on a global scale. Within the framework of the IAEA Technical Cooperation Programme, the radioligand receptor binding assay (RBA) approach to HAB toxin detection has been identified as a promising technology for use by Member States in HAB research and monitoring, and ultimately in a regulatory context since its adoption as an AOAC First Action Official Method (2011.27) http://www.aoac.org. The present document outlines the theoretical and practical aspects of the RBA method as it applies to the detection of toxins causing several human intoxication syndromes, which include the Saxitoxin group, the ciguatoxins, the brevetoxins and Domoic Acid respectively responsible in human of paralytic shellfish poisoning (PSP), Ciguatera, neurotoxic shellfish poisoning (NSP) and amnesic shellfish poisoning (ASP).

A number of detailed reviews have been published on various aspects of HAB toxins, including their chemistry, toxico-pharmacology, biosynthesis, genetics, biotransformation, distribution in the environment, detection, health effects, and the organisms that produce them (e.g. Refs [11–20]).
The information presented below constitutes a very brief summary of selected topics with the aim of providing a general understanding of the toxin groups as well as outlining the need for and challenges associated with developing reliable and efficient detection methods.

1.1. THE SAXITOXIN GROUP OR PARALYTIC SHELLFISH POISONING TOXINS

1.1.1. Organisms — ecology

The organisms responsible for production of the paralytic shellfish poisoning (PSP) toxins include marine dinoflagellates within the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium*, as well as several largely freshwater cyanobacterial genera (e.g. *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*), which are outside the scope of this brief review. Dinoflagellates associated with PSP toxin production occur throughout temperate, subtropical, and tropical latitudes as is evident on a recent map documenting the global distribution of PSP toxins (Fig. 1). PSP toxin-producing *Alexandrium* spp. (e.g. *A. tamarense*, *A. minutum*, *A. tamiyavanichi*) exhibit the widest biogeographic range, extending from temperate through tropical locations, whereas *Pyrodinium* spp. (*P. bahmense* var. *compressum* and var. *bahmense*) are restricted to subtropical/tropical areas and toxic *Gymnodinium* spp. (only *G. catenatum*) occur in temperate/subtropical regions [21]. Although some of the dinoflagellates associated with PSP can achieve concentrations causing discoloration of surface waters (e.g. Ref [22]), many taxa frequently produce sufficient amounts of toxin to cause shellfish contamination and human health concerns at concentrations of only hundreds of cells per liter of seawater — levels not visible to the naked eye. Moreover, the toxicity of these organisms is highly variable and depends on the physiological or nutritional status of the cells (e.g. Refs [23, 24]). As a result of this variation in toxicity, accurately assessing the risk associated with a bloom event requires an ability to determine the quantity of toxin produced under the prevailing oceanographic conditions.


1.1.2. Toxins

The PSP toxins comprise a group of >21 trialkyl tetrahydropurine analogues of the parent compound, saxitoxin (STX), each exhibiting a unique chemical structure and corresponding toxic potency. These toxins are generally divided into three groups according to their structure and potency, dictated primarily by substitutions at the R₄ position (Fig. 2): the highly potent carbamate derivatives, the intermediate decarbamoyl analogues, and the lower toxicity N-sulfocarbamoyl forms. As a result of their structural diversity as well as wide ranging toxicity (which varies over several orders of
magnitude), detection of the PSP toxins represents a formidable challenge. In addition, the organisms synthesizing these compounds can exhibit extensive variation in both the absolute and relative amounts produced per cell, and once incorporated into a biological matrix their metabolism/interconversion can be mediated both biologically (e.g. enzymes) and chemically (e.g. pH).

![FIG. 2. Structure, nomenclature, and toxicity equivalency factors (in brackets) (saxitoxin STX = 1; STX specific toxicity = 2483 mouse units/µmol; from Oshima 1995) of selected paralytic shellfish poisoning toxins (gonyautoxin GTX, neosaxitoxin (NEO), decarbamoy-toxins (dc-)). nd = no data (after [25]).]

### 1.1.3. Toxico-pharmacology

STX and its analogues bind to site-1 on the alpha-subunit of voltage-gated sodium (Na⁺) channels, effectively blocking the channel and preventing generation of an action potential [26]. The binding affinity and thus the toxic potency of the different PSP toxins [26] vary considerably (as noted above). The apparent dissociation constant (i.e. \( K_d \)) for saxitoxin is \(~2 \text{ nM}\). The lethal dose for humans is estimated at ~1–4 mg STX equivalents. Clinical symptoms are apparent following ingestion of as little as 0.72 mg STX eq. whereas serious intoxications are caused by 0.9–3.6 mg STX eq. [27]. The onset of symptoms following exposure is rapid (< 1 h), generally beginning with paresthesias in the lips, face, and extremities, followed by drowsiness, ataxia, paralysis, and ultimately death due to respiratory failure in extreme cases. Clearance of the highly water soluble PSP toxins occurs primarily via urine. Even in patients receiving a dose sufficient to cause respiratory arrest (and maintained on a ventilator), clearance from the blood was reported to be complete within 24 hours [28] with patients making a full recovery.

### 1.1.4. Health effects on humans and wildlife

The health effects of PSP toxins on humans and wildlife have been reviewed by Landsberg [14] and Landsberg et al. [29]. Of the human intoxication syndromes associated with shellfish consumption, PSP is the most widespread with a previously reported rate of >2000 cases per year [30], although this has undoubtedly increased considering that the number of locations worldwide reporting the detection of PSP toxins more than doubled from 1990 to 2006 (Anderson [31]; http://www.whoi.edu/redtide/page.do?pid=14899). The primary route of human exposure to PSP toxins is well documented to comprise various species of bivalve mollusc, which can differ markedly in their ability to both accumulate and metabolize these compounds [32]. Nonetheless, it is also important to note that non-bivalve invertebrates have increasingly been documented to accumulate these toxins and implicated in PSP outbreaks (reviewed by Deeds et al., [33]). The limited epidemiological data in regions where either single PSP outbreaks (e.g. Guatemala) or decades of poisoning incidents (e.g. Alaska, USA) have occurred were considered by Gessner and...
McLaughlin [34]. The PSP toxins have been implicated in wide ranging wildlife mortality events involving shellfish, fish, sea birds, and marine mammals (e.g. Refs [35, 36]), including their suspected involvement in the deaths of 14 humpback whales (*Megaptera novaeangliae*) and over 100 highly endangered Mediterranean monk seals (*Monachus monachus*) (see Ref [19]). These toxins have also been detected in the endangered North Atlantic right whale (*Eubalaena glacialis*) [37], yet adverse health/reproductive effects have yet to be demonstrated conclusively. Although not generally associated with fish kills, an intense (> 7 x 10^5 cells L^-1) bloom of *Alexandrium tamarense* was identified as the probable cause of a caged salmon mortality event in Atlantic Canada [22].

By contrast, some organisms have been documented to be highly resistant to the effects of saxitoxin. Certain populations of the softshell clam (*Mya arenaria*) exhibit a mutation in an amino acid residue that reduces the affinity of STX binding to the sodium channel by 1000-fold, thereby allowing accumulation of elevated toxin levels. Another bivalve species sequestering high PSP toxin levels is the Alaskan butter clam (*Saxidomus giganteus*), albeit the mechanism for resistance remains unknown. Interestingly, sea otters (*Enhydra lutris*) endemic to this region appear capable of detecting and avoiding ingestion of these highly toxic clams, which in turn restricts the otter’s distribution to the outer Alaskan coast where *S. giganteus* is not toxic [38].

1.1.5. Detection methods

Several authors have provided reviews describing PSP toxin detection methods, including analytical, *in vitro*, and *in vivo* approaches (see Refs [18, 39–42]). A brief summary of both analytical and bioassay methods is given below. Regardless of the toxin(s) being targeted, it has become increasingly clear that there is a strong trend away from the use of live animal bioassays for toxin testing [43] due to both ethical and performance considerations. Moreover, the issue of toxicity equivalence factors in developing alternative methods remains a challenge that must be addressed from a regulatory perspective [44].

1.1.5.1. Analytical

The primary analytical approaches developed for detection of the PSP toxins are those based on liquid chromatography (LC) coupled with fluorescence (FLD) or mass spectrometry (MS) detection. Electrophoretic (capillary) separation with ultraviolet or MS detection has also been employed, but exhibit poor sensitivity due to the very small sample volumes used. Given the absence of chromophores, PSP toxins are converted to fluorescent derivatives via oxidation either before (pre-column; e.g. [45]) or after (post-column; e.g. [46]) LC separation. Both approaches provide good sensitivity with fluorescence based detection, and both methods have been validated in AOAC collaborative trials (AOAC Official Method of Analyses 2005.06 and 2011.02) and are accepted as an alternative to the AOAC mouse bioassay (AOAC Official Method 959.08) for regulatory use. Several methods coupling LC with tandem mass spectrometry have been developed, with the most successful approaches adopting hydrophilic interaction liquid chromatography (HILIC), which provides an effective means of separating polar compounds like the PSP toxins [47]. Nonetheless, detection limits for LC-MS/MS are relatively high for these toxins and such a method has yet to be validated in an interlaboratory study.

1.1.5.2. Bioassay

A variety of *in vitro* and *in vivo* bioassays have been used to detect PSP toxins, with the AOAC mouse bioassay [48] serving as the sole regulatory method worldwide until the recent validation of an HPLC-FLD technique, as noted above. *In vitro* methods include both structure and function based assays, with the former comprising mostly immunological approaches and the latter including a range of cell and receptor based tests (see reviews cited above). Several formats have been adopted for antibody based methods, including simple, rapid lateral flow devices (LFD; [49]), standard ELISA multi-well plates [50], and surface plasmon resonance platforms [51]. The qualitative LFD developed by Jellett Rapid Testing Ltd. has been approved in the US by the Interstate Shellfish Sanitation Commission and the Food and Drug Administration as a screening method (limit of detection ~40 µg
The primary cell based approach employs N2A neuroblastoma cells that have been pretreated with ouabain (Na+/K+ pump inhibitor) and veratridine (sodium channel activator) to enhance the specificity of the assay [52]. The receptor based method, a focus of the current manual, is a competitive binding assay in which radio labelled (tritiated) STX and unlabeled toxin(s) compete for Na channel binding sites in a rat brain crude membrane preparation, which has been adapted to a high throughput, microtiter plate format [53]. First evaluated in an AOAC Single Laboratory Validation [54] the receptor binding assay was recently adopted as an AOAC First Action Official Method (2011.27) [55].

1.2. CIGUATOXINS OR CIGUATERA TOXINS

Ciguatera is an intoxication linked to consumption of fish that have accumulated ciguatoxins in their tissues. Ciguatoxins are produced by the epiphytic benthic dinoflagellate of the genus *Gambierdiscus* for which drastic changes in growth distribution and frequency are reported. Indeed, *Gambierdiscus* spp., which were described as tropical or subtropical dinoflagellates, are now encountered in temperate water of the Atlantic Ocean or in the Mediterranean [56–58], Fig. 3.). In addition the intensity and frequency of *Gambierdiscus* blooms have also increased in tropical and subtropical area and new growth areas were identified in the Atlantic and Indian Ocean coasts of Africa [59, 60].

![Map showing the global distribution of ciguatera fish poisoning (CFP) toxins as of 2009. Red circles denote locations with documented measurements of Gambierdiscus spp. and or CFP toxins in fish (modified from US National Office for Harmful Algal Blooms, Woods Hole Oceanographic Institution).](image)

**FIG. 3.** Map showing the global distribution of ciguatera fish poisoning (CFP) toxins as of 2009. Red circles denote locations with documented measurements of Gambierdiscus spp. and or CFP toxins in fish (modified from US National Office for Harmful Algal Blooms, Woods Hole Oceanographic Institution).

1.2.1. Organism — ecology

The primary source of ciguatoxins (CTX), regarded as the principal cause of ciguatera, are the epibenthic dinoflagellate species in the genus *Gambierdiscus*. Geographically *Gambierdiscus* species distribute differently. *G. belizeanus*, *G. carolinianus*, *G. ruetzleri* and *Gambierdiscus* ribotypes 1 and 2 are found exclusively in the Caribbean, whereas *G. australes*, *G. pacificus*, *G. polynesiensis*, *G. toxicus*, and *G. yasumotoi* occur only in the Pacific. *G. caribaeus* and *G. carpenteri* are found in both the Atlantic and Pacific [61]. The growth of *Gambierdiscus* has been linked to elevated water temperatures and diverse types of natural disturbance, including hurricanes, coral bleaching, and anthropogenic or natural nutrient inputs; yet, the factors that govern toxin production in *Gambierdiscus* remain poorly understood. Moreover, because the various species of *Gambierdiscus* are not easily identifiable using light microscopy, the relation between species and toxin production is not well understood.
1.2.2. Toxins

At least 50 congeners of ciguatoxins have been identified in *Gambierdiscus spp.* or in fish from the Pacific, sharing the same skeleton, which consists of contiguous cyclic ether and spiroketal rings with differences in the degree of oxidation at both extremities of the molecule (Fig. 4.). In fish from the Caribbean more than 12 ciguatoxin congeners have been identified [62–65]. Yet CTXs in *Gambierdiscus* from the Caribbean remain to be characterized by LC-MS/MS. In the Atlantic Ocean, both C-CTX and P-CTX have been identified [56] and in one case, both structures found concomitantly in a single fish [57].

![FIG. 4. Chemical structure of the P-CTX-1, one of the most toxic ciguatoxin in fish from the Pacific.](image)

1.2.3. Toxico-pharmacology

Ciguatoxins target the site-5 of the voltage-gated sodium channels, causing a shift in channel activation and inhibiting normal inactivation [66, 67]. Excitation in neurons has been reported to result in enhanced release of neurotransmitters [68, 69]. In addition to modulation of sodium channel gating, block of potassium channels in dorsal root ganglion neurons [70], frog myelinated nerve fibers [71], and skeletal muscle [72] may contribute to ciguatoxin’s repetitive firing of action potential.

1.2.4. Health effects on humans and wildlife

Ciguatoxins are transferred through the food web to reach upper predators including marine mammals [73]. The harmful effect of ciguatoxins to marine wildlife is not well known although it is believed that fish would be susceptible to ciguatoxin, which would explain why fatal cases of human ciguatera are seldom.

In humans ciguatera fish poisoning is characterized by gastrointestinal and neurological signs, and to a lesser extent cardiovascular signs. Acute gastrointestinal symptoms, such as vomiting, diarrhea, nausea, and abdominal pain usually appear within 6 h of ingestion of a toxic fish and last only few days [74, 75]. The neurological symptoms take longer to develop, with an onset between 2 to 5 days, and may involve both peripheral and central features [76] with reporting of paraesthesia and a dysesthesiae. The acute neurological signs resolve without specific treatment after 2 to 3 weeks, although relapses may occur. The disease may progress with long term neurological consequences including chronic fatigue, weakness and depression. Symptoms of ciguatera largely parallel those of neurotoxic shellfish poisoning [77] caused by the closely related family of polyether toxins called brevetoxins.

1.2.5. Detection methods

The main limitation to ciguatoxin detection is the extremely low levels of toxin present in exposed animals, which is linked to its extremely high potency (estimated LD₅₀ in mice of 0.33 µg/kg intraperitoneally [78]). Nonetheless, a variety of techniques to detect and quantify ciguatoxins in fish have been developed. Beside the mouse bioassay [79], detection methods include chemical [80, 81] and immunological [82, 83] techniques as well as pharmacological assays such as the radioligand receptor binding assay [78, 84], and the cytotoxicity assay using N2A cells [52].
Chemical liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) methods have been developed for CTX detection with a detection limit of 0.03 µg/kg fish flesh. The main impediment to this methodology is the lack of purified reference standards.

Functional assays such as the N2A assay or the radioligand receptor binding assay are based on the CTX pharmacological properties to target the voltage-gated sodium channel [85], the membrane protein responsible for the propagation of the action potential in excitable cells. Selective to voltage-gated activator toxins, this assay recognizes the various ciguatoxin congeners found in the environment, critical criteria when screening tissues with a toxin profiles that varies across the food web and within the numerous species of herbivorous, detritivorous, and piscivorous fish. This assay provides hence, with high sensitivity, a summed activity measurement expressed as equivalents of a reference toxin. Toxicity equivalency factors (TEF) values ranging from 1 to 0.05 were recently attributed to the different CTXs, based on mouse intraperitoneal toxicity ([86] Table. 1)).

**TABLE. 1. TOXICITY EQUIVALENCY FACTORS ADOPTED BY THE EUROPEAN FOOD SAFETY AUTHORITY CONTAM PANEL FOR DIFFERENT CTX GROUP TOXINS BASED ON INTRAPERITONEAL (I.P.) TOXICITY (AFTER EFSA RECOMMENDATIONS ON CIGUATERA TOXINS, 2009 [86]).**

<table>
<thead>
<tr>
<th>CTX-group toxin</th>
<th>TEF</th>
<th>LD50 (i.p. in mice) μg/kg b.w.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-CTX-1</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>P-CTX-2</td>
<td></td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>P-CTX-3</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>P-CTX-3C</td>
<td>0.2</td>
<td>1.3</td>
</tr>
<tr>
<td>2,3-dihydroxy P-CTX-3C</td>
<td>0.1</td>
<td>1.8</td>
</tr>
<tr>
<td>51-hydroxy P-CTX-3C</td>
<td>1</td>
<td>0.27</td>
</tr>
<tr>
<td>P-CTX-4A</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>P-CTX-4B</td>
<td>0.05</td>
<td>4</td>
</tr>
<tr>
<td>C-CTX-1</td>
<td>0.1</td>
<td>3.6</td>
</tr>
<tr>
<td>C-CTX-2</td>
<td>0.3</td>
<td>1</td>
</tr>
</tbody>
</table>

b.w. body weight

In the United States of America, action levels for ciguatera are being listed at 0.01 ppb (µg/kg) for Pacific ciguatoxin and 0.1 ppb for Caribbean ciguatoxin (Fish and Fishery Products Hazards and Controls Guidance April 2011). Nevertheless, besides the mouse bioassay [87], none of the biological or chemical methods for ciguatoxins have been validated so far, a step necessary to move forward toward better food safety and environmental monitoring.
Amnesic Shellfish Poisoning (ASP) was first described in connection with a human poisoning outbreak in eastern Canada in 1987 that resulted in more than 100 illnesses and three deaths. The cause of this outbreak was traced to the consumption of blue mussels (*Mytilus edulis*) contaminated with domoic acid (DA), which until this time had not been associated with either seafood intoxications or the marine microalgae (i.e. *Pseudo-nitzschia spp.*) that was later identified as the source of this toxin (see Ref [88]). More than two decades have passed since this initial ASP event and considerable effort has been directed at better understanding the ecology of the causative organisms, the chemistry, toxicology and pharmacology of domoic acid, as well as its trophic transfer and health effects. A brief outline of these topics is provided below.

### 1.3.1. Organism — ecology

The vast majority of organisms confirmed to produce domoic acid are members of the diatom genus *Pseudo-nitzschia* (see Ref [89]), although DA production has been reported for one closely related *Nitzschia* species (see Ref [90]). Several excellent reviews have been published on the ecology and physiology of DA producing *Pseudo-nitzschia* species [89–92]. Of the more than 30 described *Pseudo-nitzschia*, 12 have been demonstrated to produce DA. The distribution of *Pseudo-nitzschia* ranges from Polar Regions to the tropics and representatives of this genus occur in both coastal and oceanic environments of the world’s oceans. Taxonomically, *Pseudo-nitzschia* species are being re-evaluated at molecular and morphological levels, with descriptions of new taxa and modifications to existing descriptions continuing through the present (see Ref [89]). Identification of many species requires use of electron microscopy in order to resolve the distinguishing morphological characteristics and, as a result many studies conducted at the light microscope level distinguished between the “*delicatissima*” complex (valves ≤ 3 µm wide) and “*seriata*” complex (valves > 3 µm wide) to avoid unreliable species designations. In general, *Pseudo-nitzschia* species tend to be most abundant during the spring and summer of temperate regions and growth responds quickly to nutrient input via upwelling, river discharge, and land run of that can occur episodically at different times of the year. As is the case with other HAB species, the toxicity of *Pseudo-nitzschia* species varies markedly as a function of cell physiology and can range from negligible to highly toxic during a single bloom event. Although certain environmental factors are known to influence DA production (e.g. N, Si, Fe, light), predictive models of *Pseudo-nitzschia* toxicity remain elusive.

### 1.3.2. Toxins

Domoic acid is a crystalline, water soluble amino acid possessing three carboxylic acid groups (mol. wt. 311), and aspects of its chemistry have been reviewed by Ramsdell [93]. The three carboxyl groups have pKₐ’s of 2.10, 3.72, 4.97, and the amino group 9.82, resulting in five potential charge states of the DA molecule. At physiological pH the predominant species of DA is deprotonated at all three carboxyl groups and protonated at the amino group leading to a net charge of negative 2. Notably, the degree of protonation affects the toxicity of DA to animals. Eleven isomers of domoic acid (isodomoic acids A-G; domoilactone A & B and 5′-epidomoic acid) have been described from various sources including both macro- and microalgae, as well as shellfish. The domoic acid isomers A-G are much less potent than DA. The DA lactones are considered to be inactive and 5′-epidomoic acid is reported as similar in potency to DA.

### 1.3.3. Toxico-pharmacology

Ramsdell [93] has recently reviewed the molecular and integrative basis to DA toxicity. DA is not a highly potent toxin, with its oral effectiveness limited to well in excess of parts per thousand levels of exposure. It is neither absorbed well by the gut nor does it effectively penetrate the central nervous system. DA binds with high affinity to kainate subtypes of ionotropic glutamate receptors and also activates N-methyl D-aspartate (NMDA) ionotropic receptors, leading to neuronal excitotoxicity and degeneration [93, 94]). Molecular level interactions with the receptor prevent normal inactivation of
channel opening, which leads to enhanced ion conduction. A level of excitotoxicity is reached from an integrative action on both sides of the synapse. Intracellular calcium homeostasis is not maintained in neurons exposed to DA, leading to unrestrained intracellular Ca\(^{2+}\) influx into the neurons and ultimately causing cell damage and overt neurotoxicity. The CA3 region of the hippocampus contains a high concentration of kainic acid receptors targeted by domoic acid, which overrides the normal signaling pathway. This causes cellular and ultrastructural damage to pathways underlying spatial memory and the restraint of seizure circuitry that leads to temporal lobe epilepsy. Both memory loss and seizure activity have been linked to DA poisoning in humans and wildlife, respectively.

1.3.4. Health effects on humans and wildlife

The route of DA exposure for humans is primarily via contaminated bivalve molluscs that acquire the toxin via feeding on *Pseudo-nitzschia* cells as part of their normal diet; however, other invertebrates (e.g. crabs) as well as planktivorous fish (e.g. anchovies, sardines) are well documented to accumulate DA and may be ingested by humans. The primary symptoms of DA poisoning in humans include both gastrointestinal effects (e.g. nausea, vomiting, diarrhea) and neurological effects, including dizziness, disorientation, lethargy, seizures, and permanent short term memory loss. By examining mussels consumed during the 1987 outbreak, it was determined that 1 mg/kg was sufficient to induce gastrointestinal symptoms and 4.5 mg/kg could induce neurological effects in humans [95].

Exposure of wildlife to DA has been well documented for fish, sea birds and marine mammals [96, 97], with zooplankton and planktivorous fish being reported as the most common vectors, although it is likely that certain baleen whales may be able to directly ingest the long chains formed by *Pseudo-nitzschia* cells. The effects of DA on California sea lions have been studied intensively since a mass mortality event along the central California coast in 1998 was linked to a bloom of toxic *Pseudo-nitzschia* [98]. DA has been shown to cause reproductive failure in these sea lions [99] and Ramsdell and Zabka [100] have proposed that DA poisoning of the highly susceptible fetus may have a more prominent effect later in life, manifested as a greater likelihood of developing neurological disease. Goldstein et al. [101] reported both acute and chronic DA toxicity, documenting evidence that the acute toxicity (e.g. seizures, ataxia, or coma) leads to a novel chronic epilepsy syndrome similar to temporal lobe epilepsy in humans.

1.3.5. Detection methods

1.3.5.1. Analytical

A number of analytical methods are available for the detection of domoic acid in various sample types. The globally accepted regulatory method for DA employs high performance liquid chromatography (HPLC) coupled with UV detection (AOAC Official Method 991.26). Two other HPLC-UV methods developed by Quilliam et al. [102, 103] with lower limits of detection are currently undergoing standardization in the EU (European Committee for Standardization; CEN). HPLC methods with fluorescence detection following pre-column derivatization [104, 105] have been used extensively for the sensitive detection of DA in microalgae and seawater, but this approach requires a clean-up step when used with shellfish matrices [106]. A number of LC-MS/MS methods reported as sensitive, selective and requiring minimal or no sample clean-up have been developed for DA (see Ref [107]), but none have yet been validated for regulatory use.

1.3.5.2. Bioassay

Immunoassays for DA have been developed by several investigators, whereas only the direct ELISA of Kleivdal et al. [108] has been fully validated with an AOAC collaborative trial. This assay is recognized as AOAC Official Method 2006.02, and is now accepted in the EU as an alternative to the HPLC-UV method. The so called ‘ASP ELISA’ has a limit of quantification (LOQ) of 0.01 mg/kg, well below the regulatory action level of 20 mg/kg, and was validated for its application on mussels, scallops, and oysters. A second antibody based approach formatted for use on a surface plasmon resonance detector was developed by Traynor et al. [109] and, while sufficiently sensitive for
screening purposes, has not been validated in an interlaboratory study. A competitive microplate radioligand receptor binding assay using a recombinant rat GLUR6 glutamate receptor [110] was selected for inclusion in the current manual. Although this receptor based assay has the required sensitivity and sample throughput needed for regulatory application, it has not been validated through a comparative interlaboratory study.
2. THE RADIOLIGAND RECEPTOR BINDING ASSAY FOR ALGAL TOXINS

2.1. OVERVIEW

The radioligand receptor binding assay (RBA) is a functional bioassay developed for an array of marine toxins including saxitoxins (STXs), ciguatoxins (CTXs), brevetoxins (PbTxs), and domoic acid (DA). Specific and sensitive, this method was optimized into a high throughput format and is used as a monitoring or forecasting method [111–113] as well as diagnostic tool [114] in a number of research laboratories all over the world. In addition, over the last decade the RBA technology has been transferred and implemented through IAEA Technical Cooperation Projects to developing countries from Asia Pacific, Africa and Latin America regions.

The RBA is an excellent candidate for fulfilling the requirements of high throughput, quantitative analysis that reports a composite toxic potency in a manner analogous to the mouse bioassay. The method is based on the specific interaction between the toxins and their pharmacological target, i.e. the voltage gated sodium channel, site-1 for Paralytic Shellfish Poisoning toxins (STXs), site-5 for Neurotoxic Shellfish Poisoning (PbTxs) and Ciguatera toxins (CTXs), and the glutamate receptor for Amnesic Shellfish Poisoning (DA) toxins. All toxin congeners bind with varying affinity therefore the radioligand receptor binding assay can be used to measure the combined toxicity in a sample, independently of the particular toxin congeners present. In the assay, a radiolabeled toxin competes with unlabeled molecules from analyzed sample, for a finite number of available receptor sites. When the binding equilibrium is reached, free [3H] toxins are removed by filtration and collected receptor-bound [3H] toxins are quantified by liquid scintillation counting (LSC). The reduction in [3H] toxin binding is directly proportional to the amount of unlabeled toxin(s) present in the sample. A standard curve is generated using increasing concentrations of toxin standard solution and the concentration of toxin in samples is determined in reference to the standard curve. The PSP assay being employed in IAEA Technical Cooperation Programs has been adopted by the Association of Official Analytical Chemists (AOAC) International as an Official Method of Analysis, OMA-2011.27, following an international collaborative study of method performance [55] and is a modification of the method of Doucette et al. [53]. The domoic acid assay is a modification of the method developed by Van Dolah et al. [110] and the assay for ciguatoxins/brevetoxins is a modification of the method described in Van Dolah et al. [115]. In each case, a 96-well microtiter plate format is used to minimize error by reducing sample handling and pipetting steps. Use of the microtiter plate format, in conjunction with microplate scintillation counting, makes the assay potentially suitable for use in a high throughput regulatory setting. The RBA can potentially be applied to a variety of matrices: algal samples, fluids such as urine, blood or serum, stomach content, feces or tissue such as liver and muscles are the most commonly used. The RBA methodology from sample preparation to results analysis is illustrated in Fig. 5 and specific information related to sample size, extraction procedure and assay details will be provided under each RBA section.
2.2. PRINCIPLE

Receptors are proteins (biological macromolecules) found in plasma membranes, which are involved in receiving signals directly from outside the cell or from within the cell. Receptors play a critical role in the regulation of cellular activity by specific molecules (ligands) such as hormones, neurotransmitters and other modulators including toxins. The interaction of ligands with a receptor may influence cellular activity through a change in membrane permeability, a change in enzyme activity or an alteration in gene transcription.

In the nervous system two types of ion channels play key roles in the transmission and integration of signals: the voltage-gated ion channels that open in response to changes in voltage or the distribution of charges across the cellular membrane; and the ligand-gated ion channels that respond to specific molecules, the neurotransmitters such as acetylcholine, glutamate, etc.

The ion channels located on cell surfaces are readily accessible for interaction with extracellular molecules including drugs and toxins. Many toxins target ion channels and marine algal toxins interfere mainly with the physiological function of voltage-gated sodium channels. For instance, saxitoxins, tetrodotoxins, and µ-conotoxins interact with the same site-1 on the molecule and block the pore of the sodium channel, hindering the ion flux. Thus, radiolabeled tetrodotoxin and µ-conotoxins can be used as alternative radioligands to saxitoxin in receptor binding assays. Similarly, brevetoxin has been used as a ligand for detecting the presence of ciguatoxin by RBA because both these toxins bind to site-5 of voltage-gated sodium channels. Regarding the domoic acid, this molecule acts as an agonist of the kainate subtype of glutamate receptors and can therefore be assayed by using radiolabeled kainic acid (KA) as a ligand in RBA. Being a functional assay, the RBA will give positive results for toxins that act at the same site as the ligand being tested.

2.2.1. Receptor-ligand interaction

The simplest representation for the combination of a ligand, L with a receptor, R is:

\[ L + R \overset{k_1}{\rightleftharpoons} LR \]

where \( k_1 \) and \( k_{-1} \) are the rate constants for the forward and backward reactions.
The rate of the reaction is proportional to the product of the concentration of two reactants, and at equilibrium, \( k_1[L][R] = k_{-1}[LR] \)

Based on this relationship and its variations, several equations (Hill plot, Hill-Langmuir equation, Scatchard plot, Schild equation) have been derived allowing the quantification of ligand binding to macromolecules. With the use of radio labelled ligands, it is possible to increase the sensitivity of the assay to femto-molar \((10^{-15} \text{ M})\) levels. Radioligand binding studies can be used for measuring receptor density and association/dissociation rate constants. Binding competition experiments (as in RBA) can be applied for the determination of the toxin affinity for one receptor and then for the quantification of this toxin in a given sample.

The binding of ligands to a particular receptor is termed specific binding, whereas the binding to non-receptor tissue components is referred to as non-specific binding. As shown in Figure 6, specific binding is saturable whereas nonspecific binding is non-saturable.

\[
[L^*] = \text{concentration of unbound ligand} \\
[L^*R] = \text{concentration of ligand-receptor complex} \\
S = T - NS, \text{ where} \\
S = \text{Specific binding} \\
T = \text{Total binding} \\
NS = \text{Non-specific binding}
\]

**FIG. 6. The binding of ligand to a receptor preparation**

Competition binding assays are based on the displacement of radioligand by the unlabeled ligand or toxin as indicated below:

\[
\begin{align*}
L & \\
+ & \\
L^* + R & \leftrightarrow L^*R \\
& \\
& LR
\end{align*}
\]

\[
B_o = \text{Amount of radioligand bound in the absence of inhibitor or toxin} \\
B_i = \text{Amount radioligand bound in the presence of different amounts of inhibitor or toxin}
\]

A concentration of radioligand is chosen from the linear portion of the graph for specific binding shown in Fig. 6. The binding of the radioligand to the receptor (formation of the \(L^*R\) complex) is reduced in the presence of different concentrations of the unlabeled ligand (the inhibitor or toxin) as shown in Figure 7. This phenomenon of competitive inhibition is the basis for radioligand receptor binding assays used to quantify the amount of toxin in test samples.
2.2.2. Radioligand measurement

2.2.2.1. Scintillation counting background

The radioligand corresponds to the toxin radiolabeled with tritium $[^3H]$. Tritium is a pure $\beta$-emitter. The nucleus of tritium is composed of one proton and two neutrons. One neutron decays into a proton, with the ejection of a $\beta$ particle and an anti-neutrino, transforming tritium into the stable element helium. The maximum energy emitted by the tritium is actually quite low, i.e. 18.6 keV. This energy can be easily absorbed by the compound itself, by the surroundings and by covers on detecting equipment. As a result of difficulty of detecting the low energy levels generated by the beta emission, the technique of liquid scintillation counting was developed.

The scintillation counting process is relatively simple. The radioactive sample is added to the liquid scintillation cocktail composed of an organic solvent, a fluorescent solute (or Fluor) and an emulsifier that ensures proper mixing of aqueous samples. The $\beta$ particle emitted by $^3$H in the samples excites the solvent molecules. The energy of the solvent molecules is transferred to the Fluor, which in turn emits light in order to return to the ground state. The energy emission of the solute (the light photon) is converted into an electrical signal by a photomultiplier tube. The entire process takes about one nanosecond, so the flash of light from a single beta particle is seen by the counter as single “pulse”. The energy (voltage) is proportional to the number of photons developed by the beta particle emission event. Summation of a series of such pulses for a given isotope creates a curve proportional to its energy profile.

2.2.2.2. Scintillation counting: experimental consideration

The vial or microplate containing the scintillation cocktail and the radioactive sample is placed inside a scintillation counter for the measurement of the radioligand activity. Several physical processes interfere with the efficiency of liquid scintillation counting, i.e. chemiluminescence produced by reaction between the sample and the scintillation cocktail, photoluminescence and other general processes usually referred as background. To reduce the effects of both chemiluminescence and photoluminescence, samples should be equilibrated for several hours in the counter (in the dark) before counting. Along with these physical processes, the biggest problem encountered by scintillation counting is fluorescence quenching (process which decreases the fluorescence intensity) by chemical impurities or absorption of emitted photons by colored samples.
Scintillation signal is measured in counts per minute (cpm). Quenching and absorption are never entirely absent from a sample, meaning that the signal in cpm detected is less than the actual decay rate expressed as disintegrations per minute (dpm). The use of internal standards with the observed cpm and the known decay rate (dpm) allows the determination of the scintillation process’s counting efficiency, assuming that an added standard of the same isotope than the one present in the sample will be quenched in a fashion and in amount similar to the unknown sample.

2.2.3. Analysis of data

Curve fitting is performed using a four parameter logistic fit, also known as a sigmoidal dose response curve (variable slope, Fig. 8.), or Hill equation:

\[
y = \text{min} + \frac{\text{max} - \text{min}}{1 + 10^{(x - \log EC50) / \text{Hill slope}}}
\]

where max at the top plateau corresponds to the bound radioligand (in counts per minute -CPM) in the absence of unlabeled toxin, and min at the bottom plateau corresponds to the non-specific binding in presence of saturating unlabeled toxin, EC\text{50} is the effective concentration at 50%, also known as IC\text{50} inhibitory concentration at 50%, Hill slope is the slope of the linear portion of the curve, x is the log concentration of unlabeled toxin, and y is the bound labeled toxin expressed in CPM.

A curve fitting package such as Prism (Graph Pad Software, Inc.) or manufacturer applications (such as Wallac MultiCalc (Perkin-Elmer Wallac) or Packard Top Count software) is recommended for the data analysis.

\[
EC_{x} = \left(\frac{X}{100 - X}\right)^{1 / \text{slope}} \cdot EC_{50}
\]

Where X is the desired concentration

Example: to calculate the EC\text{20}, if given the slope and EC\text{50} values:

\[
X = 20
\]
\[
\text{Slope} = 1.1
\]
\[
EC_{50} = 3.0 \text{ nM}
\]
\[
EC_{20} = \left(\frac{20}{100 - 20}\right)^{1 / 1.1} \cdot 3.0
\]

Assay Quality Control Points

The following criteria must be met for assay acceptance:

1. For a ligand (or toxin) that interacts specifically at one receptor site, the slope of the resulting standard curve should theoretically be 1.0. If the slope of the curve for a given assay is outside of the acceptable range of 0.8–1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect. Therefore, the assay should be re-run.

2. Relative standard deviations (RSDs) of the data measured using standard toxin must be below 30% as variability will affect quantification of samples.

3. The RSDs of the quality control (QC) check should be ≤ 30% of the stated value (RSDs and QC evaluation are further detailed in the experimental section of the manual)
The following criteria must be met for acceptability of a sample measurement:

1. Sample quantification should be done only on dilutions that fall on the linear part of the
   competition curve (B/B₀ = 0.2–0.7).

2. In the case that no sample dilutions fall within the linear range (i.e., concentration is too
   high, resulting in B/B₀ < 0.2), further dilutions must be made and the sample reanalyzed. In
   the case that the sample concentration is too low to be quantified (i.e., B/B₀ > 0.7), the
   sample is reported as below limit of detection.

3. RSD of the data must be ≤30%.
3. MEMBRANE PREPARATION

3.1. RAT BRAIN MEMBRANE PREPARATION FOR CTX AND STX RBA

Apparatus
- Teflon/glass homogenizer (i.e., motorized Teflon pestle and glass tube);
- Motorized tissue homogenizer (Polytron or small handheld blender);
- High speed centrifuge and fixed angle rotor capable of 20,000 x g (rcf);
- Centrifuge tubes (12–15 mL) rated for > 20,000 x g (rcf);
- 2 mL plastic cryovials;
- 300 or 500 mL graduated beaker;
- Disposable 5 and 10 mL pipettes;
- Forceps for handling rat brains.

Reagents
- 20 rat brains, male Sprague-Dawley, six wk old (Hilltop Lab Animals);
- 1L buffer pH 7.4: 100 mM MOPS, pH 7.4 (Sigma, St. Louis) containing 100 mM choline chloride (Sigma), 0.1 mM PMSF*
  *PMSF (phenyl methylsulfonyl fluoride, Sigma) stock preparation (0.1 M): dissolve 0.0174 g PMSF in 1 mL isopropanol; a larger volume may be prepared and stored in 1 mL aliquots at -20°C (e.g. 0.174 g in 10 mL isopropanol). Prior to use thaw PMSF and thoroughly mix to redissolve completely; then add to 1L buffer fresh daily at each use. Do not refreeze aliquots.
- Protein Assay Reagent Kit: the protein concentration of the membrane preparation is determined using the Micro BCA (Pierce Micro BCA Protein Assay Reagent Kit #23235, microplate method) or #23225 (tube method) protein assay kit or equivalent protein assay.
Procedure

1. Remove medulla from each brain using ‘pointed’ forceps and discard.
2. Place cerebral cortices in ice-cold buffer pH 7.4 and place container on ice. Homogenize two brains in 25 mL buffer pH 7.4 (12.5 mL per brain) in a glass homogenizer tube with a Teflon probe. Homogenize with 10 up and down strokes at 385 rpm on ice slurry. Keep tube in ice at all times. Pour homogenized tissue into 250 mL beaker on ice and repeat procedure with remaining cortices.

3. Pool homogenized tissue and transfer to high speed centrifuge tubes. Centrifuge at 20,000 x g for 15 minutes at 4°C. Quickly aspirate (or pour off) supernatant and resuspend pellets in 10 mL buffer pH 7.4 per brain (200 mL for 20 brains).

4. Pool resuspended membrane preparation in a 500 mL beaker and keep on ice. Polytron at 70% full speed for 20 seconds.

5. Aliquot 2 mL/tube in cryogenic vials: cryotube must be kept on ice and mixed prior each aliquot. Freeze and store at -80°C. This preparation is stable for at least 6 months.

6. Determine protein concentration of the membrane preparation and hence the dilution needed for a final 1 mg/mL protein concentration in the assay.
3.2. SF9 MEMBRANE PREPARATION FOR DA RBA

Apparatus
- High speed centrifuge and fixed angle rotor capable of spinning at 21,000 RCF (x g) at 4 °C.
  - 500 W sonicator with microprobe
  - High speed centrifuge tubes (12–15 mL capacity) capable of withstanding speeds up to 21,000 RCF (x g)
  - 2 mL plastic cryovials
  - disposable pipettes for buffer handling
  - Analytical balance to ensure equal weight of tubes at high speed

Reagents
- Cell pellet from transfected sf9 cells provided by TexCell (pellet equivalent to 200 mL of cells). This is kept at -80°C until thawed.
  - Ice
  - 1L Tris buffer buffer pH 7.1 with 0.1mM PMSF

  *PMSF (phenyl methylsulfonyl fluoride, Sigma) stock preparation (0.1 M): dissolve 0.0174 g PMSF in 1 mL isopropanol; a larger volume may be prepared and stored in 1 mL aliquots at -20°C (e.g. 0.174 g in 10 mL isopropanol). Prior to use thaw PMSF and thoroughly mix to redissolve completely; then add to 1L buffer fresh daily at each use. Do not refreeze aliquots.

Procedure
1. Prec cool high speed centrifuge to 4°C. Set speed to 21,000 RCF (xg) and insert fixed angle rotor.
2. Remove 15 mL tube of cells from -80°C freezer and thaw on ice. Assumption is that these cells are equal to 200 mL Sf9 cells.
3. After cells are completely thawed, sonicate in ice bath using probe sonicator at setting 6 for short bursts adding up to 30 seconds.
4. Distribute sonicated cells evenly into 8 high speed centrifuge tubes and fill to the top with 50mM Tris buffer (pH 7.1) containing 0.1mM PMSF. It is important to fill tubes to the top and evenly because if not, they will collapse during centrifuging. Check weights of tubes on a balance.
5. Spin tubes for 20 minutes at 4°C at 21,000 RCF.
6. Retrieve tubes from centrifuge and decant supernatant into sink. Add 1mL Tris to each tube and vortex to pop pellets off the bottom of the tube. Combine pellets into one tube and break pellets into very small pieces using a small stirring rod to homogenize distribution.
7. Redistribute the 8 mL of prep into 8 new centrifuge tubes and fill each tube with Tris buffer as in step 4.
8. Repeat steps 5–7 two more times, for a total of three spins in the ultracentrifuge. Be sure to balance tubes in the centrifuge.
9. After the third spin, combine and mix pellets as in step 6, then resuspend prep to 20 mL in a 50 mL centrifuge tube.
10. Sonicate on ice bath in bursts adding up to 30 seconds at setting 6.
11. Prepare 20 1 mL aliquots in 2 mL Nalgene cryovials. Label with correct batch number and date. Store at -80°C for future use. Note: This is a suspension, not a solution, so mixing is essential when making aliquots.
4. RBA FOR PARALYTIC SHELLFISH POISONING TOXINS: SAXITOXINS

4.1. APPARATUS, SUPPLIES AND REAGENTS

Apparatus and Supplies
- Microplate scintillation counter
- Micropipettes (1–1000 µL variable volumes) and disposable tips
- 8 channel pipettor (5–200µL variable volume) and disposable tips
- 96 well microtiter filter plate with FB glass fiber filter/ 0.65 µm pore size
- Duropore support membrane (Millipore, Bedford)
- Multiscreen vacuum manifold (Millipore)
- Vacuum pump or house vacuum
- 15 and 50 mL conical plastic centrifuge tubes
- Mini-dilution tubes in 96-tube array
- Solvent reservoirs
- Ice bucket and ice
- Vortex mixer
- Plate sealing tape (Millipore)
- 1L volumetric flask
- -80°C freezer
- -20°C freezer
- Refrigerator
- pH meter or pH paper
- Hot plate
- 10 mL graduated cylinder
- Centrifuge for 15 mL tubes

Reagents
- 0.1 and 1 M hydrochloric acid (HCl)
- 0.1 M NaOH
- Water, deionized (dH₂O; 18 µOhm)
- [3H] STX (0.1 mCi/mL, 24 Ci/mmol, >90% radiochemical purity; ARC)
- STX diHCl reference standard (NIST RM 8642 (www.nist.gov)).
- 100 mm MOPS, pH 7.4, 100 mm choline chloride
- Rat brain membrane preparation
- Liquid Scintillant: Optiphase liquid scintillation cocktail (Perkin-Elmer Life Sciences)
4.2. SAMPLE PREPARATION

4.2.1. STX extraction from shellfish

1. Shuck shellfish to obtain ~ 100 g of meat. Drain on a sieve and homogenize 3 minute in a blender. Homogenate may be stored frozen at -20°C.

2. Accurately weigh 100 g tissue homogenate into a tared 500 mL beaker.
3. Add 100 mL of 0.1 N HCl, stir thoroughly and check pH (Fig. 10 Right). The pH should be < 4.0, preferably 3.0. If necessary, adjust pH as described below.

4. Heat mixture, boil gently for 5 minutes, and cool to room temperature. Adjust cooled mixture to pH 3.0-4.0 (never > 4.5) as detected by a pH meter or pH paper. To lower pH, add 5 N HCl dropwise with stirring; to raise pH, add 0.1 N NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin.
5. Transfer mixture to graduated cylinder and dilute to 200 mL.

6. Return mixture to beaker, stir to homogeneity, and let settle until a portion of the supernatant is translucent.
7. Pour approximately 15 mL of the supernatant into a centrifuge tube. Centrifuge at 3,000 x g or higher for 10 minutes.
8. Filter supernatant through 0.45 µm nylon filter (may need GF/F glass fiber pre-filter). Store extracts at -20°C until tested with receptor binding assay.
4.2.2. STX extraction from phytoplankton

1. Tare dry grinding/extraction tube, place filter at bottom, record weight.
Add desired amount of 0.1 N HCl to tube, record weight and mix.
*Note: Use 2.5 mL 0.1 N HCl for 25 mm filters, 5 mL for 47 mm filters*

2. Break up filter by grinding with Teflon pestle at 250 rpm for 1 min (2 min for 47 mm filters). Wash pestle with 0.1 N HCl between samples.
Centrifuge for 1 min at 45 x g.
Rinse upper edges of tube with supernatant.

3. Sonicate on ice slurry for 1 min for 25 mm filters (2 min for 47 mm filters) using 500 W sonic disrupter with a microprobe at the maximum allowable setting. Rinse microprobe with 0.1 N HCl between samples.

4. Heat tubes to 100°C in a boiling water bath for 7 min.
Transfer sonicated and boiled sample to a 15 mL conical tube and centrifuge for 5 min at 700 x g to pellet filter particles.
Remove supernatant using a 3 or 10 cc syringe and pass through a 0.22 µm filter into a 2 or 5 mL cryogenic vial.
Store samples at -20°C until analysis.
4.3. RECEPTOR BINDING ASSAY PROCEDURE

4.3.1. Preparation of stock solutions and standards for assay

**Assay Buffer MOPS (100nM) choline chloride (100nM), pH 7.4**
- weigh out 20.9 g of MOPS (3-morpholinopropanesulfonic acid) and 13.96 g of choline chloride and add to 900 mL dH2O
- adjust pH to 7.4 with NaOH while stirring
- transfer quantitatively to volumetric flask and bring to a final volume of 1 L with dH2O
- store at 4°C

**Radioligand solution: [3H]STX**
Stock [3H]STX is provided in 50 µCi ampoules, 24 Ci/mmol, 0.1 mCi/mL (equivalent to 4.17 µM).
- Prepare a 15 nM working solution of [3H]STX for each assay fresh daily: 14 µL of stock [3H] STX + 3.86 mL Assay Buffer (this will provide a 2.5 nM final concentration in microplate wells). Note that the amount of [3H] STX added may vary according to the specific activity of the stock [3H]STX.
- Measure total counts of each working solution prior to running an assay: add 35 µL of the [3H]STX working solution to a liquid scintillation vial with 4 mL scintillant and count using a liquid scintillation counter. A volume of 35 µL of the [3H]STX working solution should contain 27,650 dpm. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day to day and within 15% of the expected value.

**Unlabelled STX reference standard**
STX diHCl reference standard NIST RM 8642 is provided at a concentration of 268.8 µM (100µg/mL).
A “bulk” serial dilution for the standard curve is made up in advance and stored at 4°C for up to 1 month. The use of bulk reference dilutions minimizes the pipetting needed for setting up an assay routinely and improves day to day repeatability.

To make up the standard curve perform the following STX diHCl serial dilutions in 0.003 M HCl (from a 3 M stock, 50 µL in 50 mL)

| Stock | In assay
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl</td>
<td>6x10^-6 M 1x10^-6 M</td>
</tr>
<tr>
<td>500 µL 6x10^-6 M + 4.5 mL 0.003 M HCl</td>
<td>6x10^-7 M 1x10^-7 M</td>
</tr>
<tr>
<td>1.5 mL 6x10^-7 M + 3.5 mL 0.003 M HCl</td>
<td>1.8x10^-7 M 3x10^-8 M</td>
</tr>
<tr>
<td>500 µL 6x10^-7 M + 4.5 mL 0.003 M HCl</td>
<td>6x10^-8 M 1x10^-8 M</td>
</tr>
<tr>
<td>500 µL 1.8x10^-7 M + 4.5 mL 0.003 M HCl</td>
<td>1.8x10^-8 M 3x10^-9 M</td>
</tr>
<tr>
<td>500 µL 6x10^-8 M + 4.5 mL 0.003 M HCl</td>
<td>6x10^-9 M 1x10^-9 M</td>
</tr>
<tr>
<td>500 µL 6x10^-9 M + 4.5 mL 0.003 M HCl</td>
<td>6x10^-10 M 1x10^-10 M</td>
</tr>
<tr>
<td>5 mL 0.003 M HCl</td>
<td>0 M Reference</td>
</tr>
</tbody>
</table>

*All standards are diluted 1/6 in the assay.

**Unknown samples**
Perform two initial dilutions at 1:10, 1:50 and 1:200 in 0.03M HCl of unknown samples (extracted and stored in 0.1N HCl) of using dilution tube (Fig. 9.)

*Fig. 9. Dilution tubes*
Inter-assay calibration standard (QC check):
A reference standard containing $1.8 \times 10^{-8} \text{ M STX}$ ($3.0 \times 10^{-9} \text{ M STX}$ in assay) is prepared in advance in 0.003 M hydrochloric acid and kept frozen (-80ºC) in 0.5 mL to 1 mL aliquots for long term storage. Aliquots should be thawed and stored at 4ºC for routine use (stable up to 1 month) and included undiluted (35 µL) in each analysis (triplicate wells). This serves as a QC check and confirms day-to-day performance of the assay.

Rat brain membrane preparation:
- Add 2mL thawed rat membrane preparation to 18 mL MOPS, vortex
- Pour diluted prep into empty solvent reservoir

4.3.2. Performing the assay

4.3.2.1. Plate setup and incubation

When possible, use a multichannel pipette to minimize pipetting effort and increase consistency (Fig.10.). Standard curve, QC check, and sample extracts are run in triplicate. Multiple dilutions of sample extracts need to be analyzed in order to obtain a value that falls on the linear part of the competition curve for quantification.

For ease of analysis, it is convenient to use a plate layout which maximizes the number of samples and reference standards that can be analyzed on one plate (a layout example is provided in Fig. 11.) For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects while still providing a limit of quantification of approximately 30 µg/kg shellfish:

![Fig.10. Plate setup using a multichannel pipette](image)

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>$10^{-6}$</td>
<td>$10^{-6}$</td>
<td>$10^{-6}$</td>
<td>QC</td>
<td>QC</td>
<td>QC</td>
<td>U3</td>
<td>1:50</td>
<td>U3</td>
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<td>U2</td>
<td>1:10</td>
<td>U5</td>
<td>1:10</td>
<td>U5</td>
</tr>
<tr>
<td>F</td>
<td>$10^{-9}$</td>
<td>$10^{-9}$</td>
<td>$10^{-9}$</td>
<td>U2</td>
<td>1:50</td>
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<td>1:50</td>
<td>U5</td>
<td>1:50</td>
<td>U5</td>
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<td>$10^{-10}$</td>
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<tr>
<td>H</td>
<td>REF</td>
<td>REF</td>
<td>REF</td>
<td>U3</td>
<td>1:10</td>
<td>U3</td>
<td>1:10</td>
<td>U3</td>
<td>1:10</td>
<td>U5</td>
<td>1:200</td>
<td>U5</td>
</tr>
</tbody>
</table>

U = unknown sample
Add in the following order to each of the 96 wells:
- 35 μL assay buffer (assay buffer is added first in order to wet the filter membrane)
- 35 μL STX standard, QC check, or sample extract
- 35 μL [³H] STX
- 105 μL membrane preparation
- Cover and incubate plate at 4°C for 1 hour.

4.3.2.2. Assay Filtration and Counting

Assay Filtration
1. Place 96-well plate on the MultiScreen vacuum manifold. Fill empty wells with 200 μL of assay buffer to ensure even filtration across plate
2. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2-5 sec. Pull contents of all wells through until liquid is removed.
3. With vacuum still running, quickly rinse each well twice with 200 μL ice cold assay buffer using multichannel pipette

Counting
4. Remove the plastic bottom from the plate. Gently blot the bottom once on absorbent toweling
5. Place microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape
6. Add 50 μL scintillation cocktail per well using multichannel pipette
7. Seal top of plate with sealing tape.
8. Allow to sit a minimum of 1 hour at room temperature
9. Tag the plate
10. Count using a microplate scintillation counter
4.3.3. Analysis of data

Sample quantification is carried out only on dilutions that fall on the linear part of the competition curve \((B_i/B_0 = 0.2-0.7)\). Principles and assay Quality Control acceptance are detailed in the chapter 2.2.3 Analysis of Data, Page 15 of this manual.

Where more than one dilution falls on the linear part of the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in \(\mu g\) STX equivalents/kg shellfish, using the following formula:

\[
\text{(nM STX equiv)} \times \text{(sample dilution)} \times \left( \frac{210 \mu L \text{ total volume}}{35 \mu L \text{ sample}} \right) = \text{nM STX equiv in extract}
\]

\[
\text{(nM STX equiv in extract)} \times \left( \frac{1 L}{1000 mL} \right) \times \left( \frac{372 \text{ ng}}{1 \mu g} \right) = \mu g \text{ STX equiv/mL}
\]

\[
\mu g \text{ STX equiv/mL} \times \left( \frac{\text{mL extract}}{\text{g shellfish extracted}} \right) \times 1000 = \mu g \text{ STX equiv/kg}
\]
5. RBA FOR CIGUATERA TOXINS: CIGUATOXINS

5.1. APPARATUS, SUPPLIES AND REAGENT

Apparatus and Supplies
- Scintillation counter
- Micropipettors (1-1000 µl variable volumes) and disposable tips
- 8 channel pipettor (5-200µl variable volume) and disposable tips
- 96-well microtiter filter plate with fb glass fiber filter/ 0.65 µm pore size
- Duropore support membrane (millipore, bedford)
- Multiscreen vacuum manifold (millipore)
- Vacuum pump or house vacuum
- 15 and 50 mL conical plastic centrifuge tubes
- Mini-dilution tubes in 96-tube array
- Solvent reservoirs
- Ice bucket and ice
- Vortex
- Plate-sealing tape (millipore)
- 1 l volumetric flask
- -80°c freezer
- -20°c freezer
- Refrigerator
- Centrifuge for 15 mL tubes
- Tissue homogenizer

Reagents
- \[^3\text{H}]PbTx-3\) (15.3 µM) stored at -20°C
- PbTx-3 reference standard (100 µM) stored at 4°C
- PBST buffer with BSA 1mg/mL stored at 4°C
- Rat membrane prep (10.6 mg protein/mL) stored at -80°C
- Liquid Scintillant: Optiphase liquid scintillation cocktail (Perkin-Elmer Life Sciences)

5.2. SAMPLE PREPARATION
### 5.2.1. CTX Extraction from 1–3 g fish samples

**TISSUE SAMPLE PREPARATION**
- Dissect and heat sample of interest in a 70°C water bath for 15-30 min (1).
- Mince and homogenize using a tissue homogenizer (2)
- Homogenate may be stored frozen at -20°C

**ACETONE EXTRACTION**
- Add sample/homogenate into a 50-mL tube with 3 volumes of acetone (or 1:3, w:v).
- Sonicate with sonicator probe for 2 min (3).
- Centrifuge at 3,000 g for 10 min
  - Draw off supernatant S1 into a 50 mL tube
- Resuspend pellet in 3 volumes of acetone.
- Sonicate, centrifuge as above
  - Draw off supernatant S2, combining S2 + S1 in the 50 mL tube
- Resuspend pellet in 3 volumes of acetone.
- Sonicate, centrifuge as above
  - Draw off supernatant S3, combining S3+S2+S1 in the 50 mL tube
- Evaporate acetone under nitrogen flux (<40 °C, <10 psi).

**HEXANE WASH**
- Resuspend sample by adding 8 mL of 90% MeOH (sonicate if needed).
- Add 8 mL of hexane, cap and gently invert tube 15-20 times (4).
- Allow layers to separate
- Discard hexane top layer with a Pasteur pipette and keep MeOH fraction
- Re-extract MeOH layer with hexane
- Discard the top layer
- Evaporate the MeOH filtrate under nitrogen flux (<40 °C, <10 psi), (5)

**ETHER EXTRACTION**
- Resuspend the sample in 5 mL of 25% EtOH in a 20 mL tube
- Add 5mL diethyl ether. Cap and gently invert tube (15-20 times).
- Allow layers to separate
- Transfer the top ether layer into a separate 20-mL glass tube using a Pasteur pipette and keep the EtOH bottom layer.
- If sample separates in 3 layers, centrifuge the tube 5 min at 3,000g, at 4°C.
- Repeat the extraction of the EtOH layer 2 more times
- Dry the combined 3 ether fractions under nitrogen (Nitrogen flux, 40 °C, <10 psi (5)).
5.2.2. CTX extraction from large sample size

**TISSUE SAMPLE PREPARATION AND ACETONE EXTRACTION**
Dissect and heat samples in a 70°C water bath for 15-30 min (1).
Mince tissue sample and homogenize in a blender with 1mL /g tissue of acetone (or 1:1, w:v); (2).
Sonicate with sonicator probe for 2 min.
Vacuum filter through Watman #1 filter paper
Repeat acetone extraction 2 more times, combining filtrates in a glass container.
Chill overnight at –20°C to precipitate the lipids
Filter the extract while still cold and transfer to a round bottom flask
Evaporate acetone with rotovap (3) -(some liquid may remain due to water being present in the sample.

**HEXANE WASH**
Resuspend sample by rinsing round-bottom flask 3 times with 90% MeOH (total volume of 0.5 mL per gram of tissue or 2:1, w:v). Sonicate in sonicator bath if needed. Combine MeOH rinses in separatory funnel (4).
Shake with equal volume of hexane (90% MeOH:hexane 1:1 v:v).
Allow layers to separate and collect MeOH bottom fraction into a clean container. Discard the top hexane layer.
Re-extract the MeOH layer with hexane
Evaporate MeOH extract on rotovap.

**ETHER EXTRACTION**
Resuspend sample by rinsing round-bottom flask 3 times with 25% EtOH (total volume of 0.5 mL per gram of tissue or 2:1, w:v). Sonicate in sonicating bath if needed. Combine EtOH rinses in a separatory funnel.
Extract the EtOH sample 3x with diethyl ether
Combine the diethyl ether and the EtOH (1:1, v:v) sample in a separatory funnel and gently shake.
Allow layers to separate and collect the EtOH bottom layer and the ether top layer into separate containers.
Repeat the extraction of the EtOH bottom layer 2 more times, combining the ether fractions into a round bottom flask
Dry ether sample on the rotovap.
Resuspend sample by rinsing round bottom flask with 100% MeOH. Store at -20°C
5.2.3. Silica solid phase extraction cleanup

1. Dilute the sample in chloroform (2 uL/mg, min vol 100uL) ~1mL
2. Condition a 500 mg silica cartridge with 5mL Hexane followed by 5 mL Chloroform
3. Transfer the sample to the SPE cartridge using a glass Pasteur Pipette. Allow the sample to pass by gravity. Rinse the tube which contained the sample with same volume of chloroform (~1mL) and allow passing by gravity.
4. Wash the SPE cartridge with 5 mL chloroform
5. Elute with 10% MeOH in Chloroform (5 mL)
6. Dry the eluate under nitrogen flux and resuspend in 100% MeOH (~10g tissue equivalents/mL)
5.3. RECEPTOR BINDING ASSAY PROCEDURE

5.3.1. Preparation of stock solutions and standards for assay

**Assay buffer PBST-BSA pH 7.4**
Transfer 1 pack of Phosphate buffered saline pH 7.4, Tween 20 (PBST) quantitatively to volumetric flask and bring to a final volume of 1 L with dH2O. Add BSA 1g/mL. Store at 4°C

Or prepare 0.01M Phosphate buffered saline containing NaCl (0.138 M), KCl (0.0027 M), Tween 20 (0.05%). Adjust to pH 7.4 and add 1g/mL BSA. Store at 4°C

**Radioligand solution [³H] PbTx-3**
Stock [³H] PbTx-3 is provided in 50 µCi ampoules, 20 Ci/mmol, 0.1 mCi/mL (equivalent to 4.17 µM).

- Prepare a 15 nM working solution of [³H] PbTx-3 for each assay fresh daily:
  - Add 4 mL PBST-BSA to a 15-mL tube
  - Add 12 µl [³H] PbTx-3, vortex
  - Pour diluted [³H] PbTx-3 into an empty solvent basin

- Measure total counts of each working stock prior to running an assay: add 35 µL of the [³H] PbTx-3 working solution to a liquid scintillation vial with scintillant and count on a liquid scintillation counter. 35 µL of 15 nM stock should contain ~13,000 dpm. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

**Unlabelled PbTx-3 working solution:**
PbTx-3 reference standard is provided at a concentration 100µg/mL. A “bulk” serial dilution for the standard curve is made up in advance and stored at 4°C for up to 1 month. The use of bulk reference dilutions minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability.

<table>
<thead>
<tr>
<th>Stock</th>
<th>In-assay*</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 µL 100 µg/mL + 40 µL MeOH</td>
<td>60 x 10^-6 g/mL</td>
</tr>
<tr>
<td>10 µL 60 µg/mL + 90 µL MeOH</td>
<td>6.0 x 10^-6 g/mL</td>
</tr>
<tr>
<td>30 µL 6 µg/mL + 70 µL MeOH</td>
<td>2.0 x 10^-6 g/mL</td>
</tr>
<tr>
<td>10 µL 6 µg/mL + 90 µL MeOH</td>
<td>0.6 x 10^-6 g/mL</td>
</tr>
<tr>
<td>30 µL 0.6 µg/mL + 70 µL MeOH</td>
<td>0.2 x 10^-6 g/mL</td>
</tr>
<tr>
<td>10 µL 0.6 µg/mL + 90 µL MeOH</td>
<td>0.06 x 10^-6 g/mL</td>
</tr>
<tr>
<td>10 µL 0.06 µg/mL + 90 µL MeOH</td>
<td>0.006 x 10^-6 g/mL</td>
</tr>
<tr>
<td>5 mL PBST-BSA</td>
<td>0 µg/mL</td>
</tr>
</tbody>
</table>

* Standards are diluted 1:10 in microtube and 1:6 in assay to give a final curve of 3x10^-10 to 10^-6 M PbTx-3

Add 12 µL of each standard to 108 µL PBST-BSA (enough for 35µL x 3 replicates + extra)

**Unknown samples:**
Perform two initial dilutions at 1:10, 1:20 in PBST-BSA of unknown samples (extracted and stored in MeOH) using dilution microtube (FIG above.)

- 1:10 Add 10 µl sample to 90 µl PBST-BSA
- 1:20 Add 10 µl sample to 190 µl PBST-BSA to all 1:20 microtubes
Inter-assay calibration standard (QC check):
A reference standard containing 1.8 x 10^-8 M PbTx-3 standard (3.0 x 10^-9 M PbTx-3 in assay) is prepared in advance in 0.003 M MeOH and kept frozen (-20ºC) in 0.5 mL to 1 mL aliquots for long term storage. Aliquots should be thawed and stored at 4ºC for routine use (stable up to 1 month) and included undiluted (35 µL) in each analysis (triplicate wells). This serves as a QC check and confirms day-to-day performance of the assay.

Rat brain membrane preparation:
- Add 2mL thawed rat membrane preparation to 18 mL PBST-BSA, vortex
- Pour diluted prep into empty solvent basin

5.3.2. Performing the assay

5.3.2.1. Plate setup and incubation
When possible, use a multichannel pipette to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate. Multiple dilutions of sample extracts need to be analyzed in order to obtain a value that falls on the linear part of the competition curve for quantification.

For ease of analysis, it is convenient to use a standard plate layout which maximizes the number of samples and standards that can be analyzed on one plate. For fish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects.

Add in the following order to each of the 96 wells:
- 35 µL PBST-BSA assay buffer (assay buffer is added first in order to wet the filter membrane)
- 35 µL PbTx-3 standard, QC check, or sample extract
- 35 µL [³H] PbTx-3
- 105 µL membrane preparation, mix by pipetting

Cover and incubate plate at 4ºC for 1 hour.
5.3.2.2. Assay filtration and counting

Assay Filtration
1. Place 96-well plate on the MultiScreen vacuum manifold. Fill empty wells with 200 µL of assay buffer to ensure even filtration across plate
2. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2-5 sec. Pull contents of all wells through until liquid is removed.
3. With vacuum still running, quickly rinse each well twice with 200 µL ice cold assay buffer using multichannel pipette

Counting
4. Remove the plastic bottom from the plate. Gently blot the bottom once on absorbent toweling
5. Place microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape
6. Add 50 µL scintillation cocktail per well using multichannel pipette
7. Seal top of plate with sealing tape.
8. Allow to sit a minimum of 1 hour at room temperature
9. Tag the plate (9) and count using a microplate scintillation counter (10)
5.3.3. Analysis of data

Sample quantification is carried out only on dilutions that fall on the linear part of the competition curve ($B_i/B_0 = 0.2-0.7$). Principles and assay Quality Control acceptance are detailed in the chapter 2.2.3 Analysis of Data, Page 15 of this manual.

Where more than one dilution falls on the linear part of the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in $\mu g$ PbTx-3 equivalents/kg shellfish, using the following formula:

$$\left(\mu g\text{ PbTx-3 equiv}\right) \times (\text{sample dilution}) \times \left(\frac{210\mu L\text{ total volume}}{35\mu L\text{ sample}}\right) = \mu g\text{ PbTx-3 equiv in extract}$$

$$\frac{\mu g\text{ PbTx-3 equiv/mL}}{g\text{ shellfish extracted}} \times \frac{mL\text{ extract}}{kg\text{ extract}} \times 1000g = \mu g\text{ PbTx-3 equiv/ kg}$$
6. RBA FOR AMNESIC SHELLFISH POISONING: DOMOIC ACID

6.1. OVERVIEW

The radioligand receptor binding assay for domoic acid (DA), the neurotoxin responsible for the human poisoning syndrome, amnesic shellfish poisoning (ASP), as well as DA poisoning in a wide range of wildlife species, was described by van Dolah et al. [110]. This method is a competitive binding assay based on the interaction of DA with glutamate receptors and employs a radiolabeled form of the analog kainic acid (\(^3\text{H}\)-kainate) in a microplate format. While a previous version of the assay used a frog brain crude membrane preparation to provide the receptors [116], the current version utilizes a recombinant rat GluR6 glutamate receptor that is expressed in an Sf9 insect cell line following infection with a baculovirus carrying the GluR6 construct. In addition, samples are pre-treated with a recombinant \textit{Esherichia coli} glutamate decarboxylase (GAD) enzyme [117] to eliminate endogenous glutamate, which may interfere with the assay and cause a false positive response. The assay is compatible with methanol-based extraction protocols used for analytical methods (details given below). This method is capable of detecting DA well below the level of the current regulatory limit (20 mg/kg shellfish meat). Although the DA RBA has not been validated through an interlaboratory study, published data for algal extracts were consistent with those of the HPLC-FLD method [104] and results with the MUS-1 certified reference standard (mussel matrix) agreed closely with the expected value following GAD pre-treatment. The ASP RBA has also been documented to respond to DA isomers, isodomoic acids A and C, both of which exhibited markedly lower affinity than DA [118, 119].

6.2. SAMPLE PREPARATION

6.2.1. Apparatus, supplies and reagent

**Apparatus and supplies**
- Phytoplankton samples collected on 25 or 47-mm GF/F filters (or equivalent)
- Filter forceps
- Dual smooth glass grinding tube with fitted Teflon pestle
- 500 W sonicator with microprobe
- Centrifuge capable of reaching 8000 x g for 15 mL tubes
- Balance
- 0.22 \(\mu\)m filter discs
- 3 cc syringes
- 5 mL transport tubes
- Freezer -80°C and -20°C
- Fridge 4°C

**Assay Reagents**
- 10 % Methanol
- Water, double distilled and deionized
6.2.2. Domoic acid extraction from phytoplankton

- Collect phytoplankton on Glass Fiber Filters. Tare dry grinding/extraction tube on balance, carefully place sample filter at bottom of tube, record weight.
- Add desired amount of 10% methanol to grinding/extraction tubes, record weight and mix tube to homogenize cell distribution.

Note: use 2.5 mL of 10% methanol for 25mm filters, 5mL of 10% methanol for 47mm filters
- Break up filter by grinding with Teflon pestle; grind at room temperature for 1 min at 250 rpm (2 min for 47mm filters). Wash Teflon pestle with 10% methanol and water between samples and dry with kim-wipe.
- Centrifuge for 1 min at 45 x g to gently bring down filter particles and extraction solvent. Rinse upper edges of tube with supernatant.
- Sonicate on ice slurry for 1 min for 25 mm filters (2 min for 47 mm filters) using 500 W sonic disrupter with a microprobe at maximum setting. Rinse microprobe with 10% methanol and water between samples and dry with kim-wipe.
- Transfer sonicated sample to 5 mL transport tube and centrifuge for 1 min at 8000 x g to pellet filter particles. Remove supernatant using 3 cc syringe and pass through 0.22 um filter.
- Store samples in 2 or 5mL vials at -20°C until analysis.

6.2.3. Domoic acid extraction from tissue and shellfish

- Shuck oysters/shellfish to obtain approximately > 100 g shellfish meat (approximately 8-10 large oysters). Drain liquid by placing the shucked meat on a No. 8 – No. 10 sieve for 5 min. Discard pieces of shell. Weigh out 100 g drained meat. Homogenize 3 min in a blender. Store homogenate frozen at –20°C until extraction. If a limited amount of tissue is available, a portion of tissue or all can be weighed directly into the extraction tube.
- Accurately weigh 4.0 g tissue homogenate (prepared in step 1) into a tared 50 mL blender cup. Add 16.0 mL of 50% Methanol and homogenize the tissue with a blender at a medium speed for 4 min. Pour approximately 15 mL of the resulting slurry into a centrifuge tube. Centrifuge at 3,000 x g or higher for 10 min. Filter supernatant through 0.45 µm nylon filter (a glass fiber pre-filter may be necessary if sample clogs filter quickly). Store extracts at –20°C.
- SAX clean-up of extracted tissue (use Waters sep-pak manifold): condition column with 6 mL of methanol, 3 mL water and 3 mL 50% methanol, load column with 5 mL of tissue extract, wash with 5 mL 0.1 M NaCl (in 1:9 ACN:H2O) and elute with 5 mL 0.5 M NaCl (in 1:9 ACN:H2O) (place 10 x 75 mm tubes in manifold prior to elution). Transfer eluent to 5 mL volumetric flask and bring to a final volume of 5 mL with 0.5 M NaCl (in 1:9 ACN:H2O). Store cleaned extract in refrigerator.
6.3. RECEPTOR BINDING ASSAY PROCEDURE

6.3.1. Apparatus, supplies and reagent

Apparatus
- Traditional or microplate scintillation counter
- 96 well multiscreen plates (Multiscreen)
- Multiscreen sealing tape (Millipore)
- Variable volume micropipettors and disposable tips (20μl, 200μl, 1mL capacity)
- 8-channel variable volume micropipette and disposable tips (25μl to 500μl capacity)
- Vacuum supply
- 15 mL and 50 mL centrifuge tubes
- Ice bucket
- Mini-dilution tubes in 96-tube array
- Reagent reservoirs
- Vortex
- Microplate scintillation counter (Wallac microbeta)
- Filtration manifold to accommodate a 96-well plate
- 100 mL and 50 mL volumetric flasks
- -80°C freezer / refrigerator

Assay reagents
- 50 mm Tris pH 7.4
- 100 mm Sodium Acetate Buffer (pH 4.6)
- GAD Buffer
- [3H] kainic acid specific activity 30-60 Ci/mmol, 1 mCi/mL (Perkin Elmer).
- SF9 insect prep (glur6 glutamate receptors) – see membrane preparation SOP
- Scintillant: For traditional counter: Scintiverse liquid scintillation cocktail (Fisher Scientific, or equivalent) or For microplate counter: Optiphase liquid scintillation cocktail (Perkin-Elmer Life Sciences, or equivalent)

Preparation of stock solutions and standards for assay buffers
- 50 mM Tris pH 7.4 – used to dilute [3H] kainic acid and to dilute the SF9 membrane preparation
  Weigh out 6.057 g Tris (Trizma) base.
  Add 900 mL Milli-Q water, adjust pH to 7.4 using HCl (can use concentrated 12N HCl then switch to lower concentration when pH gets close to desired value). Use a pH probe that is Tris compatible.
  Bring up to 1000 mL in a volumetric flask

- 50 mM Sodium Acetate Buffer (pH 4.6) - necessary for preparing GAD buffer.

- 0.2 M Acetic Acid Solution:
  Weigh out 0.58 mL in 50 mL volumetric flask.
  Bring up to 50 mL with Milli-Q water.

- 0.2 M Sodium Acetate Stock Solution:
  Weigh out 0.82 g sodium acetate (anhydrous).
  Pour into 50 mL volumetric flask.
  Bring up to 50 mL with Milli-Q water.

- 50 mM Sodium Acetate Buffer (pH 4.6):
In 100 mL volumetric flask, combine 25.5 mL 0.2 M acetic acid stock solution with 24.5 mL 0.2 M sodium acetate stock solution. Bring volume up to 100 mL with Milli-Q water. Filter sterilize through 0.2 μm bottle top filter unit.

- **GAD Buffer** – necessary for digest reactions, only good for 2-3 days
  - 50 mM sodium acetate buffer (pH 4.6), 2 mM pyridoxal-5-phosphate, 100 mM NaCl
  - Weigh out 0.29 g NaCl in weigh boat.
  - Pour into 50 mL volumetric flask.
  - Weigh out 0.025 g pyridoxal-5-phosphate.
  - Pour into same 50 mL volumetric flask.
  - Bring dry reagents up to 50 mL with 50 mM citrate buffer, pH 4.6.

**Daily reference standard serial dilution**

Standard curve consists of 10⁻⁶ – 10⁻¹¹ M domoic acid (DA). All working stocks are 6 x (10⁻⁶ – 10⁻¹¹ M) since a 35 μl stock is added to 210 μl of total assay volume (1/6 dilution).


Use 57.2 μM DA standard working stock for preparing standard curve below:

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁶</td>
<td>6 x 10⁻⁶</td>
<td>31.5 μl CRM-DA-f</td>
<td>268 μl (A)</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>6 x 10⁻⁷</td>
<td>30 μl (A)</td>
<td>270 μl (B)</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>6 x 10⁻⁸</td>
<td>30 μl (B)</td>
<td>270 μl (C)</td>
</tr>
<tr>
<td>3 x 10⁻⁹</td>
<td>1.8 x 10⁻⁸</td>
<td>150 μl (C)</td>
<td>350 μl (D)</td>
</tr>
<tr>
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<td>6 x 10⁻⁹</td>
<td>20 μl (C)</td>
<td>180 μl (E)</td>
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<tr>
<td>10⁻¹⁰</td>
<td>6 x 10⁻¹⁰</td>
<td>20 μl (E)</td>
<td>180 μl (F)</td>
</tr>
<tr>
<td>10⁻¹¹</td>
<td>6 x 10⁻¹¹</td>
<td>20 μl (F)</td>
<td>180 μl (G)</td>
</tr>
<tr>
<td>Reference (GAD buffer only)</td>
<td></td>
<td></td>
<td>180 μl (H)</td>
</tr>
</tbody>
</table>

**Enzymes**

The current GAD enzyme has an activity of 400 Units/400ul; this is equal to 1000 Units/mL. The enzyme is stored at -20°C and should be kept on ice until ready to use. The current protocol requires 1.5 Unit of enzyme per 100 μl reaction.

**Determination of the volume of enzyme needed to digest a sample:**

1000 Units/mL (volume of enzyme concentrated stock needed) = 150 Units/mL (*total volume wanted of enzyme working stock)

* For a 96:12:12 digest, multiply 12 μL by the total number of samples plus one, for example: if 9 samples are tested, 10x12 or 120 μL total volume (18μL of enzyme stock plus 102 μL sodium acetate buffer) will be needed.

Bring up to total volume with 50 mM sodium acetate buffer, pH 4.6 (not GAD buffer).

**Quality controls**

QC check sample: CRM-DA-f working stock (57.2 μM) is diluted in 1:9 ACN:H₂O independently of the standard curve to prepare a 2.86 μM QC check standard, aliquoted into 100 μl aliquots, and stored refrigerated until use (100 μl 57.2 μM CRM-DA-f + 1900 μl 1:9 ACN:H₂O).

The QC check is then diluted 1/100 in 50mM Tris, pH 7.4 (add 5 μl 2.86 μM DA to 495 μl GAD Buffer) for analysis in each assay (28.6 nM). When 35 μl are added to total assay volume of 210 μl, the QC check contains 4.8 nM CRM-DA-f in the assay.
**[^3]H| Kainic acid**

Example using a[^3]H kainic acid purchased from Perkin Elmer with a specific activity of 47.0 Ci/mmol at 1mCi/mL, and a total activity of 250 μCi.

The calculated KA concentration is 21.276 μM

The working stock concentration of[^3]H KA is 30 nM, with a final assay concentration being 5 nM; to make up enough of 30 nM[^3]HKA for one 96 well plate, add 3.2 μL[^3]HKA (21.276μM) to 2.25 mL of Tris buffer, pH 7.4 (or 6.4 μL[^3]H] + 4.5 mL 50 mM Tris, pH 7.4 for a whole plate). In assay concentration is 5 nM after 1/6 dilution.

If possible: measure total counts of each working stock prior to running an assay: add 35 μL of the working stock[^3]H KA in buffer to a LSC vial with scintillant and count on liquid scintillation counter.

***note that the specific activity will likely change from lot to lot, so it is essential to perform a new set of calculations for each new shipment of material received.

**Sf9 membrane prep**

Dilution will depend on batch/lot of membrane used (typically 1/12-1/18). Keep membrane on ice until ready to use; example= (1mL prep + 15mL Tris buffer pH7.4 for a 1:16 dilution).

**GAD treatment of samples**

The majority of samples will have to be digested with glutamic decarboxylase (GAD) before analysis by radioligand receptor binding assay. This includes but is not limited to: shellfish, phytoplankton, serum, urine, feces, molecrabs and anchovies.

1. Arrange dilution tubes in rack and add:
   - 96 μl GAD buffer
   - 12 μl sample
   - 12 μl enzyme
2. Incubate tubes at 25°C for 30 min at room temperature.
3. GAD QC check= Add glutamate standards as samples in the digest (10 mM and 5mM glutamate in water). These samples should come up negative for domoic acid in the assay if the enzyme is working properly.

**6.3.2. Performing the assay**

**6.3.2.1. Plate setup and incubation**

When possible, use a multichannel pipette to minimize pipetting effort. Standard curve is run in triplicate. Samples are run in triplicate. Multiple dilutions may need to be analyzed before a dilution will fall on the linear part of the competition curve, which is required for quantitation. QC check sample is run in triplicate as a sample. For ease of analysis, it is convenient to use a standard plate layout, which maximizes the number of samples, and standards, which can be analyzed on one plate. Be sure to account for the 1/10 dilution in the GAD digest. Dilutions of samples should be done in GAD buffer and should be done prior to the digest if a dilution of greater than 10 is warranted (the digest dilutes the sample by 10):
<table>
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<th></th>
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<td>U8</td>
<td>U8</td>
<td>U8</td>
<td>1/50</td>
<td>1/50</td>
<td>1/50</td>
</tr>
</tbody>
</table>

U = unknown sample

1. Add 35 uL Tris buffer (pH 7.4) to each well.
2. Add the following to a 96 well multiscreen plate:
   - Standard curve: to triplicate wells, add 35 μl standard
   - QC sample: to triplicate wells, add 35 μl QC check sample
   - Samples: to triplicate wells, add 35 μl digested sample
3. Next, to each well containing sample or standard, add 35 μl [3H] kainic acid working solution. This will yield an assay concentration of 5 nM.
4. Last, to each well containing sample or standard add 105 μl glutamate receptor preparations.
5. Incubate plate at 4°C for 1 hour.

6.3.2.2. Assay filtration and scintillation counting

1. Place the multiscreen plate on vacuum manifold and filter. Optimum vacuum will pull the wells to dryness in 2-5 sec.
2. Rinse wells with 200 uL of Tris buffer, pH 7.4.
3. Remove the plastic bottom of the filter plate and blot the bottom of the plate with absorbent toweling. Cover the bottom of a carrier cassette with tape and place the plate into the cassette.
4. Add 50 uL Optiphase liquid scintillant to each well of the plate.
5. Place a multiscreen sheet (tape) on top of the plate and let incubate for 30 min at room temperature before counting on microplate scintillation counter. Timer can be set on the scintillation counter.
6. Count plate.
6.3.3. Analysis of data

The curve fitting for the binding competition curve data is carried out using a 4 parameter logistic fit, which can be carried out using commercially available curve fitting software packages. General graphing packages capable of 4PL curve fitting include Graph Pad (Lotus) and Sigma Plot (Jandel). Specialized programs for receptor assay applications include Ligand (Biosoft) and Multicalc (Wallac).

- Sample concentration is calculated in μg CRM-DA-f equivalents/ g tissue, using the following formula:

\[
(nM \text{ equiv CRM-DA-f}) \times (\text{sample dil}) \times \left(\frac{210 \mu l \text{ total volume}}{35 \mu l \text{ sample}}\right) = nM \text{ equiv CRM-DA-f in extr}
\]

\[
(nM \text{ equiv CRM-DA in extr}) \times \left(\frac{1 L}{1000 \text{ mL}}\right) \times \left(\frac{311 \text{ ng}}{1 \text{ nmol}}\right) \times \left(\frac{1 \mu g}{1000 \text{ ng}}\right) = \mu g \text{ CRM-DA-f equiv/ mL extr}
\]

\[
\mu g \text{ CRM-DA-f equiv/mL} \times \left(\frac{5 \text{ mL extr}}{1 \text{ g tissue}}\right) = \mu g \text{ CRM-DA-f equiv/ g tissue}
\]

- Sample concentration is calculated in μg DA equivalents/L seawater, using the following formula:

\[
(nM \text{ equiv CRM-DA-f}) \times (\text{sample dil}) \times \left(\frac{210 \mu l \text{ total volume}}{35 \mu l \text{ sample}}\right) = nM \text{ equiv CRM-DA-f in extr}
\]

\[
(nM \text{ equiv CRM-DA in extr}) \times \left(\frac{1 L}{1000 \text{ mL}}\right) \times \left(\frac{311 \text{ ng}}{1 \text{ nmol}}\right) \times \left(\frac{1 \mu g}{1000 \text{ ng}}\right) = \mu g \text{ CRM-DA-f equiv/ mL extr}
\]

\[
\mu g \text{ CRM-DA-f equiv/mL} \times \left(\frac{1000 \text{ mL}}{L \text{ seawater filtered}}\right) \times \left(\frac{L \text{ extract volume in L}}{L \text{ seawater filtered}}\right) = \mu g \text{ CRM-DA-f equiv/ L seawater}
\]

*Note: equiv = equivalents; dil = dilution; extr = extract*
Appendix

SAFETY

I.1. SAFE HANDLING OF TRITIUM IN LOW AND MEDIUM LEVEL LABORATORIES

It is essential in any operation involving radionuclides that appropriate provisions are made for radiation protection. This involves a commitment on the part of senior management to providing the necessary resources so that all work, experiments and applications procedures are carried out in such a way that all exposures are kept as low as reasonable achievable. Workers competence in dealing with radioactive work shall be ensured by establishing training programmes and periodical refreshers.

The isotope used in radioligand receptor binding assays is Tritium ($^3$H). Tritium emits a low energy beta particle. It can travel less than ¼ inch in the air.

The radioactive decay product of tritium is a low energy beta that cannot penetrate the human skin. The main hazard associated with tritium is internal exposure from inhalation or ingestion.

Personal protective equipment (PPE)

Radiation health risks result predominately from internal contamination, when tritium is embedded in tissue or fluids, directly exposing cells. For this reason the main protection effort should be addressed to reducing the possibility of significant intakes into the body, which may occur by inhalation, ingestion, skin absorption through contact with contaminated surfaces or with contaminated liquids.

When handling radioactive toxins change gloves frequently. Before leaving the laboratory, remove protective clothing and wash hands thoroughly.

Do not wear gloves when using computers associated with instruments or when entering or leaving the laboratory.

Special safety precautions

- Provision of adequate ventilation is required. In small laboratories it may be possible to provide the needed flow of air simply by the exhaust system of the fume hoods. Handle stock solutions and volatile forms in a fume hood or other approved ventilated enclosure.
- Bench tops and floor made of smooth, impervious material will greatly facilitate decontamination work to remove spills.
- Clearly identify all equipment used with tritium.
- Have experiments confined to trays lined with disposable absorbent materials,
- Do not pipette radioactive solutions by mouth, instead use micropipettes.
- Do not eat, drink, smoke, or use cosmetics in the lab.
- Sort radioactive waste into separate waste containers as follows:
  - Scintillation vials;
  - Radioactive glass and metal waste;
  - Radioactive dry waste;
  - Radioactive liquid waste.
- Periodic wipe tests should be performed in the radiation lab in order to make note of, and decontaminate, any radioactive areas. The method consist in swiping uniformly the surface subject to control with a swab dry or moistened with distilled water and then introduced into a vial containing scintillator cocktail and counted in standard manner.
- Minimize contact of your papers such as protocols with laboratory surfaces, which may be radioactive. Use in-lab protocols designed to stay in the radioactive lab and clip any papers in front of your work station rather than laying them down on the lab bench.
- Maintain all usage logs for radioactive material as well as logs for entering and exiting the laboratory.
I.2. SAFE HANDLING OF TOXINS, SOLVENTS, AND ACIDS

**Organic solvents and acids**
Dispensing of organic solvents and concentrated acids should be carried out in a fume hood or appropriately ventilated area. Follow standard laboratory procedures, including the use of appropriate gloves, safety glasses and protective clothing to protect against splashes and spills. Acids should be stored in a corrosives cabinet and organic solvents should be stored in a flammables cabinet. Wash hands thoroughly after use.

**Toxin handling**
Most algal toxins are potent neurotoxins, and should be handled as any dangerous laboratory chemicals. Appropriate personal protective equipment should be used when handling algal toxins to minimize exposure (ventilation via fume hood, gloves, safety glasses, lab coat) and is necessary to reduce the risk of exposure. As with any other highly toxic chemical, take extreme care to not touch, ingest, inhale, or spill these toxins. Wash hands thoroughly after use of toxins.

**Toxin Waste**
A “base bath” solution comprised of saturated potassium hydroxide in ethanol (pH > 14) is used to decontaminate/neutralize toxins on contaminated items. Use appropriate precautions (goggles, face mask/shield, rubber gloves, apron) when using the highly caustic base bath.
To decontaminate glassware, soak in the base bath for at least one hour then rinse thoroughly with water. After rinsing, follow standard laboratory washing procedures before reuse.
Disposable plastic-ware such as pipette tips, dilution tubes, filters, and other supplies contaminated with toxins can be placed in an empty container (bleach or 4L solvent bottles work well). When the bottle is full, carefully fill with base bath liquid and soak at least overnight. After soaking, carefully empty the base solution into original base bath container. Rinse out the container holding waste with water until the base is neutralized (water in bottle reaches pH 7-8). The decontaminated supplies and bottle can now be disposed of as regular waste.
For glassware contaminated with toxins, soak in base bath for at least one hour prior to placing in sonicator. Rinse thoroughly with water and allow to dry before re-use.

I.3. SAMPLE HANDLING

**Storage**
Most sample types should generally be stored as cold as reasonably possible, with -20°C being the standard. Samples can be stored inside individually labeled leakproof containers or plastic bags. Some plastics do not maintain their integrity at very low temperatures for extended periods, so use a plastic type suitable for the application (temperature, length of storage, etc).

**Shipment of samples**
Shipping requirements are country and courier specific, but the general provisions are applicable in most cases. If dry ice is unavailable, samples may be shipped overnight frozen with ice packs frozen at -80°C. Samples should be in tightly sealed leakproof containers or plastic bags, which are individually labeled. The closures should be sealed using tape and/or parafilm. Labels should identify the sample directly on the container and also inside the container sealed within a plastic bag. The tracking number provided by the overnight courier should be provided to the recipient. A letter including all sample information should accompany the samples.
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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AOAC International</td>
<td>Association of Analytical Communities</td>
</tr>
<tr>
<td>ASP</td>
<td>amnesic shellfish poisoning</td>
</tr>
<tr>
<td>CFP</td>
<td>ciguatera fish poisoning</td>
</tr>
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<td>CPM</td>
<td>count per minute</td>
</tr>
<tr>
<td>CTX</td>
<td>ciguatoxin</td>
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<td>domoic acid</td>
</tr>
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</tr>
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<td>GAD</td>
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<td>MOPS</td>
<td>3-morpholinopropanesulfonic acid</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D-aspartate</td>
</tr>
<tr>
<td>NOAA</td>
<td>National Oceanic and Atmospheric Administration</td>
</tr>
<tr>
<td>NSP</td>
<td>neurotoxic shellfish poisoning</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffer saline-tween</td>
</tr>
<tr>
<td>PbTx</td>
<td>brevetoxin</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methylsulfonyl fluoride</td>
</tr>
<tr>
<td>PSP</td>
<td>paralytic shellfish poisoning</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>RBA</td>
<td>receptor binding assay</td>
</tr>
<tr>
<td>RCF</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>STX</td>
<td>saxitoxin</td>
</tr>
<tr>
<td>TEF</td>
<td>toxicity equivalency factors</td>
</tr>
<tr>
<td>Name</td>
<td>Organization</td>
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