EVALUATING THE RISK OF CIGUATERA FISH POISONING FROM REEF FISH IN HAWAI'I: DEVELOPMENT OF ELISA APPLICATIONS FOR THE DETECTION OF CIGUATOXIN

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

CELL AND MOLECULAR BIOLOGY

MAY 2008

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Acknowledgements

The author wishes to express sincere appreciation to her major professor, Dr. Yoshitsugi Hokama, for his continual support, encouragement, and guidance throughout this entire project and beyond. The author also gratefully thanks all current and former committee members, Dr. Martin Rayner, Dr. John Bertram, Dr. Andre Theriault, Dr. Kenichi Yabusaki and Dr. Clyde Tamaru, for their valuable comments, criticism and involvement through the duration of this project. I wish to acknowledge and thank the technical staff who gave their time and energy in support of this project, as well as the many individuals including Jan Dierking, Gary Dill, and Dr. Clyde Tamaru who helped obtain fish specimens to test. Without their help, this study could not have been completed.

Special appreciation is extended to my husband Cory Campora and my three daughters, as well as my extended family for their patience and understanding in my pursuit of this goal.

This work was funded in part by grants and cooperative agreements from the Hokama-Yagawa Fund, and the United States Department of Agriculture, CSREES Agreement No. 2003-34167-14034 and 2004-34167-14801, from the National Oceanic and Atmospheric Administration, Project # A/AS-1, which is sponsored by the University of Hawaii Sea Grant College Program, SOEST, under Institutional Grant Nos. NA16RG2254, NA05OAR4171048 from NOAA Office of Sea Grant, Department of Commerce.

Abstract

Harmful algal blooms (HABs) are associated with several acute and chronic diseases in humans worldwide, which are characterized by gastrointestinal, neurological, and/or cardiovascular disturbances that can persist for many months. In the tropics, ciguatera fish poisoning, the most commonly reported marine toxin disease in the world, is the primary and most important human health manifestation of HAB and results from the consumption of fish containing high levels of ciguatoxins (CTXs), a family of complex, lipid-soluble compounds produced by the benthic marine dinoflagellate, *Gambierdiscus toxicus*.

Quantitative, reliable methods to detect ciguatoxins in fish tissue are not widely accessible, thus rendering the detection of CTX in fish destined for human consumption a serious public health concern. The recent introduction of open ocean grow-out aquaculture facilities raising reef fishes (e.g., *Seriola* sp.) that have historically been implicated in ciguatera poisoning for commercial consumption and increasing incidence of marine toxin poisonings worldwide emphasize the need for quantitative assessment of ciguatoxin exposure.

This dissertation addresses this important food safety issue by developing and validating a novel, sensitive, and specific enzyme linked immunoassay (ELISA) capable of detecting picogram quantities of ciguatoxin in fish flesh. Development of the assay represents the first ELISA for detecting ciguatoxin using chicken antibody (IgY) as an immunoanalytic tool, and further establishes a relatively rapid, reliable method for screening large quantities of fish.

The results of testing over 300 fish commonly implicated in ciguatera fish poisoning, specifically two species of amberjacks (almaco jack, *Seriola rivoliana* and greater amberjack, *Seriola dumerili,* also known as kahala), and the blue spotted grouper (*Cephalopholis argus*, also known as roi), using both the ELISA and a secondary bioassay to validate results leads to the following conclusions: a) the ELISA developed during the course of this study is a valid screening tool for evaluation of presence or absence of ciguatoxin in fish tissues with reasonable sensitivity and specificity; b) the fish farmed in open ocean aquaculture cages in Hawai'i are not likely to be susceptible to ciguatoxin bioaccumulation despite the discovery of *G. toxicus* in or on the cages; c) CTX is present in three fish species commonly implicated in ciguatera fish poisoning at overall rates lower than previously reported; and d) the prevalence of ciguatoxin in tested wild-caught fish cannot reliably be correlated to the size of the fish or the depth at which it was caught. Further, while geographic location of catch may provide some indicator of toxicity risk, the migratory nature of fish and their feeding behavior precludes it as a potential screen for ciguatoxicity. This dissertation also demonstrates that the antibody used in assay development is reactive with purified ciguatoxin and provides evidence that the AB epitope of the CTX molecule is active at the sodium channel in neuroblastoma cells.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

Fish poisoning dates back to antiquity. It was cited in Homer's Odyssey in 800 BC and was observed during the time of Alexander the Great (356-323 BC) when armies were forbidden to eat fish in order to avoid the accompanying sickness and malaise that could threaten his conquests (Halstead 1988). Marine toxins, often causing various seafood poisonings, arise naturally from marine algal sources and accumulate through the food chain, ultimately depositing in predator fish or filter feeding bivalves destined for mammalian consumption. Such seafood-borne diseases account for a large and growing proportion of all food poisoning incidents, and are associated with several acute and chronic diseases in humans worldwide, which are often characterized by gastrointestinal, neurological, and/or cardiovascular disturbances that can persist or recur for many months.

In the tropics, ciguatera fish poisoning is the primary and most important human health manifestation of harmful algal blooms (HABs). Ciguatera is the most commonly reported marine toxin disease in the world, occurring in the Pacific Ocean, Indian Ocean, and Caribbean Sea. It results from the consumption of fish containing high levels of ciguatoxins (CTXs), a family of complex, lipid-soluble, highly oxygenated cyclic polyether compounds produced by the benthic marine dinoflagellate, *Gambierdiscus toxicus*.

Quantitative, reliable methods to detect ciguatoxins in fish tissue are not widely accessible, thus rendering the detection of CTX in fish destined for human consumption a

serious public health concern. Current detection methods for ciguatoxin include a qualitative immunology-based assay, a mouse bioassay, neuroblastoma tissue culture assays, and liquid chromatography/mass spectrometry, all of which have major drawbacks which include cost, amount of time and effort involved, lack of quantifiable results, and lack of specificity. The recent introduction of open ocean grow-out aquaculture facilities raising reef fishes (e.g., *Seriola* sp.) commonly implicated in ciguatera poisoning for commercial consumption emphasizes the need for quantitative assessment of ciguatoxin exposure as a food safety issue. Data indicates that submerged cage operations offshore in Hawai'i harbors the dinoflagellate responsible for CTX production, which raises the concern that despite the contained cage environment, fish are nonetheless exposed to CTX prior to being sold for human consumption.

There is a clear need for the development of specific methodologies aimed at quantifying ciguatoxin exposure in reef fishes, which is the primary purpose of this dissertation. In the following chapters, I have attempted to address this major seafood safety issue by developing and applying various research methods to detect the toxin. Specifically, Chapter 2 reviews the current knowledge of ciguatoxin and available detection methods in detail as background information. Chapter 3 details a modified detection method for CTX, and while it is not suitable for routine screening, the study was done to show that one of the antibodies used in further assay development is reactive with several purified congeners of ciguatoxin with limited cross reactivity with other marine toxins. Chapter 4 outlines a series of experiments aimed at discovering the biologically reactive end of the ciguatoxin molecule at the sodium channel in tissue

culture. This data assisted in the development and validation of a rapid, novel immunological detection method for the detection of ciguatoxin in fish tissues using a sandwich ELISA employing chicken IgY, which is described in Chapter 5. The ELISA assay, together with a secondary neuroblastoma bioassay, was applied to evaluate the ciguatoxicity of over 300 reef fish from three different species commonly implicated in ciguatera fish poisoning which were either grown in offshore aquaculture cages, and/or wild-caught in Hawaiian waters, the results of which are reported in Chapters 6 and 7.

Chapter 2

Ciguatoxin

2.1 General Overview

Ciguatera fish poisoning, a seafood intoxication caused by ingestion of specific contaminated species of tropical and subtropical reef fishes, was described as early as 1606 in the South Pacific island chain of New Hebrides (Helfrich 1964). A similar outbreak there and in nearby New Caledonia was reported by the famous English navigator Captain James Cook in 1774 (Cook et al. 1777), who described the clinical symptoms of his sick crew – symptoms that coincide with the clinical manifestations described today for ciguatera fish poisoning (Bagnis 1964). Representing a crude bioassay, viscera from the same fishes given to Cook's crew were also given to pigs, causing their deaths (Cook et al. 1777).

The term ciguatera originated in the Caribbean area to designate intoxication induced by the ingestion of the marine snail, *Turbo livona pica* (called *cigua*), as described by a Cuban ichthyologist. Today, it is widely used to denote the most commonly reported marine toxin disease in the world resulting from the ingestion of certain fishes, primarily reef fish, encountered in tropical and subtropical regions circumglobally between the Tropic of Cancer and the Tropic of Capricorn.

Ciguatera fish poisoning stems from the consumption of fish containing high levels of ciguatoxins (CTXs), a family of small molecular weight (approximately 1,111 daltons), complex, lipid-soluble, highly oxygenated cyclic polyether compounds produced by the benthic marine dinoflagellate, *Gambierdiscus toxicus* (Hokama 2004).

They are biomagnified through the food chain, ultimately causing human and mammalian illness, as they are heat stable, colorless, odorless, and cannot be inactivated through cooking or freezing (Murata et al. 1990).

Ciguatera fish poisoning is characterized by neurological, gastrointestinal, and cardiovascular manifestations as soon as four hours after ingestion of ciguatoxic fish. Illness can occur after ingestion of as little as 0.1 ppb, or ~100 picograms/ml (Lewis et al. 1999), although the pathogenic dose is reported to be between 23 – 230 micrograms/ml by the World Health Organization (WHO 2007).

Ciguatoxins arise from biotransformation in the fish of precursor gambiertoxins (Lehane and Lewis 2000, Lehane 2000). Slight variations in CTXs can be characterized by the geographical regions in which they are commonly found; these include Pacific ciguatoxin, denoted as P-CTX, Caribbean ciguatoxin, C-CTX, and Indian Ocean ciguatoxin, I-CTX. Both P-CTX and C-CTX have unique molecular chains of 13 and 14 joined ether rings ($C_{62}H_{92}O_{19}$), respectively, as their basic structure. Nine of these transfused rings form a ladder that is similar in all CTXs, with structural modifications on different congeners seen mainly in the termini of the toxin primarily through oxidation (Pearn 2001). Caribbean CTXs differ from Pacific CTXs and are generally found to be lower in both toxicity and polarity. The main CTXs in the Pacific, present in different relative amounts in fish, are P-CTX-1 as the principal and most potent, P-CTX-2, and P-CTX-3, with more than 20 additional congeners identified in recent years (Lehane and Lewis 2000, Lehane 2000, Murata et al. 1989, 1990, Lewis et al. 1993, Yasumoto et al. 2000). Figure 2.1 depicts the molecular structures of the major Pacific and Caribbean

ciguatoxins, with the energetically less favored epimers, P-CTX-2 (52-epi P-CTX-3), P-CTX-4A (52-epi P-CTX-4B) and C-CTX-2 (56-epi C-CTX-1) indicated in parentheses.

2.2 Epidemiology

Ciguatera fish poisoning is found worldwide, typically between latitudes 35 degrees N and 35 degrees S, with Caribbean, Indian, and Pacific Ocean congeners of the toxin having been elucidated thus far (Hokama and Yoshikawa-Ebesu 2001). In the past, such toxins have been confined to seafood-dependent populations in island or coastal regions, however, with increased tourism, international fishing trade, and the emphasis on fish in the human diet there have been increasing reports of marine toxin illnesses with an estimated 50,000 cases reported each year worldwide (Baden et al. 1996). However, because of difficulties confirming cases in the absence of a reliable assay for human exposure to the toxin, the U.S. Centers for Disease Control suggest that only some 2-10% of actual cases are actually reported due to underreporting and misdiagnoses (Louisiana Department of Public Health 2007).

In the US and its territories, Hawai'i, Florida, Puerto Rico, Guam, the US Virgin Islands, American Samoa, and the Commonwealth of the Northern Mariana Islands are ciguatera endemic. In Puerto Rico it is estimated that 7% of the residents have experienced at least one ciguatera poisoning event, and a household survey in St. Thomas estimates that 4.4% of its population suffered from ciguatera annually, equivalent to approximately 600 individual cases per year (Louisiana Department of Public Health 2007). According to the Hawai'i Department of Health statistics collected over a period of 10 years, in Hawai'i there are on average a relatively low number of 25 individual

confirmed cases of ciguatera fish poisoning per year, or 0.87 cases per 10,000 population (Figure 2.2). By comparison, in archipelagos of the South Pacific and French Polynesia where populations are strongly dependent on fish resources, the number of confirmed cases rises sharply to some 5,850 cases reported per 10,000 population annually (Fleming et al. 1998). Table 2.1 shows the reported incidence and prevalence of ciguatera in selected regions, however, it is again noted that these values must be considered in the context of overall significant under-estimation of cases, and that rates are calculated based on a denominator of the entire population, not the actual population at risk.

2.3 Pharmacology

The ciguatoxins are the most potent sodium channel toxins known, with Pacific CTX-1 congener in mouse having an intraperitoneal LD_{50} of 0.25 μg/kg (Lewis 2000). Ciguatoxins and the closely related brevetoxins (a family of potent lipid-soluble polyether toxins produced by the marine dinoflagellate *Karenia brevis*) are characterized by their ability to cause the persistent activation of voltage-sensitive sodium channels (VSSCs), leading to increased cell Na^+ permeability. As a consequence, Na^+ dependent mechanisms in numerous cell types are modified, leading to increased neuronal excitability and neurotransmitter release, impairment of synaptic vesicle recycling, and induced cell swelling (Lewis 2000).

The lipid soluble ciguatoxins and brevetoxins target a common receptor site 5 on the neuronal voltage-dependent Na^+ channel, with ciguatoxin having 20 to 50 times greater affinity for the Na^+ channel than brevetoxin (Hokama and Yoshikawa-Ebesu 2001). The effects of brevetoxin and ciguatoxin on VSSCs are similar to those induced

by toxins targeting receptor site 7 which include the lipid-soluble pyrethroid insecticides (Wang and Wang 2003).

Lower doses of ciguatoxin have marked effects on both the respiratory and cardiovascular systems. Although ciguatoxin has some neuromuscular-blocking properties, the respiratory arrest induced by a lethal dose results mainly from depression of the central respiratory center (Hokama and Miyahara 1986, Miyahara et al. 1987). On the cardiovascular system, the effect is often biphasic: hypotension with bradycardia followed by hypertension and tachycardia (Miyahara et al. 1987, Li 1965). The hypotension and accompanying bradycardia are readily antagonized by anticholinergic agents (atropine, hexamethonium, and hemicholinium) (Li 1965), whereas the tachycardia and hypertension are mediated by the sympathetic nervous system and suppressed by adrenergic blockers such as propranolol and reserpine (Legrand et al. 1982).

2.4 Clinical Diagnosis and Treatment

Ciguatoxins cause gastrointestinal and neurological symptoms that typically persist for days to weeks, with vomiting, diarrhea, nausea, abdominal pain, dysesthesia, pruritus, and myalgia being common (Bagnis 1964). Severe cases of ciguatera may involve hypotension and bradycardia, although fatalities are rare. Neurological signs may persist for several months or even years (Chan 1998). Remarkably, the diagnosis of ciguatera is still largely dependent on the astuteness of the clinician. A history of recent consumption of potentially toxic fish, and at least one neurological sign and one other typical symptom are required to establish the clinical diagnosis. Other marine

poisonings, such as palytoxin poisoning, and other unrelated illnesses, such as the flu, must be excluded. Table 2.2 elaborates on clinical symptoms associated with such poisoning.

Validated tests to detect ciguatoxin exposure in humans and marine mammals are currently unavailable, although use of blood collection cards to monitor ciguatoxin exposure in mice have been tested in recent years and may be adaptable for clinical diagnosis in the future (Bottein Dechraoui et al. 2005a). In the absence of a confirmatory laboratory test, a sizable proportion of cases still go undiagnosed and unreported. Treatment is largely empiric and symptomatic. In severe cases, supportive care, particularly monitoring fluid and electrolyte balance, is paramount, and local anesthetics and antidepressants may also be useful in some instances. Following its somewhat serendipitous use for a coma victim in the Marshall Islands who was later diagnosed with severe ciguatera, intravenous mannitol is now the mainstay of therapy (Palafox 1988). Mannitol, however, is not universally beneficial, and is best when used during the acute phase of severe intoxications.

2.5 Detection Methods

Several biological, immunological, and chemical methods are available for detecting ciguatoxin in fish tissue, including a traditional mouse bioassay, radio ligand binding, high performance liquid chromatography, and mass spectrometry, however, few are well suited for routine large scale screening of fish samples. Lack of available, reliable purified CTX standards has hampered efforts to improve on current methodologies. An overview of the primary detection methods follows.

2.5.1 Bioassays

A commonly used method to detect ciguatoxins involves intraperitoneal (IP) injection of mice with crude extracts of fish (Hokama et al. 1994, Kimura et al. 1982a). Using estimates from known cases of ciguatera fishes obtained by the Hawai'i Department of Health and other laboratories, it has been found that 1 mouse unit (MU) = 7-8 nanograms of ciguatoxin (Hokama 2004), which is equivalent to the concentration of toxic extract injected IP that kills a 20 gram mouse within 24 hours. The general protocol for testing crude fish extract is as follows: Swiss-Webster mice weighing 20-25 grams are injected IP with 100 milligrams of crude fish extract re-suspended in 1 ml of 1% Tween 60 in saline. Symptoms displayed by the mouse are observed from 0.5 to 48 hours after injection and rated on a scale of 0 to 5 according to toxicity. Characteristic inotropic responses to various toxin extracts including ciguatoxin have also been established using the guinea pig atrial assay, which involves specialized dissection techniques, requires a small amount of test material, and gives some measure of specificity, as the actions are at the sites of the sodium channel (Miyahara et al. 1985). Other organisms, such as brine shrimp (Hungerford 1993), mosquitoes (Bagnis et al. 1985, 1987), chickens (Vernoux et al. 1985), and dipteral larvae (Labrousse and Matile 1996) have been used to screen for ciguatoxin, however, most have been found to be non-specific, non-quantitative, and generally unreliable for routine screening.

Directed cytotoxicity to sodium channels of neuroblastoma cells has been established for purified ciguatoxins, brevetoxins, saxitoxin, and crude seafood extracts

(Manger et al. 1993, 1995). Using a microplate high-throughput format, this assay takes several days to complete and serves as a valuable tool for marine toxin studies, detecting ciguatoxin at sub-picogram levels. A fluorescent based assay detecting sodium channel activators has also been useful in non-specific analysis of crude extract (Louzao 2004), and recently, a rapid hemolysis assay based on the neuroblastoma cell bioassay using red cells from the red tilapia (*Sarotherodon mossambicus*) has been developed for the detection of sodium channel-specific marine toxins, including ciguatoxin (Shimojo and Iwaoka 2000).

2.5.2 Immunoassays

A radioimmunoassay (RIA) (Hokama et al. 1977) and membrane immunobead assay (MIA) (Hokama et al. 1998) are advances in simple, rapid, sensitive and specific qualitative detection methods for ciguatoxin. The MIA is a field suitable assay that employs a monoclonal antibody to purified moray eel ciguatoxin (CTX-1) passively adsorbed onto colored polystyrene microbeads and a hydrophobic membrane laminated onto a solid plastic support. The membrane binds lipophilic substances such as ciguatoxin and specifically detects the toxin using the monoclonal antibody to CTX passively adsorbed onto microbeads. The intensity of the color on the membrane correlates to the concentration of toxin on the solid support by subjective scoring. This assay has a limit of detection at approximately 0.032 ng CTX/g fish tissue and has a sensitivity of 91% and specificity of 87%. However, this assay has been shown to cross react with other marine toxins including okadaic acid (Hokama et al., 1992), and has the potential to yield a high false positive rate depending on the fat content of the fish

(Tamaru et al. 2005). Recently, a sandwich ELISA was reported to detect a synthetic congener of ciguatoxin, CTX3C (Oguri et al. 2003, Tsumuraya et al. 2006), although the authors did not report testing actual fish extract or tissue using the assay.

2.5.3 Chemical methods

Because ciguatoxin and brevetoxin share a common receptor at the sodium channel receptor site 5, the use of labeled brevetoxin (^{3}H-PbTx-B) allows ciguatoxin to be quantified by competitive binding assay with sodium channel containing proteins using isolated rat brain synaptosomes (Trainer et al. 1995). The method requires a small amount of fish extract, is rapid and simple, and has a high sensitivity. Best suited for research purposes, this method is likely impractical for large-scale fish screening because of specialized equipment and the use of radiolabeled compounds.

Gradient reverse phase high performance liquid chromatography/mass spectroscopy (HPLC/MS), fast-atom bombardment tandem mass spectroscopy, and other physical-chemical methods have recently been used to elucidate the structures of ciguatoxins. While most ciguatoxins do not have a useful chromophore for selective spectroscopic detection, they do contain a reactive primary hydroxyl group that can be labeled after a clean-up step. HPLC coupled to fluorescence detection has proven effective when screening for ciguatoxin in crude fish extracts (Dickey et al. 1992, Yasumoto et al. 1993). HPLC with ionspray mass spectrometry has shown promise as a confirmatory analytical assay for ciguatoxins in fish flesh (Lewis et al. 1994). Nuclear magnetic resonance (NMR) has been used to characterize ciguatoxins in fish flesh (Lewis and Sellin 1992) and wild and cultured *G. toxicus* extracts (Murata et al. 1990, Satake et

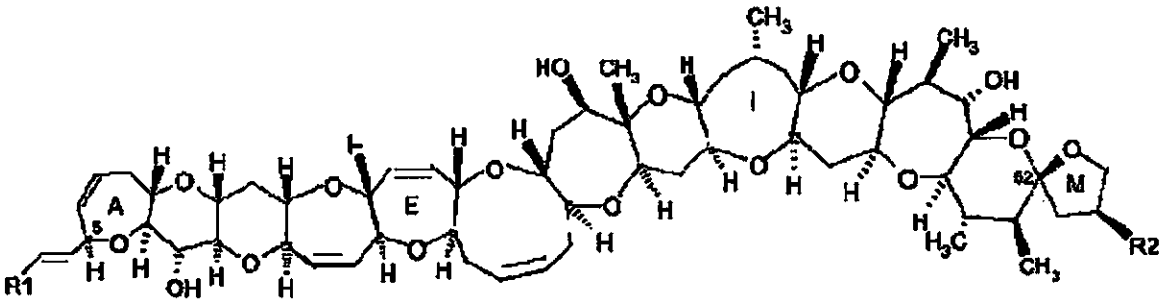
al. 1993, Lewis and Jones 1997), and Lewis and Jones (1997) used gradient reverse phase liquid chromatography/mass spectrometry methods to identify 11 new P-CTX congeners in a partially purified sample of toxic moray eel viscera. Similarly, LC-ESI-MS/MS (ESI= ElectroSpray Ionisation) was reported to detect levels of CTX equivalent to 40 ng/kg of P-CTX-1 in fish flesh and 100 ng/kg of C-CTX-1 in fish flesh (Lewis et al. 1999).

Table 2.1. Reported incidence and prevalence of ciguatera fish poisoning (adapted from Fleming et al. 1998)

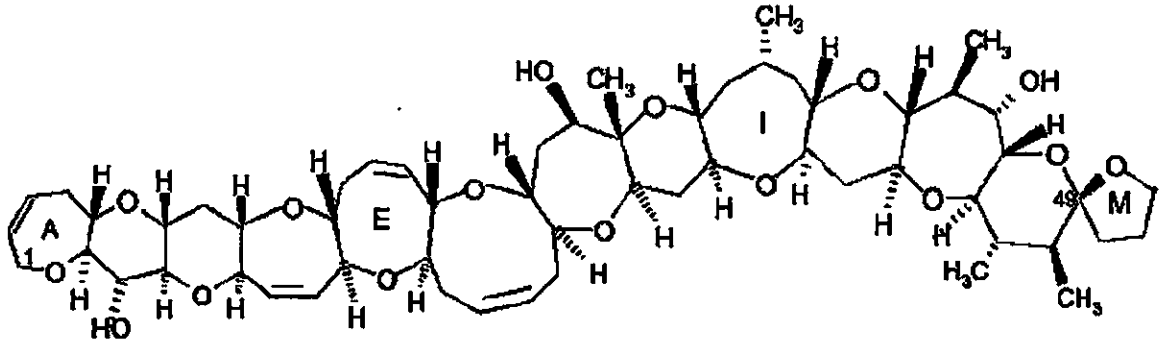
Rate	Location (Yrs.)
Annual Incidence:	
0.78/10,000/year	Reunion Island (1986-1994)
0.87/10,000/year	Hawai'i (1985-1989)
3.00/10,000/year	Queensland Australia (1965-1984)
5.00/10,000/year	Dade, Florida (1974-1976)
7.60/10,000/year	US Virgin Islands (1982)
30.0/10,000/year	Guadeloupe, Caribbean (1984)
100/10,000/year	South Pacific (1985-1990)
970/10,000/year	South Pacific (1973-1983)
5,850/10,000/year	French Polynesia (1979-1983)
Annual or Lifetime Prevalence:	
4.4%	St. Thomas, Virgin Islands (1980)
7.0%	Puerto Rico (Lifetime)
8.4%	Tahiti (1966)
10%	Niutao, Tuvalu (1990)
43%	Hao, Tuamotos (1978)
70%	Polynesian Islands (Lifetime)

Table 2.2. Clinical symptoms associated with ciguatera fish poisoning (adapted from Bagnis 1964)

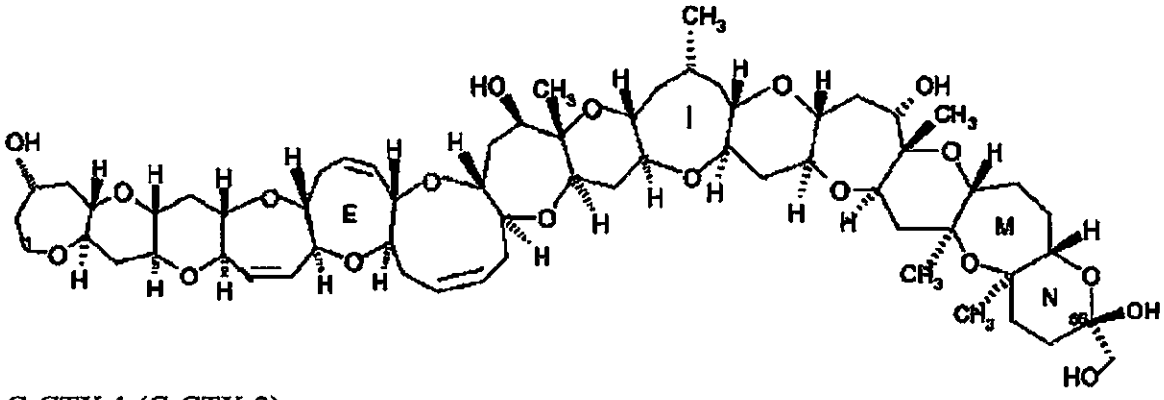
Category	Symptoms
Digestive	Nausea often followed by vomiting; diarrhea; painful defecation; abdominal pain and cramps. Symptoms general abate after 24 hours, leaving an asthenic and dehydrated patient.
Neurological	Dysethesia, principally with sensitivity to cold, temperature reversal; paresthesia, painful tingling of the palms of hands and soles of feet on contact with cold water; superficial hyperesthesia with sensation of burning and electrical discharge; mydriasis often present; patellar and Achilles reflexes sometimes diminished. Neurological symptoms generally persist for 1 week; it is not unusual to see contact dysethesia lasting a month.
Cardiovascular	Pulse slow (35-50 beats/minute) and often irregular; low arterial pressure (heart sounds distant). EKG may show dysrhythmia, from sinus bradycardia (slow heart beat) to bursts of supraventricular or ventricular extra systoles (rapid systolic heart beat). Ventricular tachycardia, excessively rapid action of the heart, may also occur. A first atricular-ventricular (A-V) block may also be observed. Cardiovascular disorders usually disappear in 48-72 hours and may be mistaken for a heart attack. Toxins from carnivorous fishes tend to cause cardiovascular problems.
General	Asthenia, making it difficult to walk, keeping patient in bed for several days; arthralgia, especially of the knee, ankle, shoulder, and elbow; dorsolumbar stiffness; myalgia, especially leg muscles; headache; marked and constant chilliness but no problems of thermal regulation; lipothymia and dizziness; itching 2 to 3 days after onset with may persist for many days; oliguria, sometimes during the first 48 hours.



	R1	R2
P-CTX-1:	$^{1}CH_2OHCHOH$	OH
P-CTX-3 (P-CTX-2):	$^{1}CH_2OHCHOH$	H
P-CTX-4B (P-CTX-4A):	$^{1}CH_2CH$	H



P-CTX-3C



C-CTX-1 (C-CTX-2)

Figure 2.1. Molecular structures of the primary congeners of Pacific and Caribbean ciguatoxins (FAO 2004).

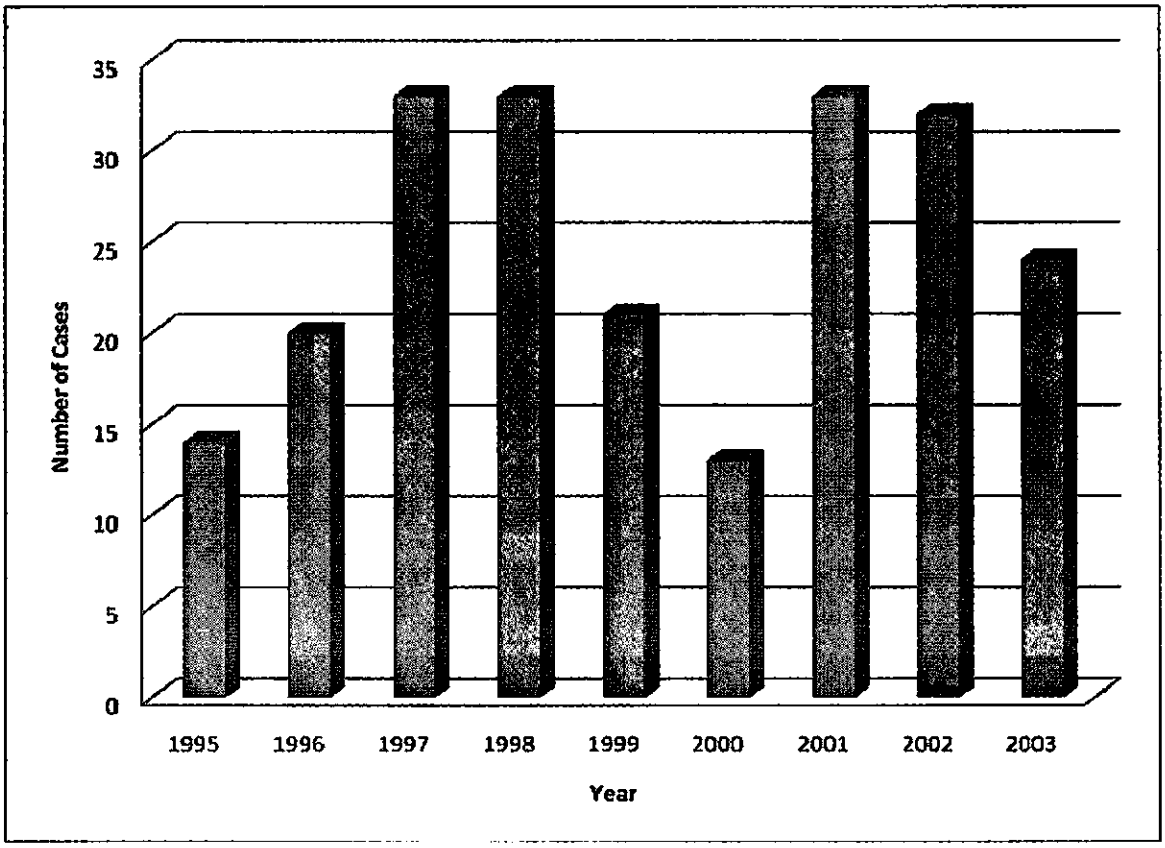


Figure 2.2. Number of ciguatera fish poisoning cases reported per year in Hawai'i from 1995 – 2003 (Source: Hawai'i Department of Health).

Chapter 3

Comparative analysis of purified Pacific and Caribbean ciguatoxin congeners and related marine toxins using a modified ELISA technique

3.1 Introduction

Ciguatera seafood poisoning, affecting more than 50,000 people annually, is caused by the ingestion of contaminated reef fishes that have accumulated ciguatoxins (CTXs) in their tissues (Daranas et al. 2001).

The main CTXs in the Pacific, present in different relative amounts in fish, are P-CTX-1, P-CTX-2, and P-CTX-3, with more than 20 additional congeners having been identified in recent years (Lehane and Lewis 2000, Lehane 2000). Other toxins, such as maitotoxins and scaritoxins can also occur in ciguatoxic fish extracts, though their potential roles in clinical poisoning are unclear. Maitotoxins, also produced by *Gambierdiscus toxicus*, are toxic via the intraperitoneal route, though they are 100 times less toxic orally, while CTX has been shown to be equipotent (De Fouw et al. 2001). Marine toxins such as domoic acid, palytoxin, and okadaic acid have also been implicated in mammalian disease outbreaks and often utilize molecular mechanisms of action similar to that of CTXs, primarily affecting cellular ion balance and regulation. Domoic acid, a neurotransmitter amino acid produced by the marine diatom *Pseudo-nitzschia*, causes amnesic shellfish poisoning and acts by binding to synaptic receptors of glutamic acid, causing ion channel permeability and subsequent cell death or dysfunction (Amzil et al. 2001). Palytoxin, one of the most deadly marine natural products, is produced by the marine dinoflagellate *Ostreopsis* sp. (Usami et al. 1995), and is found in the marine zoanthid Palythoa as well as invertebrates and fish species that live in and

around the zoanthid (Mebs 1998). Targeting the Na1/K1–adenosine triphosphatase (ATPase) pump, palytoxin converts the pump into a nonspecific ion channel, thereby short-circuiting membrane function of the cell and eventually causing cell lysis (Hilgemann 2003). Okadaic acid, a lipophilic phycotoxin produced by the marine dinoflagellate *Prorocentrum* sp. (Murakami et al. 1982), accumulates in mussels and can cause diarrhetic shellfish poisoning. Okadaic acid inhibits serine/threonine protein phosphatases, leading to hyperphosphorylation of sodium ion channels and other proteins responsible for maintaining proper ion balance and neurotransmission (Traore et al. 2003).

Current methods for the detection of CTX extracted from fish tissue include the mouse bioassay, neuroblastoma assay, and various immunological assays. Hokama (1987) developed a simple, rapid immunoassay using a mouse monoclonal antibody to CTX (MAb-CTX) passively adsorbed to colored latex beads. The test, since revised and now marketed as Cigua-Check™ (ToxiTec Inc., Honolulu, HI), has been shown to detect CTX at levels as low as 0.080 ng/mL in clinically implicated crude fish extracts (Hokama et al. 1998). This test, however, has been questionable at detecting purified CTXs at clinically significant levels, as the toxin likely requires the complex matrix of lipids that occur naturally in fish tissues in order to effectively bind to the solid phase membrane. The purpose of this study was to develop a modified enzyme-linked immunosorbent assay (ELISA) in order to determine and compare the MAb- CTX's affinity for different congeners of purified CTXs at varying concentrations, as well as to assay antibody cross-reactivity for selected marine toxins.

3.2 Materials and Methods

3.2.1 Marine toxins

Purified Pacific ciguatoxins (P-CTX-1, P-CTX-2, and P-CTX-3) and Caribbean ciguatoxin (C-CTX-1) were purchased from Richard Lewis, University of Queensland, Australia. Purified palytoxin was purchased from Hawai'i Biotech (Honolulu, HI), and pure domoic acid and okadaic acid were purchased from Sigma Chemical (St. Louis, MO). Crude extract was obtained from fish clinically implicated in ciguatera outbreaks as defined by the Hawai'i Department of Health. Fish tissue was extracted according to Kimura et al. (1982a) and extract was tested using the mouse bioassay to confirm toxicity. All toxins were stored at –80°C until use and were serially diluted using methanol for experimentation.

3.2.2 Monoclonal antibody conjugation and modified ELISA development

A monoclonal antibody to ciguatoxin from hybridoma culture was purified by salt fractionation and conjugated to horseradish peroxidase according to Voller and Bidwell (1986) using the glutaraldehyde method. The modified ELISA procedure was adapted from Hokama et al.'s (1985) protocol. In short, the sharp end of bamboo sticks were dipped in Pentel correction fluid (Pentel of America, Ltd., Torrance, CA) and allowed to air-dry overnight. Sticks were dipped in varying concentrations of purified toxins dissolved in methanol, or methanol alone as a control for 30 seconds, removed, and air-dried for 5 minutes. Sticks were subsequently dipped in a 1:500 MAb-CTX horseradish peroxidase conjugate (MAb-CTX-HRP) in Tris buffer with 1% nonfat dried milk to block nonspecific binding for 30 seconds, followed by three thorough washes in Tris buffer. Sticks were placed in o-phenylenediamine dihydrochloride (OPD) substrate for 15

minutes. The reaction was stopped by addition of an equal volume of 1M sulfuric acid. The resulting color was read using a microplate reader (Bio-Tek Instruments, Winooski, VT) at an absorbance of 490 nm. Five replicates of each sample concentration were assayed.

3.2.3 Data analysis

Sticks were treated using purified toxin dissolved in methanol at 0.078, 0.156, 0.625, 1.25, and 5.00 ng/mL, and methanol alone to serve as a control. The average of the methanol blanks was subtracted from the value of each individual data result (n=5 sticks tested per concentration) for the various concentrations of toxin. Outliers were determined using the Grubbs test and, if present, were eliminated from the data sets. These subtracted results were then averaged to obtain the data points used in linear and non-linear regression analysis using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA), with the methanol blank being scored as the value "0.00".

3.3 Results and Discussion

After optimizing the experimental conditions and obtaining linear curves for data points using ciguatera implicated positive fish extract in correlative milligram per milliliter ranges (Figure 3.1), pure P-CTX-1 was assayed in the 20–40 ng/mL range to verify the procedure using purified product. Because the MAb-CTX proved effective in detecting P-CTX-1 at these levels, the concentration of toxin was decreased in an effort to determine the lower limits and sensitivity of MAb-CTX using the MAb-CTX-HRP conjugate.

The MAb-CTX-HRP conjugate was shown to consistently detect purified P-CTX-1, P-CTX-2, and P-CTX-3 at levels ranging from 0.078 to 5.00 ng/mL (Figures 3.2 and 3.3),

exhibiting strong R^2 and P values (Table 3.1). C-CTX-1 was also reliably detected in the 0.078 to 1.25 ng/mL range, however, the optical density values level off slightly at the higher testing limit of 5.00 ng/mL, giving lower R^2 and P values, which at the outset make it appear to look statistically insignificant. Given that a saturation effect was observed, data were also analyzed using non-linear regression analysis which yielded more reliable R^2 values for both P-CTX-3 ($R^2 = 0.974$) and C-CTX-1 ($R^2 = 0.988$). In contrast, low, nonspecific affinity of MAb-CTX for okadaic acid, palytoxin and domoic acid is shown in Figure 3.5 with statistically insignificant R^2 and P values being demonstrated (Table 3.1). It is interesting to note that in other studies performed, okadaic acid appeared to be detected using this method at toxin concentrations higher than 25 ng/mL (data not shown), suggesting that concentration plays some role in the reactivity and specificity of the MAb-CTX.

The only commercially available ciguatera detection kit, Cigua-Check, employs a polyvinylidene difluoride (PVDF) membrane-based solid phase to successfully allow for crude toxin binding following a methanol extraction from fish flesh. The assay, however, has not been able to consistently detect purified CTXs in methanol. This is likely due in large part to the matrix of lipids that exist in conjunction with crude methanol fish extracts, allowing for more efficient binding of the small CTX molecule to the membrane. Purified toxins lack this diverse matrix essential for adequate membrane adherence and detection using this method. I adapted the original immunoassay developed by Hokama (1987), which effectively utilizes bamboo sticks coated in Pentel correction fluid, the porosity and composition of which appears to better bind both crude extract and purified toxins, to verify that the MAb-CTX used in testing crude fish can

adequately detect purified CTXs, thus providing the rationale for protocol modification. The data presented in this chapter confirms previous AOAC collaborative studies (Hokama et al. 1998) showing that the MAb-CTX used in the Cigua-Check™ membrane immunobead assay can detect as little as 0.080 ng/mL of CTX in crude fish extract standard.

Efforts were made to minimize error, however, there does exist some variation and nonspecific binding in the optical density readings for different toxins, which can be accounted for in large part by visually undetectable differences in bamboo stick diameter and coating of substrate on the stick, and possible differences in the estimated actual purity of "purified" compounds purchased or received through third-party sources. The modified ELISA assay as described is not suitable for commercial purposes, given that the above factors largely contribute to variations in selectivity and specificity; however, it does serve as a rapid, reliable means for verifying and comparing cross-reactivity with the immunological methodology used in the marketplace for detecting CTX in methanol extracts of fish flesh.

Table 3.1. Linear regression analyses of MAb-CTX vs. various compounds using modified ELISA.

Compound	Linear Regression	
	R^2	P Value
Ciguatera-implicated extract	0.9646	0.0029
P-CTX-1	0.9451	0.0012
P-CTX-2	0.9574	0.0007
P-CTX-3	0.8511	0.0088
C-CTX-1	0.4940	0.1348
Palytoxin	0.1042	0.5327
Okadaic acid	0.0188	0.7956
Domoic acid	0.3652	0.2039

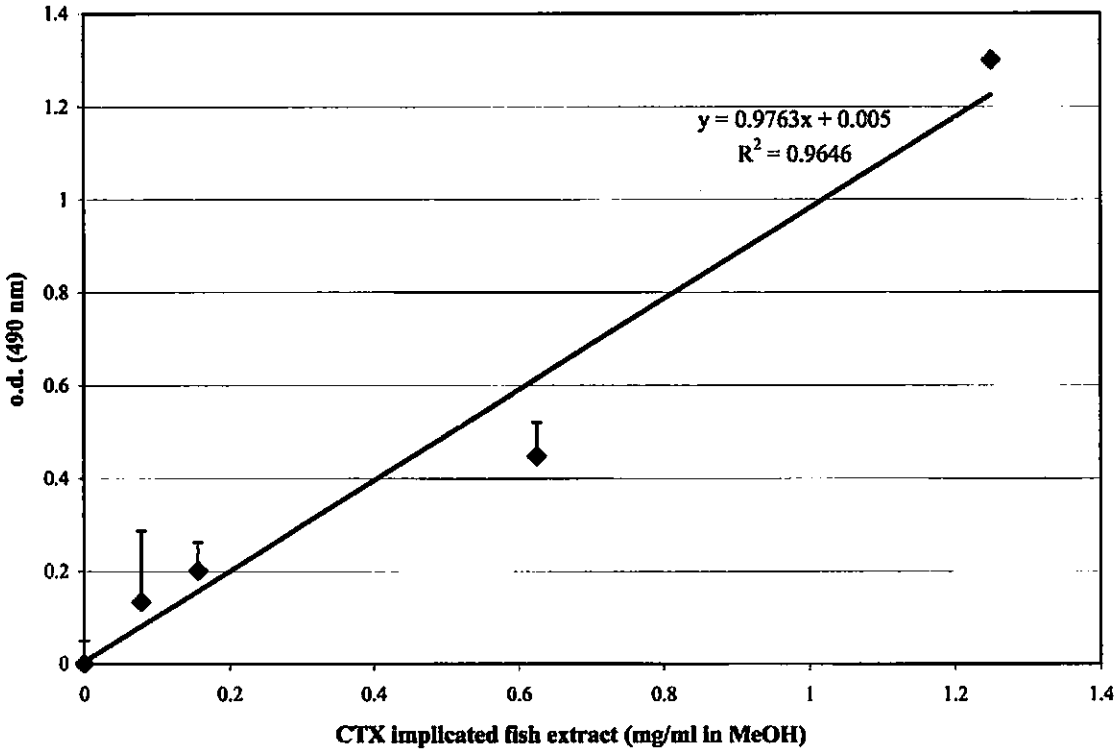


Figure 3.1. Results from modified ELISA showing specificity of MAb-CTX for CTX-implicated positive fish extract.

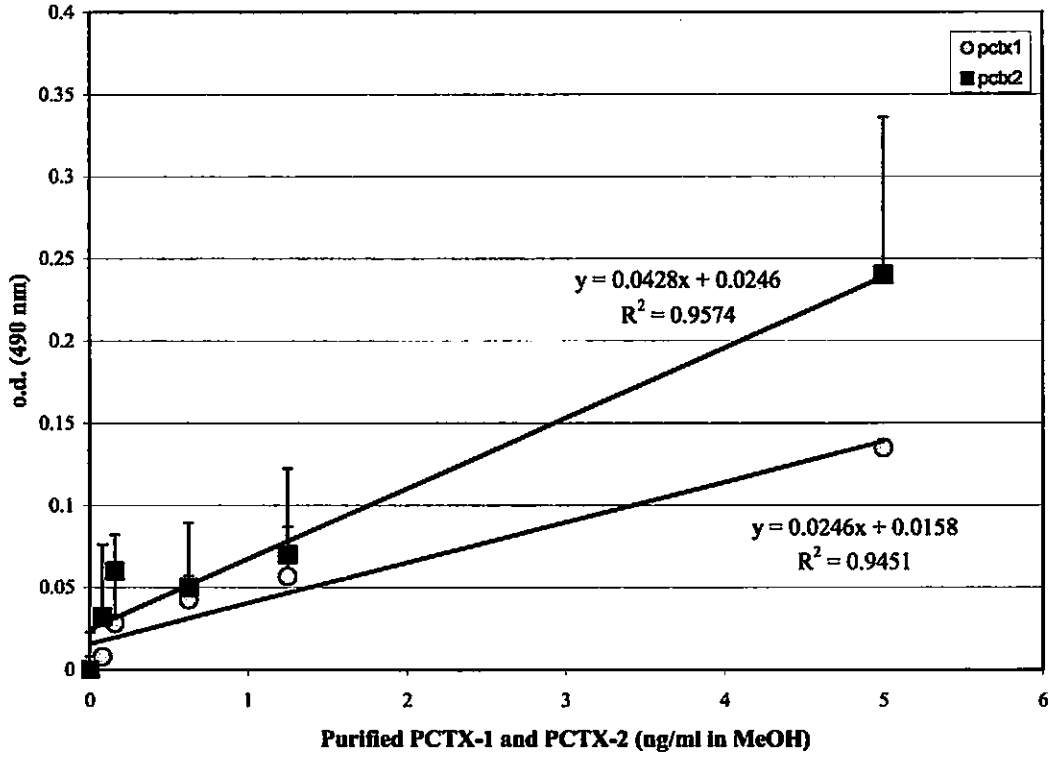


Figure 3.2. Results from modified ELISA showing specificity of MAb-CTX for purified P-CTX-1 and P-CTX-2.

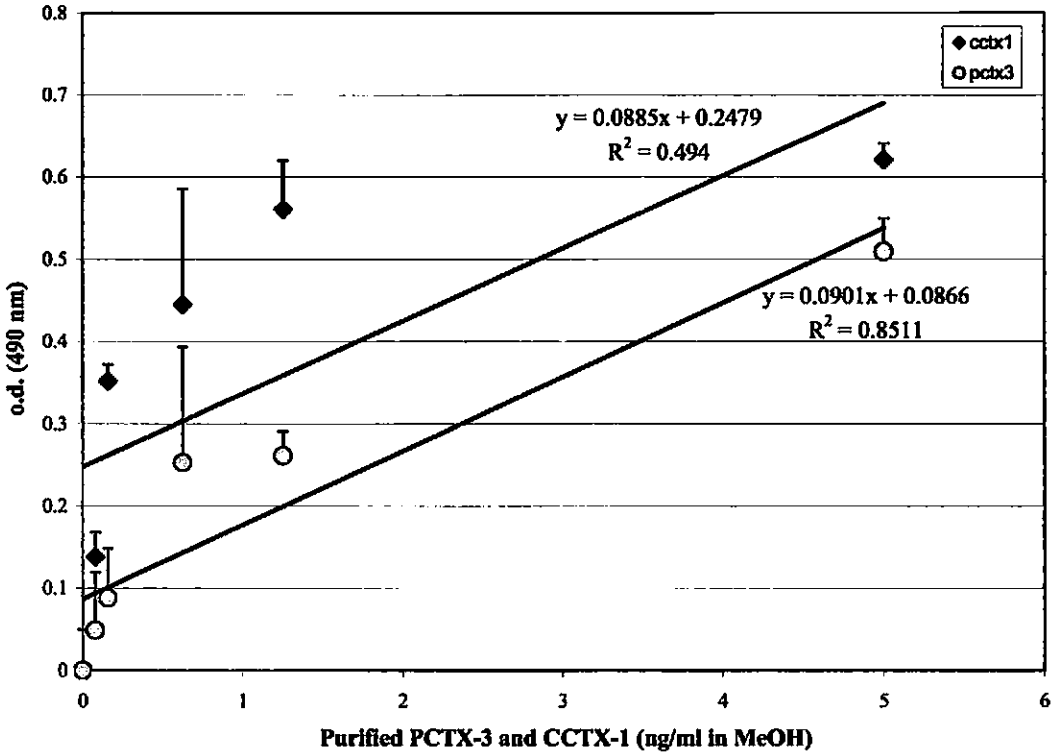


Figure 3.3. Linear regression analysis results from modified ELISA showing specificity of MAb-CTX for purified P-CTX-3 and C-CTX-1.

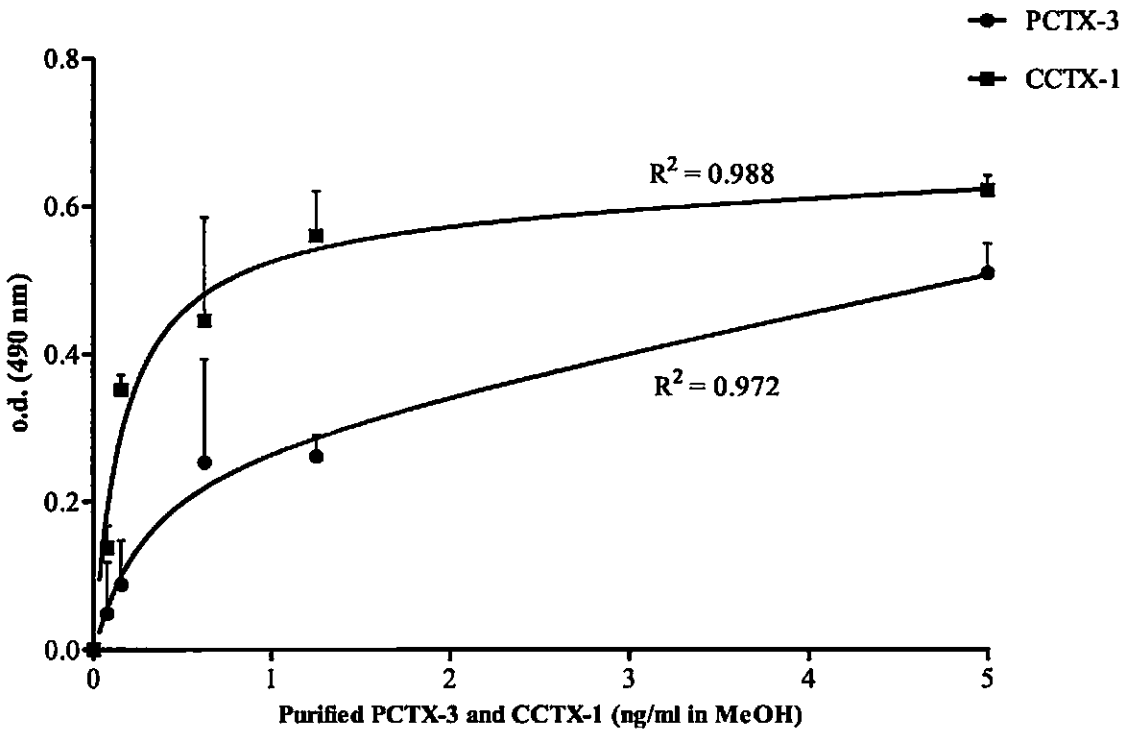


Figure 3.4. Nonlinear regression analysis results from modified ELISA for purified P-CTX-3 and C-CTX-1.

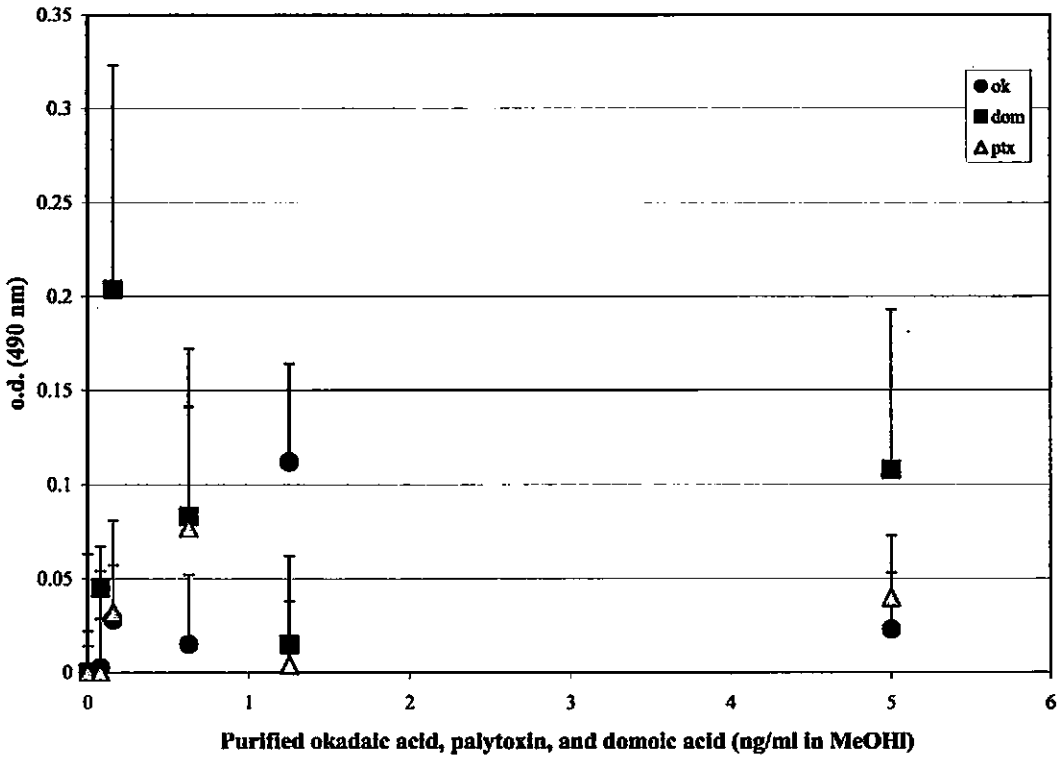


Figure 3.5. Results from modified ELISA showing specificity of MAb-CTX for purified okadaic acid (OA), palytoxin (PTX), and domoic acid (DA).

Chapter 4

Biological activity of the ciguatoxin fragment AB on the neuroblastoma sodium channel in tissue culture

4.1 Introduction

It is well established that ciguatoxin (CTX) acts on the sodium channel of various cells and increases action potential by influx of sodium into the cells via the sodium channel (Miyahara et al. 1985). This has been demonstrated in pharmacological in vitro assays using guinea pig atrial tissue (Hokama and Miyahara 1986) and through the cytotoxic effects of CTX in combination with chemical agonists ouabain and veratridine on mouse neuroblastoma (NB) cells in tissue culture (Manger et al. 1993, 1995). In this study, the action of synthetic fragments of ciguatoxin on the sodium channel were analyzed using the guinea pig atrium and neuroblastoma cell assays, providing evidence that the major ciguatoxin epitope associated with the activation of the sodium channel is the AB region, depicted in Figure 4.1a. Competitive inhibition experiments with the AB fragment of CTX and sodium channel receptor proteins extracted from epithelial membrane of pig intestine also demonstrate that the AB region of CTX is likely associated with the activation of the sodium channel. Conversely, the JKLM fragment of CTX (Figure 4.1b), showed no measurable effects on the sodium channel in guinea pig atrial tissue and NB cells in tissue culture.

4.2. Materials and Methods

4.2.1 Ciguatoxin extraction

The organic solvent extraction and partial purification of CTX from fish flesh and viscera were performed as previously reported (Kimura et al. 1982a). The partial purification steps included separation in silica gel chromatography and further partitioning into four major fractions: fraction 1 was partitioned in 100% chloroform; fraction 2 in 10% methanol/90% chloroform; fraction 3 in 50% methanol/50% chloroform; and fraction 4 in 100% methanol. A mixture of fractions 2 and 3 were further purified chromatographically on the C18 solid phase column and eluted with ethyl acetate and hexane (Kimura et al. 1982b). Both fractions were shown to be active in the membrane immunobead assay (MIA) test (Hokama et al. 1998) and were examined using the NB assay.

The AB and JKLM fragments of ciguatoxin were synthetically produced and generously provided by Professor Minoru Isobe (Nagoya University, Japan, Department of Chemistry). A previous study using the MIA immunological inhibition test of CTX fragments with partially purified CTX extracted from fish clinically implicated in ciguatera outbreaks by the Hawai'i Department of Health (DOH-CTX) demonstrated that the monoclonal antibody to CTX (MAb-CTX) reacted with the JKLM but not the AB fragment (Hokama et al. 2003).

4.2.2 Guinea pig atrial assay for sodium channel activity

Inotropic effects of partially purified DOH-CTX and other marine toxins were determined by the method developed by Miyahara et al. (1985) and Hokama and

Miyahara (1986). Atrial tissue dissected from adult male Hartley guinea pigs was suspended in a physiobath containing 25ml Krebs bicarbonate solution, pH 7.4 maintained at 30°C temperature and aerated with 95% air and 5% CO_2. After 15 to 30 minutes and established equilibrium of the heart beat, partially purified DOH-CTX (10 mg/bath), pure maitotoxin (MTX; 50 ng/bath), palytoxin (PTX; 32 ng/bath), and brevetoxin (PbTx-3; 1 µg/bath) were added to the physiobath to obtain inotropic response curves. Ten minutes after the initial inotropic response, solutions of various inhibitors were added to the physiobath using separate segments of atrium for each toxin. These included 0.15 ml of the following at $1x10^{-7}$ M: tetrodotoxin (TTX), verapamil, propanol, and phentolamine. The physiobath was rinsed with Krebs solution to complete the assay.

4.2.3 Neuroblastoma cell assay

The NB cell assay was modified after the method outlined by Manger et al. (1993, 1995) and preparation of toxins at various concentrations were performed as previously described (Hokama 1995). Mouse neuroblastoma cells (neuro-2a, CCL131, ATCC) were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 2mM glutamine, 1mM sodium pyruvate, 50 µg/ml streptomycin, and 50 units/ml penicillin. Cultures were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere.

Neuroblastoma cells from stock culture were seeded in complete media into 96 well microtiter plates at a concentration of 5×10^5 cells/ml in 100 µl volumes per well and maintained for 24 hours at 37°C. After 24 hours, 10 µl each of 10mM ouabain, 1mM veratridine, and dilutions of synthetic fragments were added to replicate wells. Control wells of ouabain/veratridine (OV) and untreated controls received incomplete RPMI

media to make up for volume differences. Plates were incubated at 37°C for 16-20 hours for the detection of sodium channel activity indicated by increased cytotoxicity when compared to the control wells. End-point assessment with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoleum) was carried out and color end-point was read at 490 nm with a microplate reader (BioTek, Winooski VT). Reagents were obtained from Sigma Chemical, St Louis MO unless otherwise indicated.

4.2.4 Extraction of sodium channel receptor from pig gut epithelial cell layer

Fresh, cleaned pig gut was obtained from a commercial food market. It was cut longitudinally to expose the epithelial layer. The layer was scraped with a scalpel and the free tissue layer was soaked in distilled water and mixed with a magnetic stirrer for one hour at room temperature. The suspension was centrifuged at 2500 rpm for 30 minutes. The clear supernatant containing the soluble sodium channel receptors was decanted for use in the NB assay. In addition, three concentrations of the pig gut extract containing the sodium channel receptor were added to the physiobath containing the guinea pig atrial tissues suspended in Krebs bicarbonate solution.

4.3 Results and Discussion

CTX action on sodium channels causes positive inotropic (increasing contractile force) responses in cardiac tissues is shown to be a composite of two effects: an initial rapid effect resulting from catecholamine release from adrenergic nerves and a slow developing sustained effect arising from direct depolarizing action on the myocardium, which is antagonizable by adrenoreceptor blocking agents and sodium channel blockers

such as TTX (Lewis and Endean 1986). Other marine toxins show differing inotropic effects depending on their mechanism of action.

Typical inotropic curves for the guinea pig atrial assay are shown in Figures 4.2a-d for four marine toxins: maitotoxin at 50 ng (Figure 4.2a), palytoxin at 32 ng (Figure 4.2b), brevetoxin (Figure 4.2c) at 1 μg, and partially purified DOH-CTX at 10 mg (Figure 4.2d). MTX reactivity in Figure 4.2a shows a sloping ascending curve with slight block with TTX 10^{-7} M, moderate block with 10^{-7} M verapamil, and slight block with 10^{-7} M with propanol and phentolamine, which serve as adrenergic blockers. Pure PTX response shows a moderated ascending slope with a long descending curve, showing inhibition with verapamil 10^{-7} M and no inhibition with TTX, 10^{-7} M and adrenergic blockers 10^{-7} M. Brevetoxin shows a strong rapid ascending curve with a strong block with TTX 10^{-7} M but none with verapamil 10^{-7} M, while the partially purified DOH-CTX shows an initial moderate ascending slope which was strongly inhibited by TTX 10^{-7} M and a moderate block by verapamil. Brevetoxin and ciguatoxin show characteristic patterns of sodium channel activators, the effects of which can be readily reversed by addition of TTX. Maitotoxin and palytoxin, although greater in overall toxicity, tend to be more destructive in the lysis of erythrocyte and muscle tissues, causing severe tissue destruction as suggested by pathological studies (Mossman 1983).

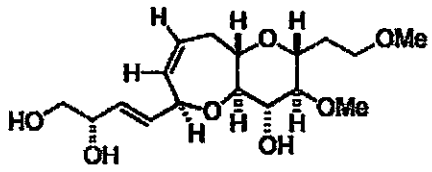
Using the partially purified DOH-CTX, sodium channel activity was analyzed using competitive inhibition in the presence and absence of MAb-CTX and pig gut sodium channel receptors in the guinea pig atrial trial assay. Figure 4.3a-c shows the inhibition of the inotropic response by pig gut extract containing soluble sodium channel

receptors at three graded concentrations, where competitive binding of the AB region of the CTX molecule is likely occurring, thereby inhibiting or reducing the typical response. Conversely, MAb-CTX, which has previously been shown to react immunologically with the JKLM region of CTX, added in high concentrations to the guinea pig atrial assay showed no inhibition of the inotropic effect, indicating that competitive binding at the JKLM region of the molecule has little or no effect on sodium channel activity. The TTX effect also appeared to be nullified by the pig gut epithelial extract.

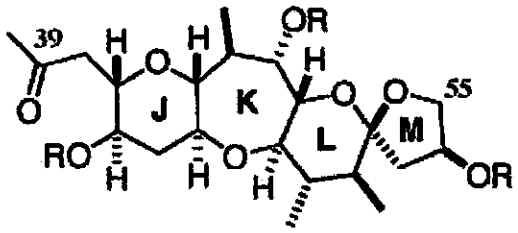
In the presence of chemical agonists ouabain and veratridine (OV), ciguatoxin will cause increased cytotoxicity, or inhibition of cell proliferation, in NB cells due to specific interactions at the sodium channel. The NB assay colorimetrically measures the dehydrogenase activity of live cells; thus cytotoxicity when compared to controls can be quantitatively determined by decreased absorbance, or optical density, when read using a microplate reader. The synthetic fragments from both regions of the molecule were tested using the NB assay to determine which is responsible for activity at the sodium channel. Figure 4.4 shows increasing cytotoxicity when treated with varying nanogram concentrations of the synthetic AB fragment of CTX, showing the most pronounced effects at a concentration of 1.5 ng/ml. Two tailed t-tests showed that concentrations of 1 ng/ml and 1.5 ng/ml were significantly different from the OV control ($P=0.042$ and $P=0.005$, respectively). The addition of MAb-CTX at 10 ng/ml, which reacts with the JKLM region of the molecule according to previous studies, did not neutralize the action of the AB fragment ($P<0.001$), also shown in Figure 4.4. Conversely, competitive inhibition when adding varying concentrations of pig extract sodium channel receptors to

0.5 ng/ml, 1.0 ng/ml, and 1.5 ng/ml of the AB fragment of CTX with ouabain and veratridine completely antagonized or neutralized the effects of AB fragment cytotoxicity as shown in Figure 4.5. Figure 4.6 demonstrates that the addition of CTX synthetic JKLM fragment at concentrations of 0, 0.5 ng/ml, 1.0 ng/ml, and 1.5 ng/ml in the presence of ouabain and veratridine and MAb-CTX had no observed cytotoxic effects on the NB cells. Similarly, addition of pig gut extract shows no effect (data not shown).

Data presented in the guinea pig atrial and NB cell assays strongly support the contention that ciguatoxin acts via the AB region of the CTX molecule on sodium channels of tissue, cardiac, and muscle cells. It is interesting to note that the MAb-CTX, which binds to the JKLM region of the CTX molecule shows no inhibition of the sodium channel effect, though it is useful in detection of whole ciguatoxin by the MIA procedure from ciguatoxic fish tissues (Hokama et al., 1998). It is concluded that the AB region of the CTX molecule is associated with important sodium channel activation and that the JKLM region of the CTX molecule is the antigenic epitope for the MAb-CTX used in the MIA test for identifying ciguatoxic fish.



AB-ring methyl ether



(a) (b)

Figure 4.1. Synthetic fragments of ciguatoxin: (a) the AB fragment, and (b) the JKLM fragment (Hokama et al 2006).

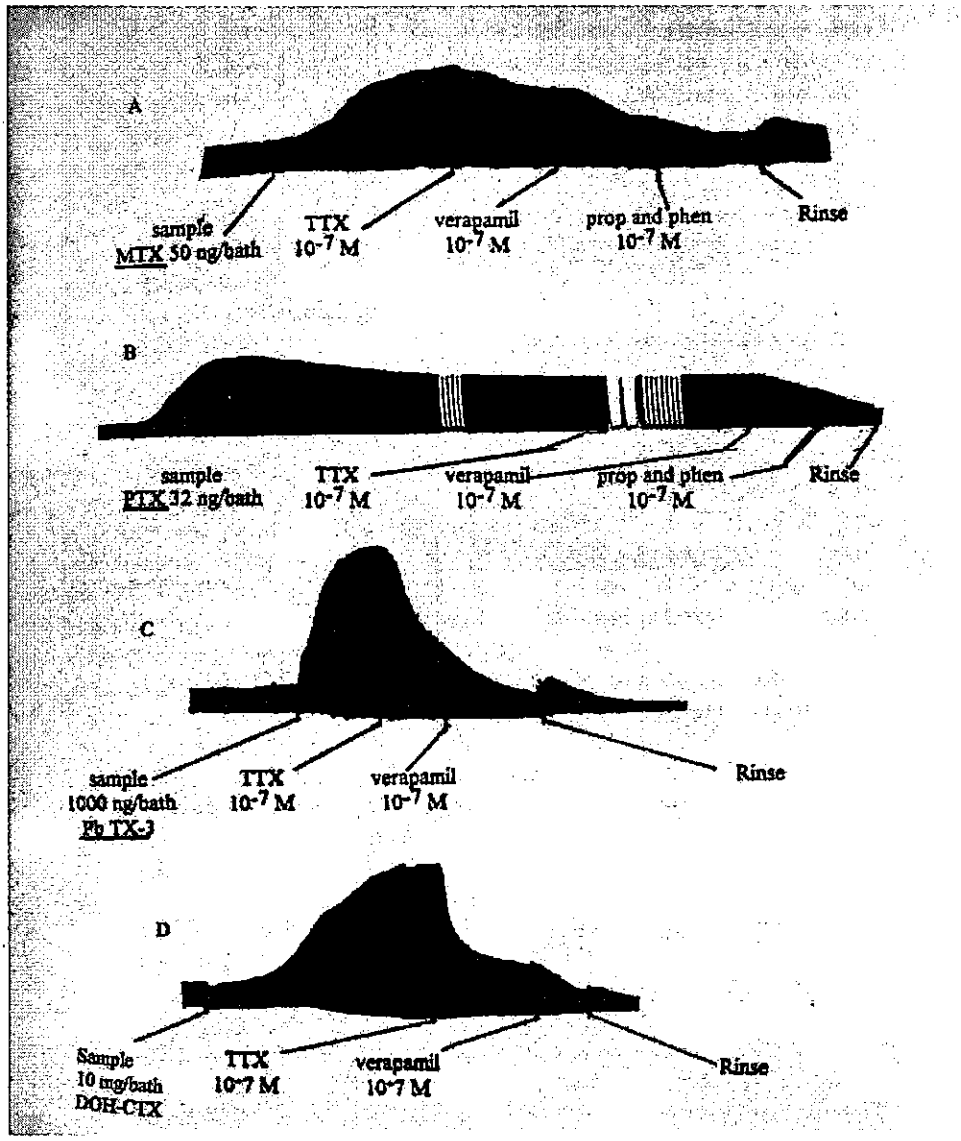


Figure 4.2. Typical inotropic curves for the guinea pig atrial assay are shown for (a) maitotoxin at 50 ng, (b) palytoxin at 32 ng, (c) brevetoxin–3 at 1 μg, and (d) partially purified DOH-CTX at 10 mg. Response is measured as the intensity of contractile force over time. (Source: Hokama et al 2006).

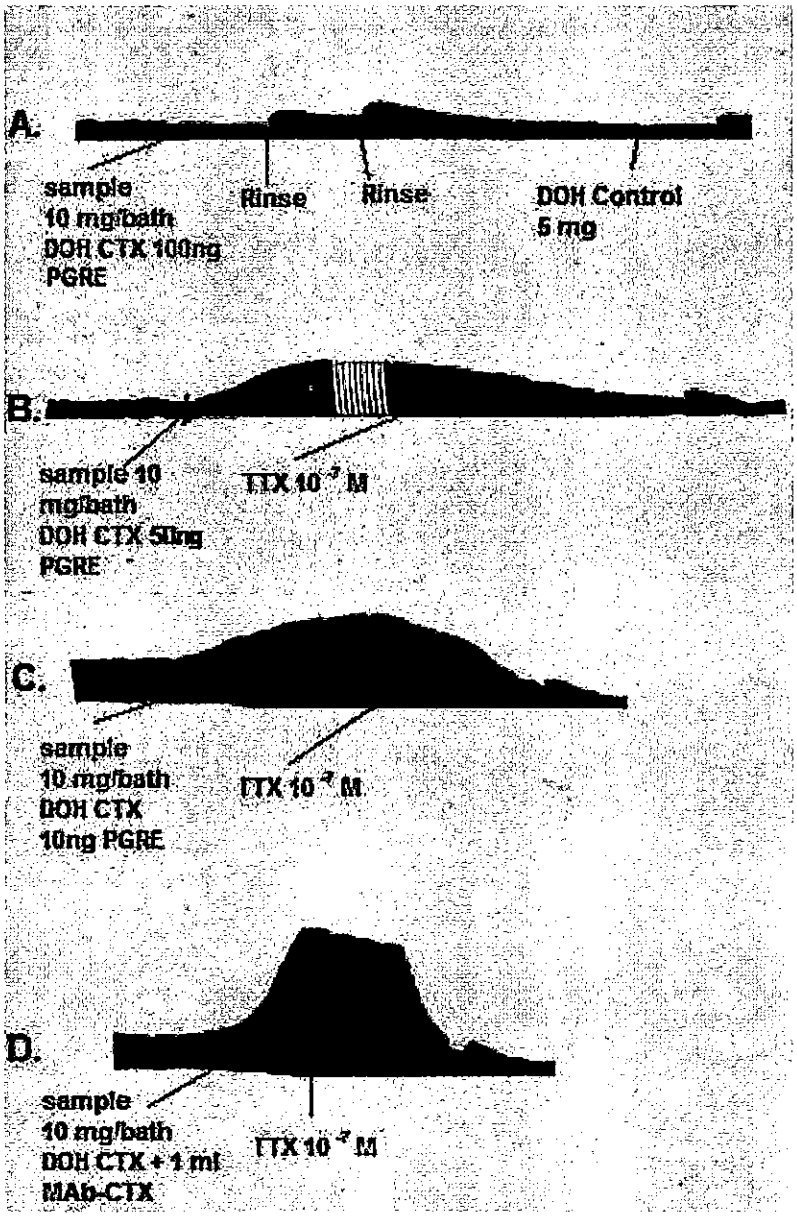


Figure 4.3. Dose response inhibition of the inotropic response by pig gut extract (PGRE) containing (a) 100 ng, (b) 50 ng, and (c) 10 ng of soluble sodium channel receptors when using 10 mg of partially purified CTX; (d) lack of inhibition of inotropic response when MAb-CTX is incubated with 10 mg partially purified CTX. Response is measured as the intensity of contractile force over time. (Source: Hokama et al 2006).

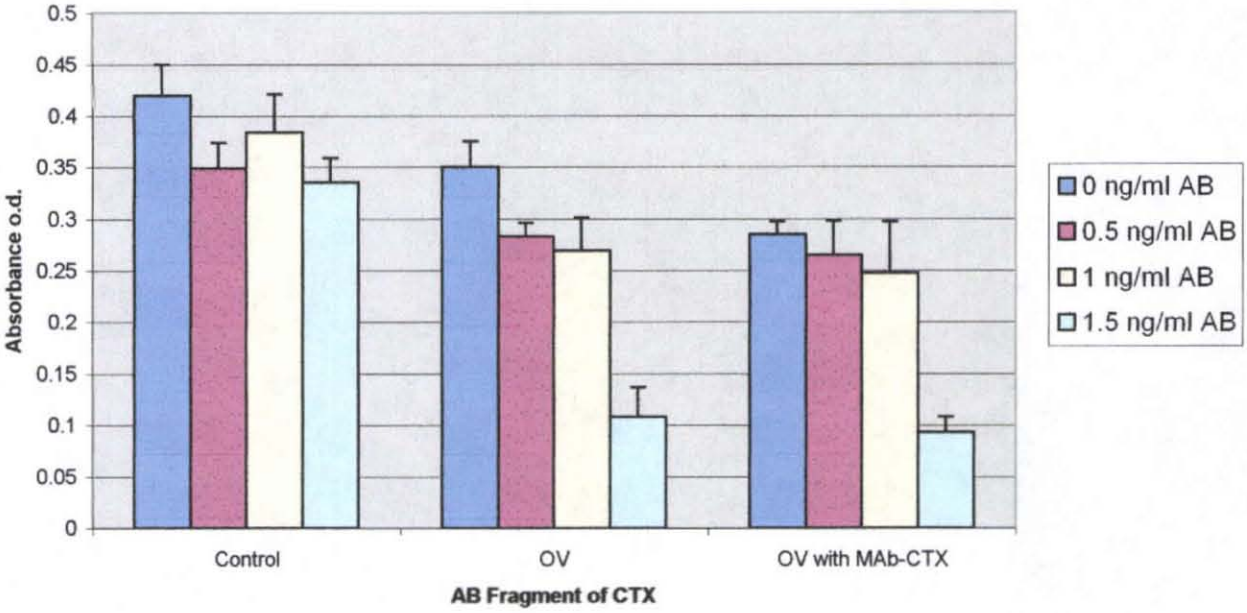


Figure 4.4. The effects of the AB fragment of CTX on the sodium channel using the neuroblastoma cell assay in the presence of ouabain and veratridine (OV) with and without the addition of 10 ng/ml MAb-CTX. Data represents the average optical density of triplicate wells measured at 490 nm, and vertical bars indicate + standard deviation.

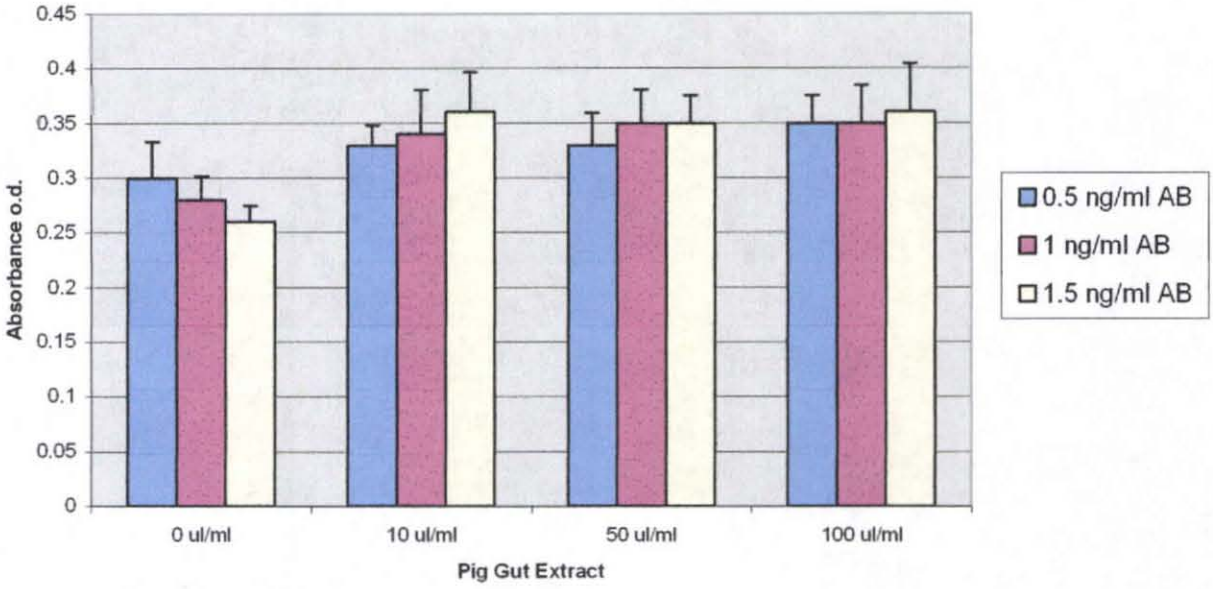


Figure 4.5. Competitive inhibition effects of the AB fragment of CTX on the sodium channel using the neuroblastoma cell assay with various concentrations of pig gut extract containing sodium channel receptors. Data represents the average optical density of triplicate wells measured at 490 nm, and vertical bars indicate + standard deviation.

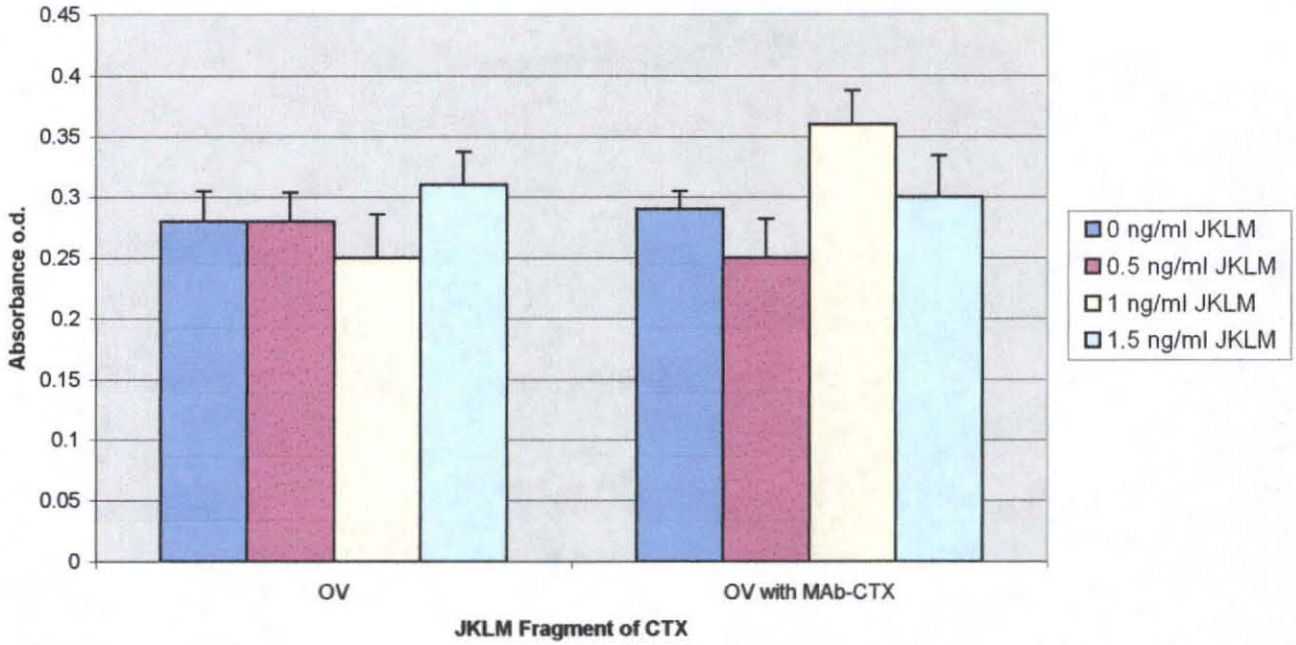


Figure 4.6. The effects of the JKLM fragment of CTX on the sodium channel using the neuroblastoma cell assay in the presence of ouabain and veratridine (OV) with and without the addition of 10 ng/ml MAb-CTX. Data represents the average optical density of triplicate wells measured at 490 nm, and vertical bars indicate + standard deviation.

Chapter 5

Development and validation of an enzyme-linked immunosorbent assay (ELISA) for the detection of ciguatoxin using chicken immunoglobulin Y

5.1 Introduction

In the past, illness from the ingestion or inhalation of toxins such as ciguatoxin have been confined to seafood-dependent populations in island or coastal regions, however, with increased tourism, international fishing trade, and the importation of various reef fish by the seafood industry, there have been increasing reports of marine toxin illnesses in inland parts of the world. An estimated 50,000 cases of ciguatera fish poisoning are reported each year worldwide (Baden et al. 1996), although that figure is likely a low conservative estimate due in part to misdiagnosis and underreporting.

Drawbacks to currently available screening methods for ciguatoxin in fish tissues include non-specificity, costly routine screening, and questionable reliability for yielding quantitative results. While enzyme-linked immunoassays specific for ciguatoxin or synthetic fragments of the toxin have recently been reported in the literature (Oguri et al. 2003, Tsumuraya et al. 2006), none have been shown to adequately screen fish extracts, nor have any utilized chicken IgY as an immunoanalytic tool.

ELISA is a sensitive, specific method that has been used to successfully screen for small molecules in seawater, fish tissue extracts, and mammalian body fluids. To counter some of the disadvantages of mammalian antibodies, avian egg yolk antibody has been identified as a convenient, non-invasive, inexpensive source of antibodies (Polson et al. 1980, Jensenius et al. 1981). Immunoglobulin Y (IgY) is a counter part of mammalian

IgG present in the egg yolk of aves, reptiles, and amphibians (Warr et al. 1995), differing in molecular weight (IgG = 150 kDa, IgY = 180 kDa) from IgG. Like IgG, IgY has two heavy chains and two light chains; however, IgY has four constant regions compared to three for IgG (Chiou 2003). Chicken IgY antibodies have been used in recent years to develop ELISA assays in a variety of disciplines and are a promising new tool in assay development.

A sandwich ELISA was developed for the detection of CTX using a IgY immunoglobulin produced in chickens immunized with the ABCD fragment of CTX (anti-CTX ABCD) and a mouse anti-CTX IgG previously described (Hokama 1990) that recognizes the JKLM fragment of CTX (anti-CTX JKLM). Figures 5.1a and 5.1b show the molecular structures of the CTX fragments targeted by the antibodies.

5.2 Materials and Methods

5.2.1 Antibody preparation

Mouse anti-CTX JKLM was produced as previously described (Hokama 1990), and maintained as a hybridoma cell line in tissue culture flasks (Costar, Corning, NY) in DMEM media (Sigma Chemical Company, St. Louis, MO), kept at 37 ° C with 5% CO_2. Antibody was fractionated and affinity purified using Protein A column chromatography for use in assay development. This antibody was conjugated to horseradish peroxidase (HRP) using the EZ Link Activated Peroxidase Kit (anti-CTX JKLM-HRP) (Pierce Chemical Company, IL).

The ABCD fragment of the CTX molecule was chemically synthesized and generously provided by Professor Minoru Isobe (Nagoya University, Japan). Five

hundred micrograms of the fragment was conjugated to 50 milligrams of human serum albumin (HSA) using an 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Sigma Chemical Co., St. Louis, MO) mediated conjugation as described by Hokama et al. (1977). The resulting immunogen conjugate was sent to Aves Labs, Inc. (Tigard, OR) for injection into chickens. The IgY fraction containing the ABCD fragment of CTX, as well as pre-immune chicken IgY, was collected and stored at 4° C for use in ELISA development.

5.2.2 CTX standards and other marine toxins

Toxic fish samples previously implicated in ciguatera fish poisoning incidents were obtained from the Hawai'i Department of Health (DOH). Fish were submitted from patients with clinical symptoms verified by an attending physician or the DOH Epidemiology Branch staff and tissues were extracted and partially purified according to the method of Kimura et al. (1982a). The DOH CTX positive extract was evaluated for the presence of CTX using the membrane immunobead assay (MIA), mouse toxicity, neuroblastoma, and guinea pig atrial assays (Hokama et al. 1994). Based on the neuroblastoma assay and inhibition studies using the MIA technique, the DOH CTX positive extract was calculated to contain approximately 0.32 ng of CTX per mg of extract (Manger et al. 1995, Hokama et al. 1998). The same extraction and purification procedures for processing fish extract were followed for a deep water pelagic fish that is not expected to harbor ciguatoxin (Pacific Blue Marlin, *Makaira nigricans*) to serve as a non-toxic control for use in experiments.

Purified brevetoxin-3 (PbTx-3), okadaic acid, and domoic acid were purchased from Calbiochem, (San Diego, CA) for cross-reactivity studies.

5.2.3 Sandwich ELISA for detection of CTX

A sandwich ELISA was developed in the following manner: 96-well ELISA plates (Costar, Corning, NY) were coated with 0.25 mg/ml anti-CTX ABCD IgY antibody in phosphate buffered saline (PBS), pH 7.4, 100 µl/well, and incubated overnight at 4 ° C. Non-specific binding sites were blocked with a milk-free blocking solution, Superblock (Pierce Chemical Company, IL), 200 µl/well for 5 minutes followed by three washes with PBS wash buffer, pH 7.4. Partially purified CTX positive extract was diluted in 20% methanol : 80% PBS buffer at concentrations ranging from the methanol:PBS buffer alone as a control, and 0.004 mg/ml to 0.5 mg/ml. One hundred microliters of the control and standards were added to each well in quadruplicate, and plates were incubated for 1 hour at room temperature followed by three washes with PBS to remove excess antigen. Anti-CTX JKLM-HRP monoclonal antibody (30 µg/ml in PBS, pH 7.4) was added to each well, 100 µl/well, and incubated for 1 hour at room temperature followed by six thorough washes with PBS to remove residual antibody. Bound antibodies were visualized by the addition of 100 µl/well *o*-phenylenediamine (OPD; Sigma Chemical Company, St. Louis, MO). Reaction was allowed to develop for 5 minutes before being stopped by the addition of 50 µl/well 1N sulfuric acid. Absorbance was measured at 490 nm using a microplate reader (BioTek Instruments, Winooski, VT). Results are expressed as relative absorbance units.

The specificity of the assay was further assessed using a competitive ELISA method in which the anti-CTX ABCD antibody was coated onto the microplate overnight and 1 mg/ml of CTX positive extract in 20% methanol : 80% PBS buffer and a buffer control were applied as described in the previous section and incubated for 1 hour. Simultaneously in a separate test tube, CTX positive extract at 0.032 mg/ml, 0.125 mg/ml, 0.5 mg/ml, and 1.0 mg/ml were added to 30 µg/ml of anti-CTX JKLM-HRP antibody in PBS and incubated for 1 hour at room temperature. A control was also performed in which antibody was incubated alone without any added extract. After washing the microplate with PBS, one hundred microliters per well of each of the above extract-antibody mixtures was transferred to the CTX treated ELISA plate and incubated for 1 hour. The assay proceeded as described previously, and signal obtained was plotted showing percent inhibition against the free CTX concentration. All experiments were performed in triplicate and mean values were obtained.

5.2.4 Neuroblastoma cell assay

The neuroblastoma (NB) cell assay was modified after the method of Manger et al. (1993, 1995) as described in section 4.2.3 of this dissertation, with minor modifications. Briefly, mouse neuroblastoma cells (neuro-2a, CCL-131, ATCC, Manassas, VA) were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2mM glutamine, 1mM sodium pyruvate, 50 µg/L streptomycin, and 50 units/mL penicillin. Cultures were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere. NB cells from stock culture were seeded in complete media in 96 well microtiter plates (Costar, Corning, NY) at a

concentration of 1.5 x 10^5 cells/ml in 100 µl volumes per well and maintained at 24 hours at 37°C. After 24 hours, 10 µl each of 10mM ouabain and 1mM veratridine and dilutions of both partially purified CTX positive and non-toxic fish extracts were added to wells in triplicate. Control wells of ouabain/veratridine alone and untreated controls received media to make up for volume differences. The plates were incubated at 37°C for 16-20 hours for the detection of sodium channel inhibition. End-point assessment using MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleum) was carried out as described previously and color end point was read at a wavelength of 490 nm using a microplate reader (BioTek, Winooski, VT). Reagents were purchased from Sigma Chemical unless otherwise indicated. Data was analyzed to obtain the mean and standard deviation of triplicate wells.

Statistical analyses were performed using Microsoft Excel and GraphPad Prism 5.0 software (San Diego, CA).

5.3 Results

5.3.1 ELISA development, optimization, and validation

Several different ELISA platforms and antibody combinations were tested, and a checkerboard titration was performed to determine the optimal reagent concentrations. Because CTX may be susceptible to conformation changes in a purely aqueous solution, various ratios were tested in order to determine the optimal solvent to buffer ratio for use in the assay, which was found to be 20% methanol: 80% PBS. In order to show that the anti-CTX ABCD antibody was reactive when compared to other coating materials, bovine serum albumin (BSA), pre-immune chicken IgY, and anti-CTX ABCD chicken

IgY at 0.25 mg/ml in PBS were used to coat the microplate and the assay was performed as described. BSA and pre-immune IgY showed partial reactivity as a coating substrate, however, statistical two tailed unpaired t test analysis showed that the anti-CTX ABCD IgY antibody is the optimal solid phase antibody in the ELISA as indicated by a higher relative absorbance when detecting CTX positive extract at levels above 0.016 mg/ml ($p<0.05$) (Table 5.1). The anti-CTX JKLM antibody was preliminarily tested as a coating substrate and the assay was attempted using an anti-CTX ABCD-HRP conjugate, however, data (not shown) did not show promise and was not pursued further. It is theorized that the anti-CTX ABCD IgY, with higher protein concentrations, adheres to the surface of the wells more efficiently than the anti-CTX JKLM antibody, which may explain the difference in reactivity.

The neuroblastoma cell assay, a commonly accepted method of assessing the activity of marine toxins on sodium channels in vitro using the agonistic effects of ouabain and veratridine, was performed on both the partially purified CTX positive extract and a non-toxic marlin extract used to develop the assay. The results of the assay show that the CTX positive extract induces cytotoxicity in a dose response manner as expected for extracts containing ciguatoxin when compared to a ouabain (O) and veratridine (V) control sample (Figure 5.2), while the non-toxic marlin extract shows no cytotoxicity when compared to the OV control (Figure 5.3). Comparing CTX positive extract with the OV control, one way analysis of variance (ANOVA) indicates statistical significance at $p< 0.0001$ and Tukey's multiple comparison test indicates significance ($p<0.05$) at extract concentrations at and above 0.125 mg/ml (equivalent to approximately

40 pg of CTX), while the non-toxic marlin extract shows no statistical variation between samples when compared to the control ($p=0.1299$, Tukey's multiple comparison test shows $p>0.05$ for all data points).

As a further validation of the reactivity between the CTX positive extract and the anti-CTX JKLM antibody, an inhibition study was performed wherein anti-CTX ABCD antibody was coated to the plate overnight and 1 mg/ml of CTX positive extract was applied. The anti-CTX JKLM-HRP antibody was pre-incubated for 1 hour in a separate test tube with varying concentrations of the CTX positive extract. A control was also performed in which antibody was incubated alone without any added extract. Antibody and extract mixtures were added to the assay plates and tested for reactivity. Results show the following: 46% reduction when blocking with 0.032 mg/ml (10 pg CTX equivalent); 52% reduction in reactivity at 0.125 mg/ml (40 pg CTX equivalent); 69% reduction at 0.5 mg/ml (160 pg CTX equivalent); and 72% reduction in reactivity was noted when testing extract at 1 mg/ml (320 pg CTX equivalent) concentrations with respect to the control, indicating that the extract is binding and effectively blocking the antibody from reacting with available sites in the assay (Figure 5.4). One way ANOVA indicates that each of the concentrations assayed is significantly different ($p<0.0001$) when compared to the control, and one tailed unpaired t test shows statistical significance between the 0.032 mg/ml and 1.0 mg/ml data points ($p=0.0326$).

5.3.2 Limit of detection and range

Figure 5.5 shows the ELISA result when comparing partially purified CTX positive extract and non-toxic marlin fish extract, and Figure 5.6 shows dose response linear regression log-log transformed ELISA results for the partially purified CTX positive extract ($p=0.004$, $r^2=0.89$, slope=0.353±0.050) and non-toxic marlin fish extract ($p=0.400$, $r^2=0.12$, slope=0.013±0.015), demonstrating the specific affinity of the ELISA for CTX in extract. When statistically evaluated using a two tailed t test in comparison with the non-toxic marlin, the lower limit of detection was determined to be 0.016 mg/ml of CTX positive extract ($p=0.0003$), or approximately 5 pg/ml of equivalent CTX per mg of extract. The upper limit of the working range was found to be 2 mg/ml of CTX positive extract, or approximately 640 pg/ml of equivalent CTX per mg of extract. CTX equivalents, where 1 mg/ml of crude extract equates to approximately 320 pg/ml of actual CTX, are based on previous work shown in Hokama et al. (1998).

5.3.3 Precision

The repeatability (intra-assay variation) and reproducibility (inter-assay variation) of the sandwich ELISA was performed using concentrations of CTX positive extract at 0.016 mg/ml, 0.032 mg/ml, 0.065 mg/ml, 0.125 mg/ml, 0.25 mg/ml and 0.5 mg/ml, plus a control consisting of the 20% methanol : 80% PBS buffer alone. The coefficient of variation (CV) for intra-assay using said concentrations ($n=10$) was found to be in the range of 4% - 22%, while inter-assay ($n=8$) showed a range of 5% - 24%, with most values coming in at approximately 10%. Tables 5.2 and 5.3 summarize the data.

5.3.4 Recovery

Various concentrations of CTX positive extract, from 0.016 mg/ml to 0.5 mg/ml, were spiked to a non-toxic marlin fish plug (weight=approximately 0.2 g) characterized as negative for ciguatoxin, acetone extracted, and subsequently assayed using the ELISA for recovery of sample. Percent recovery ranges from 75% to 106% (Figure 5.7). The data was analyzed using two tailed paired t tests to assess significance between the recovery and control at each concentration. The results indicate no significant differences between the control and the percent recovered ($p>0.05$) at each of the data points assayed.

5.3.5 Cross reactivity

Purified marine toxins brevetoxin-3, okadaic acid, and domoic acid were spiked into 0.25 mg/ml of non-toxic marlin extract and tested for cross reactivity with the ELISA as described in the concentration range of 0.065 ng/ml to 20 ng/ml. While brevetoxin-3 showed a slight reaction, statistically significant cross reactivity was not observed for any of these marine toxins in the specified range (Figure 5.8).

5.4 Discussion

Enzyme immunoassays are used widely in both clinical and research fields because they are rapid, simple, accurate, cost effective, and specific. This assay represents an improvement on current detection methods in that it detects low levels of ciguatoxin in fish samples with no detected cross reactivity with structurally similar polyethers and other marine toxins, including brevetoxin-3 which shares a similar

molecular backbone to CTX, and okadaic acid, which has shown cross reactivity with antibody based assays previously (Hokama et al. 1992, 1998).

The ELISA, which takes approximately 4 hours to complete and can be performed using 96-well assay plates to accommodate a large number of samples, is capable of detecting between 5 pg/ml and 2 mg/ml of CTX according to CTX equivalent data. Comparing the detection limits of other common methods of CTX detection, the neuroblastoma cell assay is capable of detecting as low as 1 pg/ml of purified P-CTX-1 and 3 pg/ml of CTX3C after 7 hours of incubation (Hokama 2004), but the method is not conducive to large scale rapid screening, nor is it necessarily specific to ciguatoxin. The mouse bioassay can detect CTX as low as 5 ng/ml, depending on the congener and extraction techniques used (Lewis and Sellin 1992, Lewis et al. 1991), however, its drawbacks include lack of specificity and requires the maintenance of live animals. The membrane immunoassay (MIA) shows a reasonable limit of detection for CTX at approximately 32 pg/g tissue of CTX (Hokama et al. 1998), while the liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry application (Lewis et al. 1999) has been reported to detect levels of CTX equivalent to 40 ng/kg of PCTX-1 in fish flesh, and 100 ng/kg of CCTX-1 in fish flesh.

A limiting factor in the modified immunoassay (Hokama et al. 1998) is the background noise or non-specific binding that has the potential to produce false positive results. The development of a two site ELISA circumvents this issue while maintaining sensitivity and low cross reactivity. The ability of the described ELISA to detect low toxin levels is likely due to several factors, including the use of a synthetic fragment to

create antibodies which may have reduced the lethality of the toxin such that an appropriate immune response in chickens could be achieved, and the selection of purified IgY as the immobilized antibody in the ELISA which can reduce background interference. This specificity may be a disadvantage, however, in screening for other congeners of the ciguatoxin molecule not present in the CTX positive extract used to develop this assay.

Because reliable, purified CTX was not available in a sufficient quantity to serve as standards, the assay was optimized for the detection of ciguatoxin using a partially purified CTX positive extract that had been previously characterized using a number of bioassays accepted in the marine toxin research community, including the mouse toxicity and guinea pig atrial trial. The use of such extracts in assay development is routine when purified or synthetic toxin is scarce (Lewis et al. 1999, Hokama et al. 1998, Hokama 1990, Bottein Dechraoui et al. 2005a, Bottein Dechraoui et al. 2005b, Shimojo and Iwaoka 2000), and simple recalibration can be performed following purification and characterization of new extracts to maintain assay consistency. Additional steps were taken to standardize the assay, including the extraction of a deep water pelagic fish that is not expected to not harbor ciguatoxin, *M. nigricans*, to rule out that the lipid matrix or extraction solvents might affect the assay result. The lack of availability of reliable purified ciguatoxin standards to further validate the assay may affect the interpretation of the results with respect to the exact sensitivity of the assay and what true levels of ciguatoxin present in the partially purified fish extract are being detected as opposed to estimates of equivalence, and leaves the door open for further studies to be done toward

improvement of the method. However, because the ELISA was developed using CTX positive fish extract rather than purified compounds, the transition into screening wild caught fish samples is more streamlined, as the assay has already been optimized for use with the complex matrix of lipids and other background that accompanies testing fish tissues.

In conclusion, the development of this ELISA demonstrates the usefulness of chicken IgY as an immunoanalytic tool and provides a sensitive, specific methodology to test for ciguatoxin in fish extracts, which has broad implications for consumers and commercial fishing operations alike.

Table 5.1. Comparison of pre-immune IgY and anti-CTX ABCD IgY as the capture antibody in the ELISA. Two-tailed P value results from unpaired t-test show statistical significance where $p < 0.05$. Data represents the average optical density of triplicate wells measured at 490 nm.

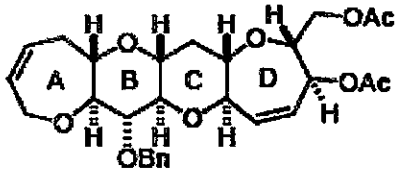
CTX positive extract in mg/ml	Pre-immune IgY 0.25 mg/ml	Anti-CTX ABCD IgY 0.25 mg/ml	P value
0 control	0.142	0.138	–
0.004	0.145	0.141	**0.2921**
0.008	0.158	0.171	**0.1202**
0.016	0.229	0.259	**0.0514**
0.032	0.363	0.435	**0.0018**
0.065	0.453	0.515	**0.0483**
0.125	0.466	0.594	**0.0014**
0.25	0.489	0.603	**0.0025**
0.50	0.542	0.638	**0.0014**

Table 5.2. Intra assay coefficient of variation. Data represents the average optical density of triplicate wells measured at 490 nm and standard deviation.

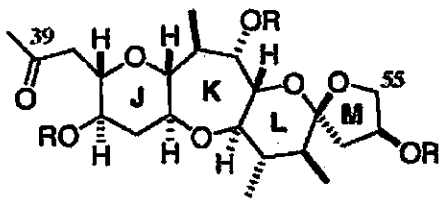
CTX positive extract in mg/ml	CTX equivalents in pg/ml	Number of Determinations	Mean Absorbance	CV (%)
0 control	0	10	0.039 ± 0.009	**22.3**
0.008	2.5	10	0.071 ± 0.011	**15.5**
0.016	5	10	0.082 ± 0.008	**9.55**
0.032	10	10	0.145 ± 0.013	**9.24**
0.065	20	10	0.256 ± 0.016	**6.31**
0.125	40	10	0.305 ± 0.013	**4.35**
0.25	80	10	0.316 ± 0.020	**6.45**
0.50	160	10	0.383 ± 0.022	**5.80**

Table 5.3. Inter assay coefficient of variation. Data represents the average optical density of triplicate wells measured at 490 nm and standard deviation.

CTX positive extract in mg/ml	CTX equivalents in pg/ml	Number of Determinations	Mean Absorbance	CV (%)
0 control	0	8	0.058 ± 0.013	**22.9**
0.008	2.5	8	0.094 ± 0.020	**21.4**
0.016	5	8	0.171 ± 0.042	**24.6**
0.032	10	8	0.286 ± 0.043	**15.3**
0.065	20	8	0.321 ± 0.026	**8.18**
0.125	40	8	0.365 ± 0.031	**8.72**
0.25	80	8	0.375 ± 0.033	**8.85**
0.50	160	8	0.338 ± 0.018	**5.42**



(a)



(b)

Figure 5.1. Ciguatoxin synthetic fragments, (a) the ABCD fragment and (b) the JKLM fragment, targeted by the antibodies in assay development.

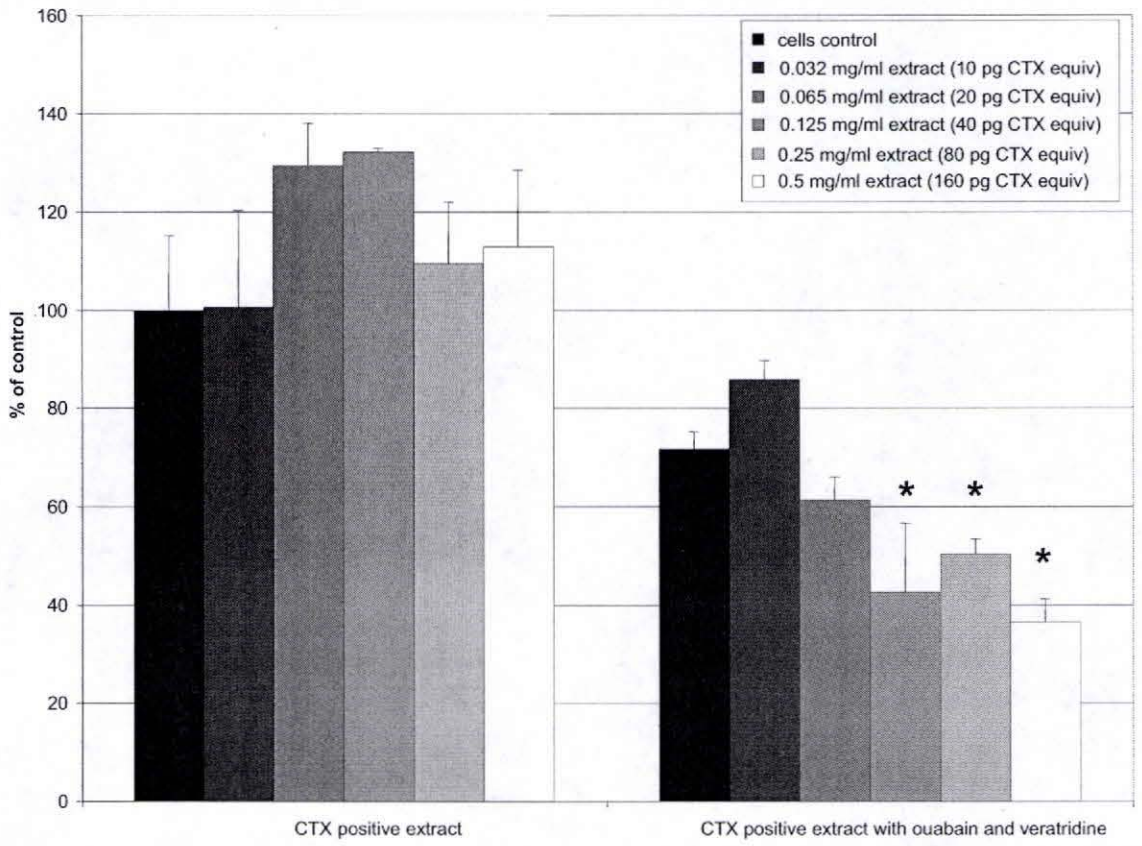


Figure 5.2. Neuroblastoma cell assay results for DOH CTX positive extract, where 1 mg/ml of extract contains an equivalent of approximately 320 picograms of CTX. Results are expressed as percent of the baseline CTX extract cell control where each point represents the mean of triplicate determination with vertical bars indicating + standard deviation. Statistical significance is represented as * ($P<0.05$).

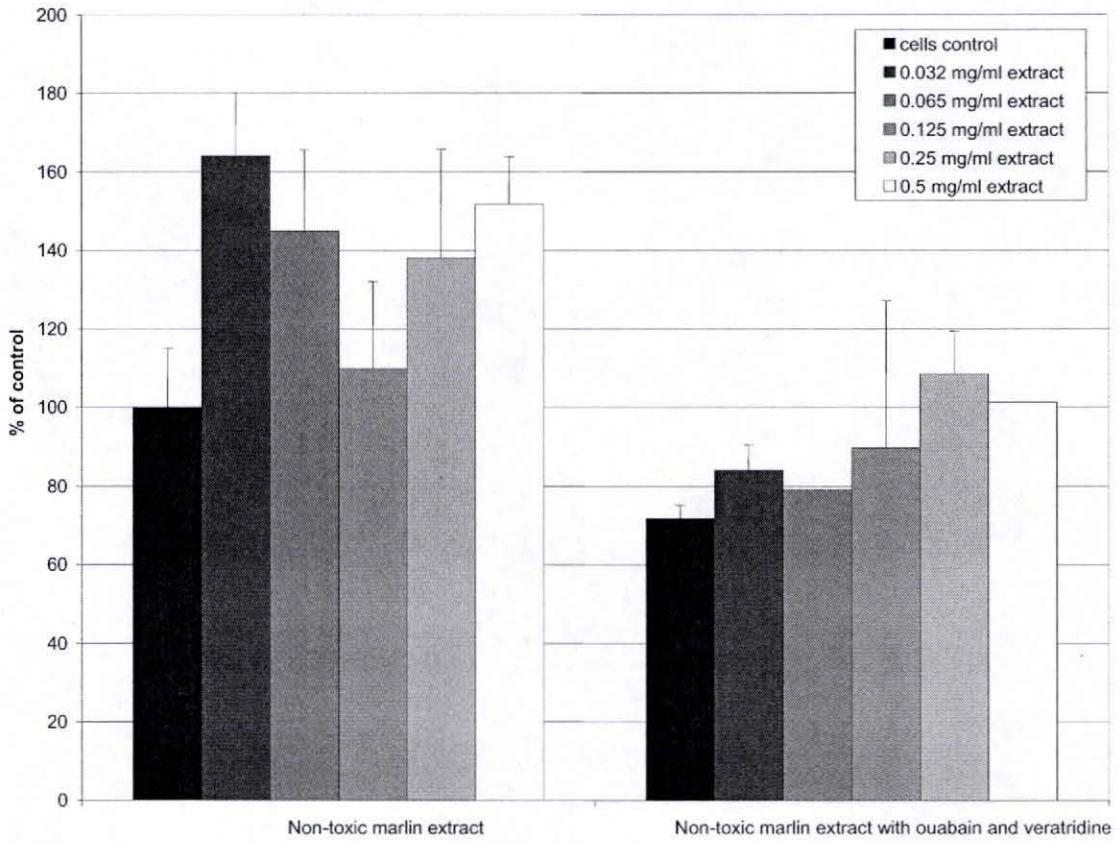


Figure 5.3. Neuroblastoma cell assay results for non-toxic marlin extract. Results are expressed as percent of the baseline marlin extract cell control where each point represents the mean of triplicate determination with vertical bars indicating + standard deviation. Statistical significance was not observed.

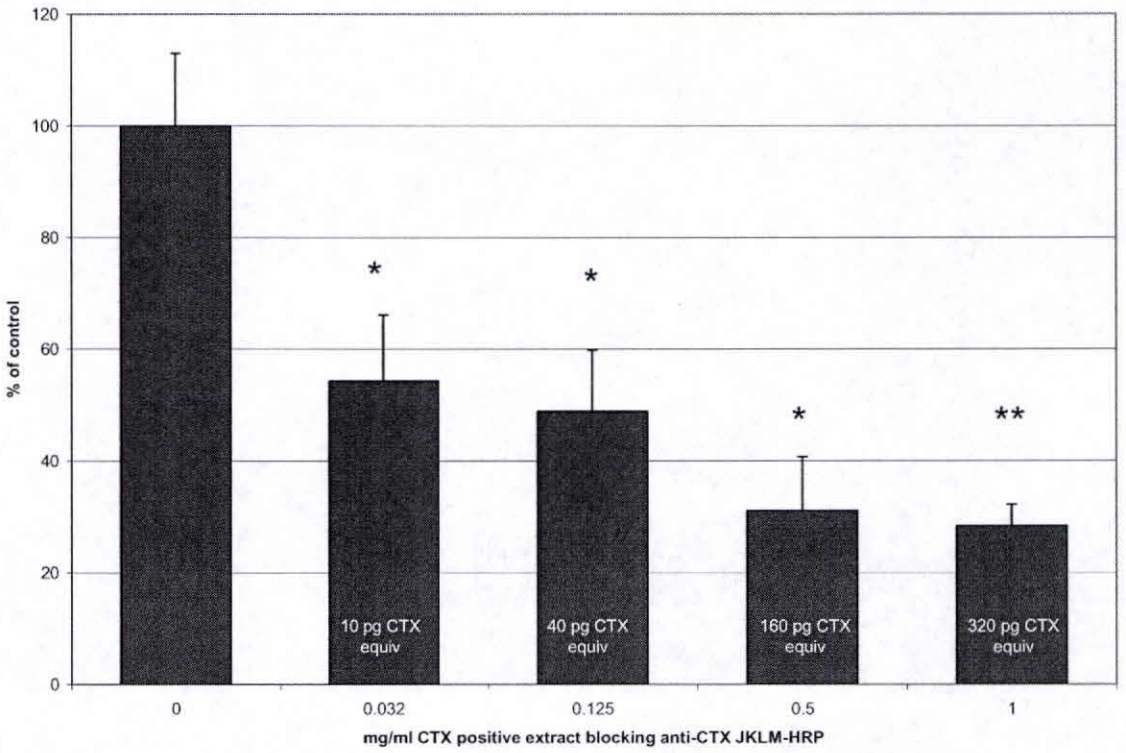


Figure 5.4. Competitive inhibition showing that CTX positive extract blocks the reactivity of anti-CTX JKLM-HRP when incubated together for one hour prior to the assay. Each data point represents the mean of triplicate determination with vertical bars indicating + standard deviation. Statistical significance indicating inhibition when compared to the zero control is represented as * ($P<0.05$), and statistical significance when comparing the 0.032 mg/ml and 1.0 mg/ml results is represented as ** ($P<0.05$).

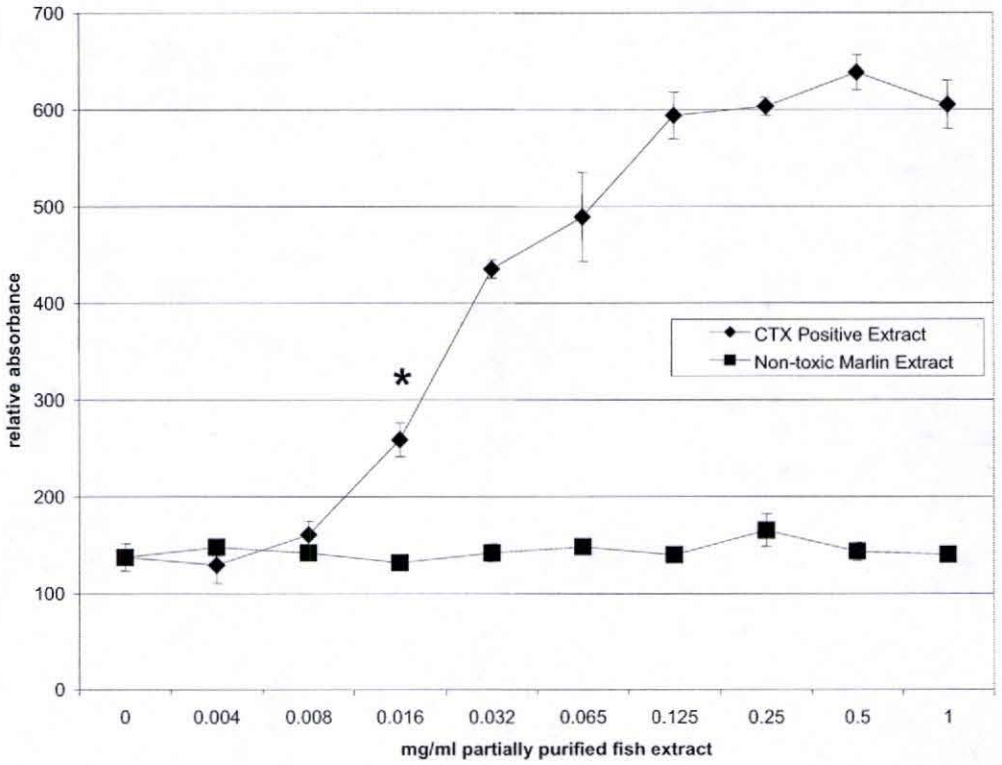


Figure 5.5. ELISA results evaluating the effects of matrix and extraction background for partially purified CTX positive extract compared with marlin extract that is negative for CTX, including a data point for the 20% methanol: 80% PBS buffer as a zero control. Each data point represents the mean of triplicate determination at 490 nm with vertical bars indicating +/- standard deviation. Statistical significance indicating the lower detection limit of CTX positive extract when compared to the non-toxic marlin extract is represented as * (P=0.0003).

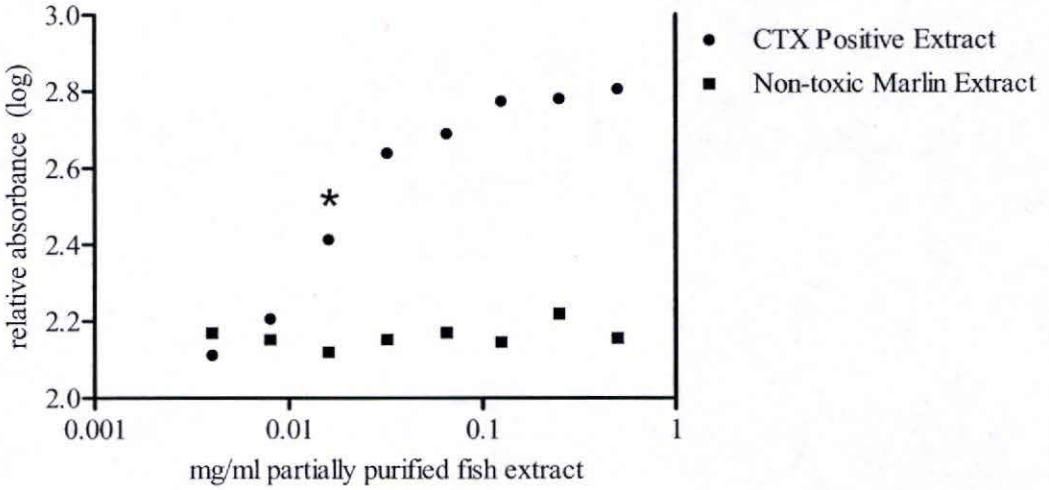


Figure 5.6. Log-log transformed ELISA results for partially purified CTX positive extract and non-toxic marlin extract. Each data point represents the mean of triplicate determination.

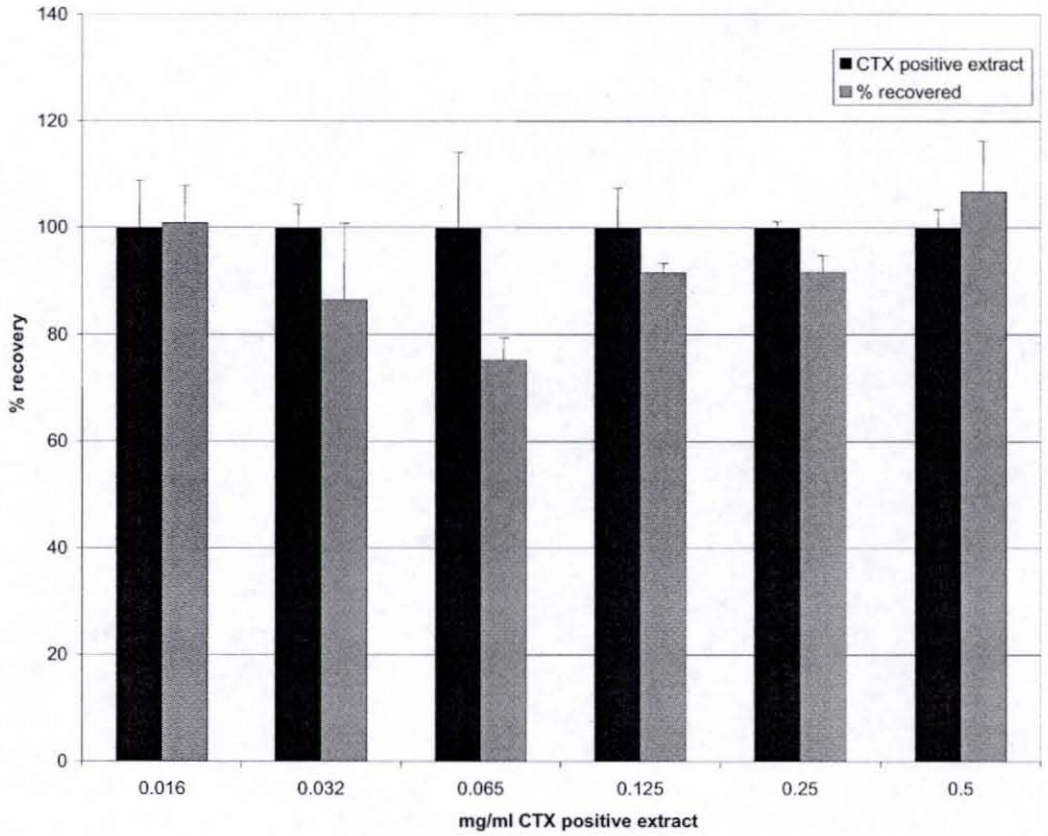


Figure 5.7. Percent recovery of DOH CTX positive extract after spiking a non-toxic marlin fish sample. Data represents the mean of triplicate evaluations and vertical bars indicate + standard deviation. The results indicate no significant differences between the control and the percent recovered ($P>0.05$) at each of the data points assayed.

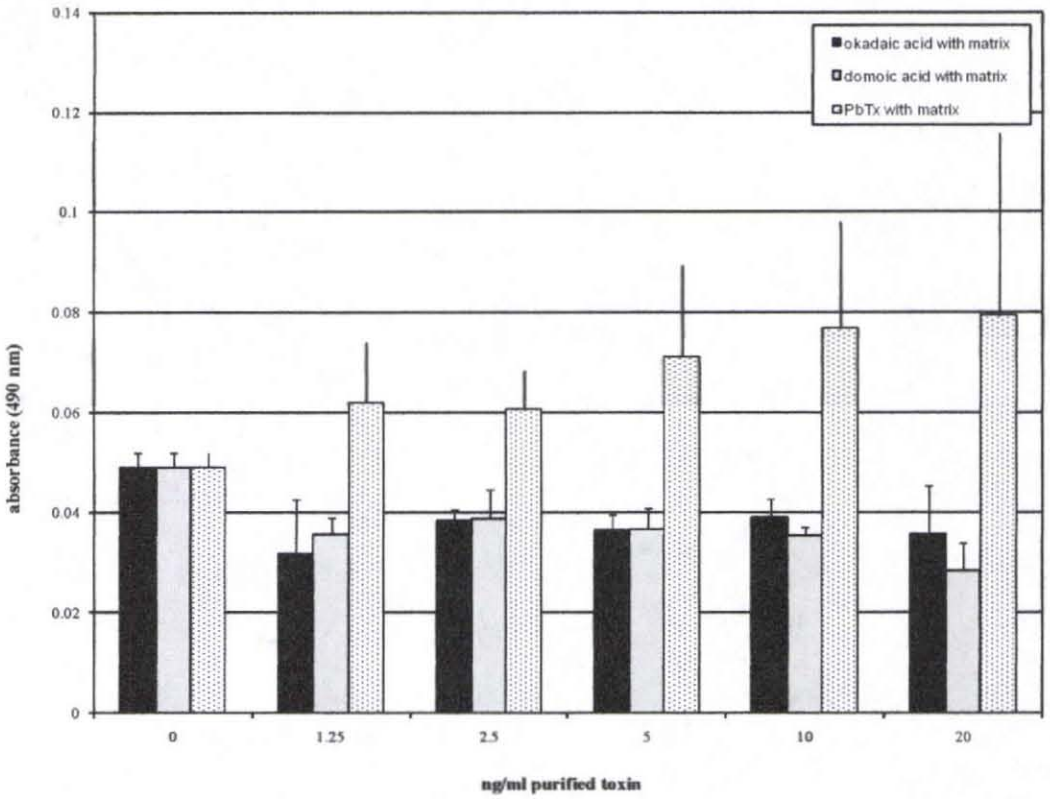


Figure 5.8. Cross reactivity of brevetoxin-3, domoic acid, and okadaic acid using the ELISA.

Chapter 6

Evaluating the risk of ciguatera fish poisoning from reef fish grown at marine aquaculture facilities in Hawai'i

6.1 Introduction

Ciguatera fish poisoning is an intoxication resulting in characteristic gastrointestinal and neurological symptoms following the ingestion of fish containing ciguatoxin (CTX) or one of its congeners. In Hawai'i, a total of 126 incidents of ciguatera fish poisoning were reported to the Hawai'i State Department of Health during a four-year period (Sasaki 2001). It is a major concern for both commercial and recreational fishermen in tropical and subtropical waters, and of particular interest to those involved in raising various species of fish at environmentally susceptible open ocean aquaculture facilities.

Ciguatoxin is produced by the marine dinoflagellate, *Gambierdiscus toxicus,* (Yasumoto et al. 1980) which is found on marine macroalgae associated with coral reefs. It is capable of bio-accumulating through the food chain through herbivorous and predatory reef fish (Lehane and Lewis 2000, Mines et al. 1997, Swift and Swift 1993).

Several biological, immunological, and chemical methods are available for detecting ciguatoxin in fish tissue, including a traditional mouse bioassay, radio ligand binding, high performance liquid chromatography, and mass spectrometry, however, none are well suited for routine large scale screening of fish samples. A membrane immunoassay (MIA) developed by Hokama et al. (1998) is a field suitable qualitative assay used to detect ciguatoxin, although it has been shown to cross react with other

marine toxins (Hokama et al. 1992), and has the potential to yield a high false positive rate depending on the fat content of the fish (Tamaru et al. 2005). A novel sandwich enzyme linked immunoassay (ELISA) recently developed (Campora et al. in press) to detect CTX in partially purified fish extract is semi-quantifiable and shows promise in eliminating some of the problems observed when using other methods. In addition, a directed cytotoxicity assay that records sensitive changes in the sodium channel of neuroblastoma cells in the presence of sodium channel activators or inhibitors has been established for the detection of ciguatoxins in crude seafood extracts (Manger et al. 1995). Using a microplate high-throughput format, the neuroblastoma assay serves as a valuable tool for marine toxin studies, capable of reliably detecting ciguatoxins at sub-picogram levels.

The incidence of ciguatera fish poisoning in Hawai'i is relatively low, with approximately 3.6 cases per 100,000 people. The leeward coasts of the main Hawaiian Islands often report the majority of cases, with west Oahu, the north shore of Kauai, west Maui, and the Kona and north Kohala coasts on the Island of Hawai'i leading the statistics (Anderson et al. 1983). Currently, west Oahu and the Kona coast house open-ocean aquaculture facilities (Helsley 2000). The almaco jack (*Seriola rivoliana)*, also known as kahala, is cultivated at the Kona Blue Water Farms (KBWF) open ocean aquaculture facilities on the Kona coast. In the 1970's, the amberjack (*Seriola rivoliana and Seriola dumerili*) was the fish most frequently associated with ciguatera fish poisoning in Hawai'i, responsible for 21 percent of ciguatera incidents reported to the State Department of Health between 1975-1981 (Anderson et al. 1983). Wild-caught

amberjacks were subsequently excluded from commercial markets by retailers and restaurants due to the risk of ciguatera, however, there is increasingly renewed interest in the farmed product.

Polydactylus sexfilis, commonly known as Pacific threadfin or moi, were a rare delicacy in ancient Hawai'i reserved for only the nobility, but are widely popular on restaurant menus today. Hukilau Foods (formerly known as Cates International Inc.) cultured moi from 1999-2005 in large offshore cages in Hawai'i 's first open ocean farm located three miles off of Barbers Point on the leeward coast of the island of Oahu. At one time, there were four offshore cages deployed producing over 300,000 pounds of cultured product annually, with an estimated capability of producing up to 900,000 pounds (Helsley 2003). While only one case of ciguatera poisoning from wild caught moi was reported during the period 1996-2002 according to the Hawai'i Department of Health, the potential for toxin accumulation in a partially enclosed environment prompted evaluation of cultured moi.

The objective of this study is two-fold: first, to examine open ocean aquaculture facilities for the presence of *G. toxicus,* and second, to test aquacultured fish for ciguatoxins and compare the results using a novel immunoassay and a well established sensitive bioassay for marine toxin detection. The results of the study will enable a more thorough evaluation of the risk of ciguatera fish poisoning from consumption of reef fish grown at open ocean facilities in Hawai'i. Demonstration that farmed fish do not harbor ciguatoxin when grown in the presence of *G. toxicus* would boost consumer confidence

that the products are safe to be eaten, particularly in light of rising concerns about the safety of consuming farmed fishes (Hites et al. 2004).

6.2 Materials and Methods

6.2.1 Assessment of G. toxicus in aquaculture facilities

Algae specimens were collected by staff members of Hukilau Foods and Kona Blue Water Farms using SCUBA on the surface of the submerged cages at a depth of approximately 25 meters. Algae samples and one liter of seawater from four separate locations within each cage (north, south, east and west faces) were placed into separate five liter ziplock bags and brought to the surface. Samples were kept in the dark on board the tending vessel and transported to the University of Hawai'i at ambient temperatures. Algal samples were obtained from Hukilau Foods moi cages #1, #2 and #3 in August 2004, and cage #3 was sampled on three additional occasions (December 2003, May 2004, and October 2004). Algal samples from Kona Blue Water Farms open ocean cage #6 were obtained in June 2007.

In the laboratory, the algae and accompanying seawater were placed in a five liter beaker and shaken vigorously for two minutes to loosen epiphytic dinoflagellates from the algae. The macroalgae was then removed from the beaker and total wet weight was recorded. The remaining salt water-algal suspension was passed through a 125 μm sieve to remove larger algal fragments followed with the passage through a 25 μm sieve. The residue collected on the 25 μm mesh was back-washed with filtered seawater and transferred into a 100-ml sterile glass bottle and capped loosely to provide aeration. The contents in the sample bottle were shaken vigorously and 3 mls were transferred onto a

Sedgewick Rafter cell counting slide for *G. toxicus* quantification. Cell counts were performed in triplicate using a compound light microscope to determine the number of dinoflagellate cells/ml of seawater. The average number of cells/ml per sample location was extrapolated to obtain the total number of dinoflagellate cells collected. This value was divided by the total wet weight of the macroalgae and is reported as the number of *G. toxicus* cells/gram algae. All four samples from each cage were used to determine the average number of cells/gram algae that was found on each cage.

Small samples of the macroalgae were kept refrigerated for later identification based on the field guides by Abbott (1999) and Magruder and Hunt (1979).

6.2.2 Preparation of extract from fish samples

Fresh fish specimens were obtained through the normal marketing distribution channels used by each commercial farm and stored at –80° C pending analysis. A total of 60 individual cultured kahala and 10 individual cultured moi were obtained at various times over the course of this investigation. Sampling dates for the cultured kahala were in November 2004 and May through August 2007. Sampling dates for cultured moi were in November 2004. Originally, 40 moi samples were collected for testing, however, 30 of the samples were lost after a laboratory flood, thus the lower representation. A total of 81 wild-caught kahala fished from the various locations in the Northwestern Hawaiian Islands were obtained from May 2007 through August 2007 for ciguatoxin assessment both as a validation for the assay parameters, and for comparison with the aquacultured fish samples. In addition, muscle tissue from 15 individual tilapia farmed in freshwater at

the Windward Community College Aquaculture Complex located on the island of Oahu was sampled as negative controls.

A 2.5-cm by 2.5-cm cube of muscle tissue without the skin was excised from the left side or head region of each fish. The cube of muscle tissue was placed in a clean test tube and lipids were extracted by soaking in 3 mls of acetone overnight. The acetone extract was transferred to a clean, tared test tube, blown down using a laboratory air source, and weighed. The extract was re-suspended in 100% methanol at a concentration of 10 mg/ml and stored at 4° C for further analysis. To rule out that the lipid matrix or extraction solvents might affect the ELISA assay result, the same extraction procedures were followed for a deep water pelagic fish purchased from a commercial market that is not expected to harbor ciguatoxin, Pacific Blue Marlin (*Makaira nigricans)*, referred herein as non-toxic fish extract.

6.2.3 Enzyme linked immunoassay for ciguatoxin detection

A novel sandwich enzyme linked immunoassay (ELISA) was recently developed to detect picogram levels of ciguatoxin in partially purified fish extract (Campora et al. in press, and as described in Chapter 5 of this dissertation) and was evaluated as a preliminary tool to assess the lipid extracts from each fish for CTX. Briefly, 96-well ELISA plates (Costar, Corning, NY) were coated with anti-CTX capture antibody specific to the ABCD region of the CTX molecule in phosphate buffered saline (PBS) and incubated overnight at 4 degrees C. Non-specific binding sites were blocked with a milk-free blocking solution, Superblock (Pierce Chemical Company, IL) followed by three washes with PBS. The lipid extract from each fish sample was diluted in 20%

methanol : 80% PBS buffer for use in the assay at a concentration of 1 mg/ml. One hundred microliters of each sample was added to wells in triplicate, and plates were incubated for 1 hour at room temperature followed by three washes with PBS to remove excess antigen. The detecting anti-CTX antibody specific to the JKLM region of the CTX molecule conjugated to horseradish peroxidase was added to each well and incubated for 1 hour at room temperature followed by six thorough washes with PBS to remove residual antibody. Bound antibodies were visualized by the addition of 100 µl/well *o*-phenylenediamine (OPD; Sigma Chemical Company, St. Louis, MO). Reaction was allowed to develop for 5 minutes before being stopped by the addition of 50 µl/well 1N sulfuric acid. Absorbance was measured at 490 nm using a microplate reader (BioTek Instruments, Winooski, VT).

6.2.4 Neuroblastoma cell assay for ciguatoxin detection

The neuroblastoma (NB) cell assay for the detection of ciguatoxin was modified after the method of Manger et al. (1993, 1995), and was carried out as described in section 5.2.4 of this dissertation.

6.2.5 Statistical analysis

All statistical analyses were carried out using Microsoft Excel or Prism 5.0 (GraphPad Software, USA). Differences were regarded as significant when $p<0.05$ unless otherwise indicated.

To determine the borderline and positive threshold scores for ELISA, the mean optical density (OD) and standard deviation of triplicate wells run with fish extract using the ELISA were obtained and compared to the 6-8 replicate wells run on each plate using

the previously described non-toxic fish extract. Results were extrapolated to reflect results obtained from multiple intra and inter assay runs using the non-toxic fish extract in which the average OD was found to be 0.100 (n=42). A 'positive' cut-off score was established for CTX by multiplying the mean OD of the non-toxic fish extract by 2, and samples were considered positive using the ELISA if the mean value scored above the positive threshold or 0.200 OD. Borderline or '+/-' values for CTX presence included values that fell within 0.020 OD of the established positive threshold, or between 0.180 and 0.200 OD. Samples were considered 'negative' using the ELISA if the mean value fell below the borderline threshold of 0.180 OD.

Using the neuroblastoma cell assay, the cytotoxicity of each fish sample was measured as the percentage of surviving cells as indicated by optical density readings where decreased OD values indicate increased cytotoxicity. Data were obtained for analysis by comparing the average OD of wells containing cells, fish extract, and ouabain and veratridine with the averaged OD of control wells containing cells and ouabain and veratridine alone. Data were statistically assessed by one way analysis of variance (ANOVA) followed by Tukey's post-test for multiple comparisons. P values below 0.05 were considered to be significant for CTX activity, and were scored as 'positives' using the neuroblastoma assay. Post-test critical values for significance that were above 3.50 but that fell below the established criteria for significance at $P<0.05$ were scored as 'approaching significance', as the triplicate averages of these samples showed cell death when compared to the OV control, indicating some measure of toxicity within the

sample. Values that scored 'not significant' using these criteria were scored as 'negative'.

6.3 Results

6.3.1 Assessment of G. toxicus in aquaculture facilities

The predominant macroalgae that was obtained from the open ocean cages at Kona Blue Water Farms was *Ulva fasciata, and Sphacelaria furcigera*, and macroalgae harvested from the Hukilau Foods open ocean cages was identified as *Tolypiocladia glomerulata.* Processing the macroalgae samples revealed low levels of *G. toxicus* on all of the Hukilau Foods moi cages #1-3 sampled in August 2004 (Figure 6.1) and KBWF cage #6 sampled in June 2007. There were no statistical differences ($p = 0.125$, ANOVA) that could be detected between the average number of *G. toxicus* cells/g algae on each of the Hukilau Foods cages (average range: 85 - 201 cells/g algae). The KBWF kahala cage #6 had an extremely low average of 2.51 cells/g algae.

Hukilau Foods moi cage #3 was sampled quarterly over the course of one year and the temporal *G. toxicus* changes are summarized in Figure 6.2. The densities of the dinoflagellate fluctuated between sampling dates and locations within the same cage (range: 2 - 214 cells/g algae), however, there were no statistical differences ($p = 0.504$, ANOVA) detected in the number of *G. toxicus* cells/g algae found over the course of the sampling period.

6.3.2 Ciguatoxin detection in fish samples

A total of 60 cultured kahala obtained from KBWF (average fork length = 46.1 ± 5.3 cm, average body weight = 2.2 ± 0.9 kg), 10 cultured moi from Hukilau Foods

(average fork length = 25.2 ± 4.0 cm, average body weight = 0.34 ± 0.07 kg), 15 freshwater farmed tilapia, and 81 wild-caught kahala (fork length = 73.15 cm ± 14.42, 4.4 ± 0.8 kg) were evaluated for the presence of ciguatoxin using both the ELISA and neuroblastoma cell assays. Wild-caught kahala, fished from various banks in the Northwestern Hawaiian Islands, were caught at an average depth of 22.82 ± 4.48 meters.

The described ELISA is a new tool for CTX screening, and shows promise for its capabilities of efficiently and economically screening a large number of fish samples using a unique two-site antibody configuration. However, the assay was developed to test partially purified fish extract, and recent studies employing the assay to test crude acetone fish extracts have indicated a percentage of 5.2% false positives which may be attributed in part to assay parameters, or to cross-reactivity of extraneous lipids in the crude extract. As such, the results obtained from the ELISA assay are considered preliminary. Of the aquacultured kahala, moi, and tilapia, 2 of the 95 crude fish extracts gave an OD reading that was at or above the established positive threshold, zero scored in the borderline range, and 93 were scored as negative using the ELISA, with both of the positive results being kahala. Of the 81 wild-caught kahala, 5 scored at or above the established positive threshold, zero scored in the borderline range, and 76 were scored negative using the ELISA (Table 6.1).

Because of the known false positive component to the newly developed ELISA, a widely known neuroblastoma assay commonly used to screen fish extracts for marine toxins was employed as a secondary assay to confirm or refute the results obtained using the ELISA. Using the accepted NB assay, 0 of the 95 cultured crude fish extracts showed

any signs of significant cell death or cell death approaching significance, and all scored negative using the secondary bioassay. Of the 81 wild-caught kahala, 4 screened positive using both the ELISA and secondary neuroblastoma cell assays (4.9%), and an additional 2 samples that scored negative using the ELISA showed cell death approaching significance when using the neuroblastoma bioassay, implying a potential low 1.4% false negative rate when using the ELISA (Table 6.2). Data from the neuroblastoma bioassay alone indicate that 0% of the aquacultured fish and approximately 7.4% of wild-caught kahala tested positive for ciguatoxin. Differences in the ELISA and NB assay results imply a percentage of 2.1% false positives when assessing the aquacultured fish using the ELISA alone, and show a percentage of 1.2% false positives when screening the wild-caught kahala when compared to the NB results.

In Figure 6.3 the distribution of ELISA and NB results for each fish sampled compared to the non-toxic CTX negative control and a partially purified fish extract previously shown to be positive for CTX at 0.016 mg/ml, where 1 mg/ml of extract corresponds to approximately 0.32 ng/ml of CTX equivalents (Hokama et al. 1998) is shown. Two-sided Chi square analysis using Fisher's exact test comparing the toxicity results obtained from the cultured kahala and wild-caught kahala show a statistical significance at $p=0.0382$.

4. Discussion

Dinoflagellates that are associated with ciguatera fish poisoning typically occur with certain macroalgae (Tindall and Morton 1998, Lehane and Lewis 2000). The most widely studied specific habitat supporting dinoflagellates associated with ciguatera are

sessile macroalgae belonging to the phyla Rhodophyta, Phaeophyta, Chlorophyta and Cyanophyta (Tindall and Morton 1998). The spectra netting used in the open ocean cages employed by both KBWF and Hukilau Foods was found to provide adequate substrate to support the growth of an epiphytic community, including macroalgae that was found to house variable amounts of *G. toxicus*.

The presence of *G. toxicus*, especially at low levels, at any farm site does not necessarily mean that the fish produced have the potential to become ciguatoxic. Considerable variation in the cell densities were observed on Hukilau Foods cage #3 and between the different cages sampled. The high degree of temporal variation in *G. toxicus* cell density over the course of the study period is likely due to differences in the maintenance procedures being employed as well as the relatively turbulent offshore environment that the cages are located in. Housing cages at a distance several miles offshore and proper cage cleaning and maintenance appear to aid in keeping dinoflagellate counts and the risk of ciguatoxin accumulation low. Understanding the distribution of ciguatoxins in a natural environment requires the understanding of the ecology of *G. toxicus*, its algal hosts, and the complexities associated with herbivore-algae interactions. A recent study by Cruz-Rivera and Villareal (2006) points out that quantifying dinoflagellate counts as a risk assessment in ciguatoxin prone areas is not necessarily valid, as it is operating on the assumption that the algae sampled is a readily consumable resource for herbivorous grazers when in fact some species of macroalgae that harbor *G. toxicus* may be unpalatable, have poor nutritional quality, or utilize chemical or structural defenses against such grazers.

As shown in Figures 6.1 and 6.2, the cell densities from all of the cages sampled are considered to be extremely low, averaging at or well below 250 cells/g algae with the exception of cage #2 at a single time point. For comparison, areas at high risk for ciguatera fish poisoning such as the Gambier Islands in French Polynesia have reported *G. toxicus* cell densities as high as 5×10^5 cell/ g algae, with some 23,000 ciguatera cases per 100,000 individuals (Yasumoto et al. 1980, Lehane and Lewis 2000).

Using growth data from laboratory trials (Laidley and Shields 2004) and average body weight of harvested kahala, which are placed in the open ocean cages as fingerlings, cultured individuals are estimated to be between seven and eight months of age at harvest and consumption. The smaller size and/or young age of the cultured kahala may be contributing factors in the absence of ciguatoxin, as there is limited time for potential toxin bioaccumulation in the muscle to occur. Although commercial fish meal diets used to feed cultured fish product could in theory contribute to marine toxin poisonings, the commercial diets used by KBWF and Hukilau Foods employ fish meal that belongs to the clupeid family (e.g., anchovies, herrings) which feed on zooplankton rather than macroalgae, reducing the risk of ciguatoxins entering the culture process through the feed to nearly zero.

Reliable, specific assays available for the routine screening of crude fish extracts for low levels of ciguatoxin are not widely available for a number of reasons. The scarcity of purified ciguatoxin has hampered the definitive use of immunological applications, and other methods are often non-specific, require the maintenance and use of live animals, or utilize equipment and technical expertise that is not available to a

broad audience. This study is one of the first to use the novel ELISA in an attempt to detect ciguatoxin in crude fish extracts as opposed to more purified product. While the assay is specific to ciguatoxin by employing two distinct antibodies to different regions of the toxin in a sandwich method, the establishment of a somewhat arbitrary threshold (indicated as two times the mean OD value recorded for a non-toxic fish extract run at the same time as the samples and compared to intra and inter-assay repetitions previously collected) for positivity may inadvertently score fish as positive or borderline that are truly negative, leading to an overall estimated 5.2% false positive rate. Conversely, significantly altering the threshold may lead to an increase in false negatives, the implications of which are much greater. Due to the preliminary and novel nature of the ELISA and to circumvent the risk of false positives, the neuroblastoma cell bioassay was performed as a secondary screen, and while it lacks specificity in that the score results from a functional mechanism rather than structural recognition, it is a more acceptable result at this stage due to its applied use in the literature (LePage et al. 2005, Garrec et al. 2005, Bottein Dechraoui et al. 2005b, Dechraoui et al. 1999, Manger et al. 1993, 1995).

The neuroblastoma cell bioassay is accurate in detecting sodium channel activity, although it is not necessarily suitable for mass routine screening of fish extracts due to the intensive nature of the assay and specialized equipment and reagents required. When administered in picogram quantities, ciguatoxin, a potent sodium channel activator, in combination with the chemical agonists ouabain and veratridine, will lead to quantifiable neuroblastoma cell death. Although 2 of the aquacultured kahala samples were given a positive score using the ELISA, all 95 of the cultured fish samples run using the NB

assay were shown to be negative for cytotoxicity, implying false positive results from the ELISA.

When using the ELISA in combination with results from the neuroblastoma assay, 7.4% of the wild-caught kahala were shown to score positive for CTX, which represents a statistic that is lower than anticipated for CTX incidence in wild-caught kahala, which was initially reported to be closer to 20% when using a different immunological detection method (Hokama et al. 1990). While both the ELISA and NB assays have similar reported sensitivities, this study suggests that the ELISA may require more refinement with respect to definition of threshold values, and may necessitate further extract purification to reduce the incidence of false positives. However, it appears to show promise as a simple, rapid tool for initial routine screening of crude fish extracts for ciguatoxin.

Despite the presence of *G. toxicus* on the cages and a slight false positive rate when testing fish for CTX using a new immunological technology, the low dinoflagellate cell counts on several open ocean cages combined with dual assay results indicate that none of the aquacultured fish tested were found to contain even sub-clinical picogram levels of ciguatoxin. The results obtained demonstrate that the risk of cultured fish becoming ciguatoxic is extremely remote. KBWF produced more than 700,000 pounds of cultured kahala during 2004-2006 for consumption on the open market (Neil Sims, KBWF President, personal communication), and Hukilau Foods cultured an average of nearly 200,000 pounds of moi annually from 2002-2005 (Helsley 2005) without a single reported complaint of marine toxin illness. The success rate of the farms combined with

the data presented in this paper should provide a boost to consumer confidence with regards to the safety of consuming these relatively new-farmed products.

Table 6.1. ELISA positive and borderline results for kahala. ^ indicates the average OD and standard deviation of the fish that scored negative for comparison purposes (sample ID numbers not included).

Aquacultured Kahala *Sample ID*	Established CTX Threshold for Positive and Borderline (average OD ± SD)	ELISA Result (average OD ± SD)	ELISA Score
Kahala #6	0.200 ± 0.020	0.284 ± 0.045	Positive
Kahala #19	0.200 ± 0.020	0.221 ± 0.025	Positive
Kahala (n=58)	0.200 ± 0.020	0.102 ± 0.032^	Negatives
Wild Kahala			
NWHI #3	0.200 ± 0.020	0.205 ± 0.004	Positive
NWHI #5	0.200 ± 0.020	0.208 ± 0.018	Positive
NWHI #6	0.200 ± 0.020	0.229 ± 0.013	Positive
NWHI #7	0.200 ± 0.020	0.271 ± 0.017	Positive
NWHI #11	0.200 ± 0.020	0.200 ± 0.005	Positive
NWHI (n=76)	0.200 ± 0.020	0.086 ± 0.048^	Negatives

Table 6.2. Results of the ELISA and neuroblastoma assays for detecting ciguatoxin in crude fish extract. Neuroblastoma scoring derived from the following statistical criteria (ANOVA and Tukey's posttest): NS: no significant change in cell death when compared to the control (P>0.05); NS^: cell death occurred with respect to the control approaching levels of significance; (*) and (**): significant increase in cell death when compared to the control (0.01<P<0.05, 0.001<P<0.01, respectively).

Aquacultured Kahala, Moi and Tilapia n=95	ELISA Result (1 mg/ml)	NB Result (0.1 mg/ml)	Final Result
Kahala #6	Positive	NS	Negative
Kahala #19	Positive	NS	Negative
Kahala #1-5, 7-18, 20-60 (n=58)	Negative	NS	Negative
Moi #1-10 (n=10)	Negative	NS	Negative
Tilapia #1-15 (n=15)	Negative	NS	Negative
Wild Kahala n=81			
NWHI #K3	Positive	**	Positive
NWHI #K5	Positive	**	Positive
NWHI #K6	Positive	*	Positive
NWHI #K7	Positive	*	Positive
NWHI #K11	Positive	NS	Negative
NWHI #L22	Negative	NS^	Positive
NWHI #KP14	Negative	NS^	Positive
NWHI#K1-2,4,8-10,12-31,#L1-21,21-37, #KP1-13,15,16 (n=74)	Negative	NS	Negative

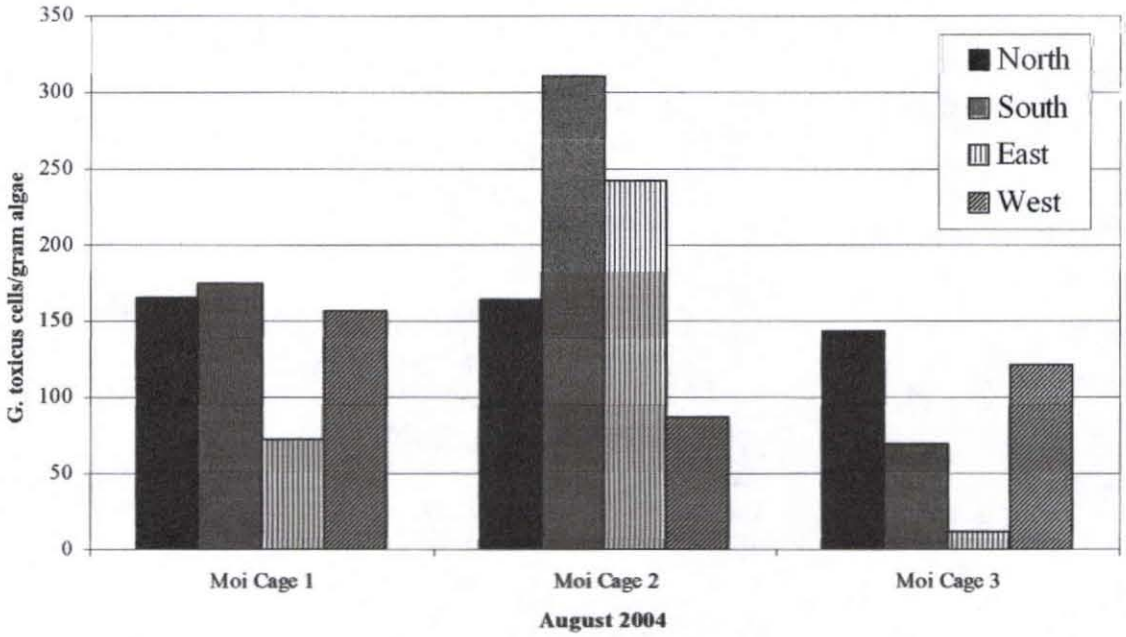


Figure 6.1. Cell counts of *G. toxicus* per gram of algae from open ocean moi cages sampled in August 2004. Average ± SD cells/g algae are: Cage 1 = 142 ± 47; Cage 2 = 201 ± 96; Cage 3 = 86 ± 58. Not depicted is the KBWF kahala cage #6 that was sampled in June 2007, yielding a negligible 2.5 ± 2 average cell count.

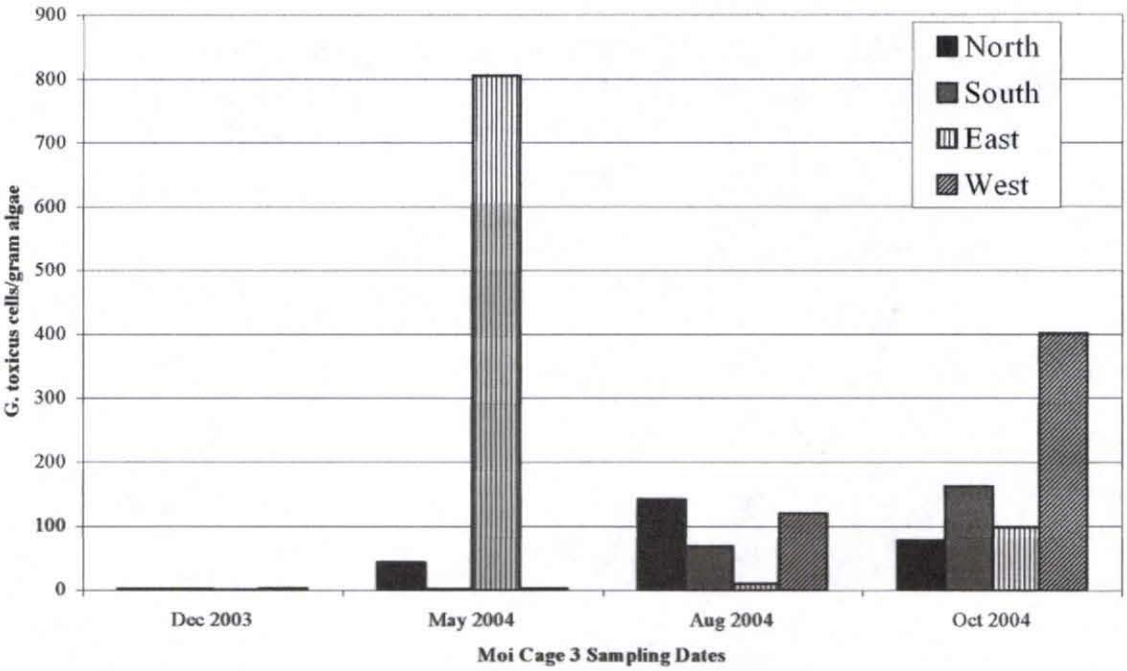
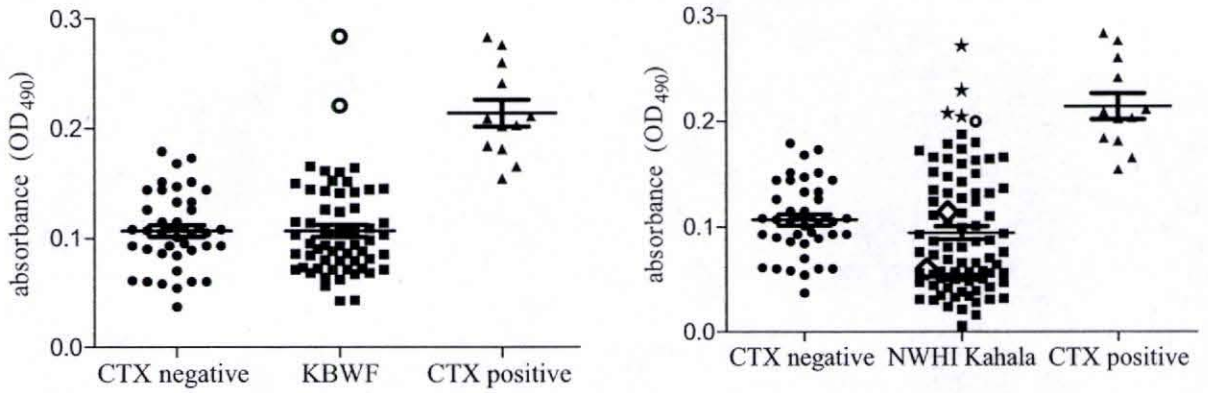


Figure 6.2. Cell densities of *G. toxicus* per gram of algae from moi cage #3 sampled four times over the course of the study period. Average ± SD cells/g algae for each sampling period are: Dec 2003 = 2 ± 1; May 2004 = 214 ± 395; Aug 2004 = 86 ± 58; Oct 2004 = 185 ± 148.



(a) (b)

Figure 6.3. Distribution of ELISA and neuroblastoma results in aquacultured kahala samples and wild-caught kahala samples. (a) Individual ELISA results from KBWF aquacultured kahala (n=60) and (b) wild-caught kahala (n=81) are shown compared to the CTX negative control and CTX positive fish extract (0.016 mg/ml). Stars indicates results that were positive using both the ELISA and NB, open circles indicate false positive ELISA results, and data points that indicated cell death approaching significance using the neuroblastoma assay but were negative using the ELISA are shown as open diamonds.

Chapter 7

A survey of ciguatoxin in *Cephalopholis argus* (roi) and *Seriola spp.* (kahala) in the Hawaiian Islands

7.1 Introduction

The incidence of ciguatera fish poisoning in Hawai'i is approximately 3.6 cases per 100,000 people annually, which is considered to be low compared with other regions in the tropics such as French Polynesia. The majority of cases are often reported from leeward coasts of the main Hawaiian Islands (MHI), with west Oahu, the north shore of Kauai, west Maui, and the west and northwestern coasts on the Island of Hawai'i leading the statistics (Anderson et al. 1983).

Fishes of the grouper family (Serranidae) are among the most common predators on coral reefs worldwide (Parrish 1987), and play an important role in shaping reef fish communities (e.g., Goeden 1982, Macpherson et al. 1997, Hixon and Jones 2005, Mumby et al. 2006). Groupers support important commercial, artisanal, and recreational fisheries over much of the tropics and subtropics, and are among the most commercially valuable species on coral reefs (Heemstra and Randall 1993). The blue spotted grouper (*Cephalopholis argus*), or roi, was introduced to the MHI from Moorea in 1957 with the intent to establish a new fishery (Randall 1987). After an initial time-lag in population growth, roi has established itself as one of the dominant large nearshore predatory reef fish in the MHI (Randall et al. 1993). However, despite the fact that roi is an economically valuable species in many of its native habitats, the envisioned fishery did not develop after roi caused a number of ciguatera fish poisoning incidents in Hawai'i

and was rejected by consumers (Bruno and Effler 2001, Earle 2005). Twenty-one incidents of ciguatera from roi consumption were reported in Hawai'i between 1996 and 2000, representing 17 percent of all reported incidents (Bruno and Effler 2001).

Similarly, during the 1970's, the amberjack, also known as kahala, (greater amberjack, *Seriola dumerili* and almaco jack, *Seriola rivoliana)*, was the fish most frequently associated with ciguatera fish poisoning in Hawai'i, responsible for 21 percent of ciguatera incidents reported to the State Department of Health between 1975-1981 (Anderson et al. 1983). Wild-caught amberjacks were subsequently excluded from commercial markets by retailers and restaurants due to the risk of ciguatera fish poisoning. Kahala was harvested commercially in Hawai'i for decades with peak landings of over 68 metric tons (MT)/year and average landings of 40 MT/year recorded prior to 1980. It remained a significant bycatch in the handline fishery targeting high-value deep-sea snappers, and although the price for kahala was low ($0.83 ± 0.36/kg), it could be harvested in sufficient quantities to offset fishing costs when the more valuable snappers were hard to catch (DAR 2005, NOAA 2006). Historical data on kahala catch and sales in Hawai'i over time is summarized in Figure 7.1 and illustrates the sharp decline in marketability of this species concurrent with associated outbreaks of ciguatera fish poisoning from the mid-1970's to the present.

Several biological, immunological, and chemical methods are available for detecting CTX in fish tissue, including a traditional mouse bioassay, radio ligand binding, high performance liquid chromatography, and mass spectrometry, however, few are well suited for routine large scale screening of fish samples. The membrane immunoassay

(MIA) developed by Hokama et al. (1998) is a qualitative field assay used to detect CTX, although it has been shown to cross react with other marine toxins (Hokama et al. 1992), and has the potential to yield a high false positive rate depending on the fat content of the fish (Tamaru et al. 2005). A novel sandwich enzyme linked immunoassay (ELISA) recently developed (Campora et al. in press) to detect CTX in partially purified fish extract is semi-quantifiable and shows promise in eliminating some of the problems observed when using other methods. In addition, a directed cytotoxicity assay that records sensitive changes in the sodium channel of neuroblastoma cells in the presence of sodium channel activators or inhibitors is an established method for the detection of marine toxins, including CTXs, in crude seafood extracts (Manger et al. 1995). Using a microplate high-throughput format, the neuroblastoma assay serves as a valuable tool for marine toxin studies, capable of reliably detecting CTXs at sub-picogram levels.

Due to the lack of suitable detection methods for large-scale evaluation of CTX, very few comprehensive field studies on fish toxicity at various geographic locations have been reported. While the general principle of CTX accumulation is understood (Banner 1974), the presence and distribution of ciguatoxic fish individuals often appears random and unpredictable, and the patterns behind CTX accumulation remain unknown (Lewis 2001). In some locations, certain stretches of coastline or specific reefs have been found to consistently produce ciguatoxic fish, while neighboring coastlines or reefs may house fish that can be considered safe for consumption (Lewis 2001).

The objective of this study is to assess the presence of CTX in three fish species commonly implicated in ciguatera fish poisoning in Hawai'i at various sites using two

different methods. The survey will also be useful in evaluating the potential for the newly developed ELISA as a screening tool for CTX when compared to the more widely accepted neuroblastoma cell bioassay. Because fish were caught in diverse areas around the MHI and NWHI, comparative analysis of location, fish size, depth of catch, and ciguatoxicity is also presented. The results of the study will enable a more thorough evaluation of the risk of ciguatera fish poisoning from consumption of kahala and roi throughout the Hawaiian Islands.

7.2 Materials and Methods

7.2.1 Fish collection and sample preparation

Roi individuals were caught by spearfishing on SCUBA at two different sites in the MHI in July and August 2003: off of the west (Kona) coast of the Big Island of Hawai'i (n=93; average standard length: 28.8 cm ± 5.6) and at various locations off of Oahu (n=82; average standard length: 26.5 cm ± 5.3). Kahala were obtained by hook and line from two different locations between December 2006 and August 2007: off the Penguin Banks in the MHI (n=83; average fork length: 75.1 cm ± 9.5; average body weight: 6.0 kg ± 2.4), and from various sites in the NWHI (n=81; average fork length: 73.5 cm ± 14.4; average body weight: 4.4 ± 1.8 kg).

Fish were stored on ice during transport back to a laboratory where a 2.5-cm by 2.5-cm cube of muscle tissue without the skin was excised from the left side or head region of each fish and stored at –20°C until lipid extraction could be performed. The cube of muscle tissue was subsequently placed in a clean test tube and lipids were extracted by soaking in 1 ml 100% acetone per 2.5 grams of fish tissue overnight. The

acetone extract was transferred to a clean, tared test tube, blown down using a laboratory air source, and weighed. The extract was re-suspended in 100% methanol at a concentration of 10 mg/ml and stored at 4°C for further CTX analysis. To rule out that the lipid matrix or extraction solvents affect the ELISA or neuroblastoma assay results, the same extraction procedures were followed for a deep water pelagic fish purchased from a commercial market that is not expected to harbor CTX, Pacific Blue Marlin (*Makaira nigricans)*, referred herein as non-toxic fish extract.

7.2.2 Enzyme linked immunoassay for CTX detection

A novel sandwich enzyme linked immunoassay (ELISA) was recently developed to detect picogram levels of CTX in partially purified fish extract (Campora et al. in press) as described in Chapter 5, section 5.2.3 and Chapter 6, section 6.2.3 of this dissertation and was evaluated as a preliminary tool to assess the lipid extracts from each fish for CTX.

7.2.3 Neuroblastoma cell bioassay for CTX detection

The neuroblastoma (NB) cell bioassay for the detection of CTX was modified after the method of Manger et al. (1995), and was carried out as described in Chapter 5, section 5.2.4 of this dissertation.

7.2.4 Statistical analysis

All statistical analyses were carried out using Microsoft Excel or Prism 5.0 (GraphPad Software, USA). Differences were regarded as significant when $p<0.05$ unless otherwise indicated.

Statistical analyses for the ELISA and neuroblastoma assays are as described in Chapter 6, section 6.2.5 of this dissertation.

Chi square analysis and Fisher's exact test were used to calculate the sensitivity, specificity, and positive and negative predictive values of the ELISA when compared to the neuroblastoma assay. Chi square analysis and Fisher's exact test were also used to compare differences in toxicity between locations. Regression analysis and Pearson's correlation analysis were used to observe associations between relative toxicity based the results of the ELISA assay with standard length of roi, fork length of kahala, and depth of catch for both species. Pearson's correlation was also used to compare the results of the ELISA and NB assays.

7.3 **Results**

7.3.1 ELISA and neuroblastoma assay results

Roi from the Kona coast showed the highest rate of ciguatoxicity when evaluated with the ELISA, with a total of 21.5% yielding positive or borderline results (13/93, or 14.0%, and 7/93, or 7.5% respectively). Using the ELISA, roi from Oahu resulted in a total of 6.1% positive or borderline results (2/82, or 2.4%, and 3/82, or 3.7%, respectively).

Using the NB cell bioassay, 11.8% of the roi from Kona (11/93) tested significantly positive using strict statistical criteria when compared to the controls. An additional 6.5% (6/93) gave results indicative of cell death 'approaching significance' when compared to the control and were classified as borderline. Thus, a total of 18.3% of roi from Kona gave results indicative of CTX presence using this method. Of the roi

from Oahu, 3.7% (3/82) showed significantly positive results, with an additional 1.2% (1/82) showing signs of cell death approaching significance when compared to the control, for a total of 4.9% considered positive for CTX. The recorded OD values obtained for borderline and positive scores using the ELISA and neuroblastoma assays for roi are summarized in Table 7.1.

Of the kahala from the MHI tested with the ELISA, 2.4% gave positive or borderline results (1/83, or 1.2%, and 1/83, or 1.2%, respectively), and kahala caught from locations in the NWHI yielded a total of 6.2% positive or borderline results (5/81, or 6.2%, and 0/81, or 0.0%).

None (0/83) of the kahala from the MHI tested significantly positive or showed signs of cell death approaching significance using the neuroblastoma cell bioassay. Of the kahala from the NWHI, 4.9% of samples (4/81) tested significantly positive, and an additional 2.5% showed signs of cell death approaching significance (2/81) for a total of 7.4% considered positive for CTX. The recorded OD values obtained for borderline and positive scores using the ELISA and neuroblastoma assays for kahala are summarized in Table 7.2.

7.3.2 Assay comparison

Of a total of 32 positive or borderline scores using the ELISA results from all fish species (n=339), 23 were scored as significantly positive or having cell death approaching significance using the neuroblastoma assay. The remaining 9 were scored as 'not significant' using the neuroblastoma assay, leading to a 2.7% implied false positive rate when using the ELISA. Additionally, 4 of the samples tested negative using the

ELISA, but were scored as significantly positive or having cell death approaching significance using the neuroblastoma assay, implying an overall 1.2% false negative rate when using the ELISA. Using the data from Kona roi, correlation analysis demonstrated that the results of the ELISA and the results of the NB assay are statistically significant at $P<0.001$ (Figure 7.2). The distribution of ELISA and neuroblastoma results from both species when compared to the CTX non-toxic negative extract and CTX positive extract (described in Chapter 5 of this dissertation, see Figure 5.6) are summarized in Figure 7.3.

The positive percent agreement of the ELISA as calculated using the NB and ELISA data was 85% (95% CI: 0.65 to 0.95), and the negative percent agreement was 96% (95% CI: 0.93 to 0.97).

7.3.3 Fish size and CTX

A significant correlation was observed between the standard lengths of roi from Kona ($P = 0.048$, $R = -0.21$) and relative toxicity and the fork lengths of kahala from NWHI ($P = 0.017$, $R = -0.27$) and relative toxicity. However, there was no significance noted between size and toxicity when evaluating roi from Oahu ($P = 0.71$, $R = -0.04$) and kahala from MHI ($P = 0.74$, $R = -0.08$) (Figure 7.4).

7.3.4 Catch location and CTX

The proportion of positive samples from each location scored with the neuroblastoma cell bioassay are compared to the total number of fish caught and tested at each site in Table 7.3. Figures 7.5 and 7.6 graphically depict specific kahala and roi sampling sites in the MHI and NWHI. As shown, there is considerable spatial variation between sampling sites. Differences in toxicity between roi from Kona and Oahu (P =

0.009), as well as between kahala from the MHI and NWHI (P = 0.013) were shown to be statistically significant using Chi square analysis.

7.3.5 Depth of catch and CTX

No significant correlations were observed between the mean depth of catch of roi from Kona (R = -0.15) and Oahu (R = -0.09), or kahala from the NWHI (R = -0.14) and relative toxicity, depicted in Figure 7.7. Depth of catch data is not available for kahala from the MHI.

7.4 Discussion

ELISA has become an indispensable tool for quantitative and semi-quantitative analysis for the detection of specific antigens in a variety of matrices. Several models for determining positive-negative thresholds using ELISA have been reported, including 2 and 3 times the mean of a non-toxic negative control, and the mean of the non-toxic negative control plus 2, 3, and/or 4 standard deviations (Sutula et al. 1986, Fenlon and Sopp 1991). Receiver-operator characteristic (ROC) curves have also been used to give a statistically optimal cut offs and to assist in visualizing the tradeoff between high sensitivity and specificity. However, the selection of actual cut off determinations using immunological methods is generally mandated by practical realities rather than statistical optimization. In practice, cut offs may need to be lowered or adjusted in order to not misclassify samples as false negatives. While such an action may decrease the specificity of the test, thereby increasing the incidence of false positive results, in situations where devastating economic or health implications are possible, this situation is preferred. It is recognized that the common use of such thresholds are arbitrary and generally should

rely on a secondary assay to assist in determining the optimal threshold, which in this study was implementation of the neuroblastoma cell bioassay.

ELISA threshold was established in this study to be two times the mean OD of the non-toxic fish extract based in part on multiple assay runs using both the non-toxic fish extract and a previously characterized partially purified positive CTX extract (Hokama et al. 1994). The ELISA shows statistically significant detection of CTX at 0.016 mg/ml (Figure 5.6) using the partially purified CTX positive extract when compared to a non-toxic fish extract, or negative control, which, according to previous studies, correlates to approximately 5 pg/ml of equivalent CTX per mg of extract (Hokama et al. 1998). At 0.016 mg/ml, the CTX positive extract gives an average OD of 0.200 (n=15), which is approximately double the average OD reading obtained when running the non-toxic fish extract with the ELISA (0.100, n=42), which was useful in establishing a cut-off for positivity. One of the pitfalls of ELISA is the sequential accumulation of small random errors such as small differences in incubation times, calculation of standards and samples, and slight differences in reagents through the course of the experiment, which culminates in color development and optical density measurements that are consequences of all the previous steps. While experiments were closely controlled to maintain uniformity and minimize such errors, data from each plate was extrapolated to reflect the 0.100 OD negative control reading and the cut-off for CTX positivity was named as 2 times the negative control, or 0.200 OD in this study. The lack of availability of reliable purified CTX standards to further validate the ELISA may affect the interpretation of the results with respect to determination of the true levels of CTX present in the partially purified

CTX positive fish extract and crude fish samples, and leaves the door open for further studies to be done toward improvement of the method.

Due to the preliminary and novel nature of the ELISA, the neuroblastoma cell bioassay was performed as a secondary screen. As a potent sodium channel activator, CTX, if present in crude fish extract even in picogram quantities, in combination with the chemical agonists ouabain and veratridine (OV), will lead to quantifiable neuroblastoma cell death (Manger et al. 1995). The NB assay lacks specificity in that the results represent a functional mechanism rather than structural recognition of a particular antigen or molecule, however, the NB results are currently more acceptable at this stage of testing as gauged by its applied use reported in the literature (LePage et al. 2005, Garrec et al. 2005, Bottein Dechraoui et al. 2005b, Dechraoui et al. 1999, Manger et al. 1993, 1995).

According to the US Food and Drug Administration (FDA), when reference standards are not available, estimates of sensitivity and specificity can be made using a secondary method, and are reported as positive and negative percent agreements (FDA 2007). Results of the ELISA and NB were highly correlated ($P<0.001$, Figure 7.2), and comparison of results indicated that the ELISA has an positive percent agreement of 85% and negative percent agreement of 96%, which can be considered reasonable for a biological assay of this type. In this study, relatively low false positive (2.7%) and false negative (1.2%) values were low. The occurrence of false positives likely results from the somewhat arbitrary determination of the positive threshold; however, it is preferable to err on the side of caution and report false positives rather than false negatives when detecting a potentially harmful substance such as CTX. It is important to note that using

the NB assay results to confirm or refute the ELISA results assumes that the NB assay results are completely accurate, which in practice is open to discussion due to the non-specific nature of the assay and the potential for error during administration. A tertiary evaluation of a subset of the positive and negative samples using a chemical evaluation method for CTX such as liquid chromatography-mass spectrometry could be used in the future to further narrow down estimates for precision and accuracy, and to provide improve sensitivity and specificity values by refining the cut-off value.

The determination of ciguatera fish poisoning risk from roi in Hawai'i is important for both recreational and subsistence fishermen and for commercial operations that may have an interest in culturing the popular reef fish for human consumption. Understanding the factors correlated with roi ciguatoxicity could allow resource managers and fishermen to predict if certain roi, e.g., individuals below a certain size limit or from a particular geographic area, are predictably free of CTX and safe for consumption. While the frequency of occurrence of toxic individuals in roi was much higher than the frequency observed for Hawaiian reef fishes in general, reported as 4% positive in 1998 by Hokama et al., results of the ELISA alone are in accordance with a more recent immunological assessment of the Kona and Oahu caught roi using the MIA test in which 24.7% (45/182) of roi from Kona and 6.6% (7/106) of roi from Oahu were found to be positive (Dierking, 2007). Interestingly, the current report uncovered individuals that were positive for CTX using both ELISA and NB assays that were detected in the waters surrounding Kaneohe, located on the windward coast of Oahu, as it had been previously reported that the preponderance of ciguatoxic poisonings reported in

Hawai'i were from fishes that were predominantly caught on the leeward side of the MHI (Anderson et al. 1983).

A two-year longitudinal study of CTX in kahala caught in Hawai'i using a radioimmunoassay reported that an average of 15% of samples were scored as positive (824/5,529) (Kimura et al. 1982b), and also noted that there was no significant rejection pattern expressed over the course of the study. Discrepancies between the trends may be due to seasonality of sample collections, and differences in methods of analysis. The lower percentage of ciguatoxic individuals presented in this study suggests that with proper screening in place, wild-caught kahala could once again be safe for sale on the open market. A testing regimen could lead to an increase in utilization of the kahala resource, which may in fact have a positive effect on other bottomfish fisheries by reducing predation and competition for food and habitat (Tagawa and Tam 2006)

There was an inverse relationship noted between fish size and toxicity for roi from Kona and kahala from the NWHI, although correlation coefficients from both data sets indicate a weak association. While roi from Oahu and kahala from the MHI did not show significant associations, the smaller number of samples that scored positive from both species in these locations likely contributed to the statistical outcome. Differences in food sources, feeding habits of both small and large individuals, and their proximity and exposure to *G. toxicus* may serve to partially explain the differences in observed toxicities both between size classes and sampling locations. Mackerel scad, or opelu (*Decapterus* sp.), has been observed to be the predominant food source for larger kahala (Uchida et al. 1982), however, one study suggests that kahala in the NWHI primarily feed

on octopus and bottom associated prey, while kahala in the MHI tend to feed on prey associated with the water column (Humpreys 1986). Depth of catch was not statistically significant relative to toxicity at any of the sites examined, and despite the weak association between size and toxicity, neither size nor depth of catch can be considered a reliable indicator of the potential for ciguatoxicity in kahala or roi. Clearly, the distribution and abundance of ciguatoxic fishes throughout the Hawaiian Islands will require further study.

Specific locations have some correlation to toxicity in that fish caught at certain sites all test positive for CTX while fish caught at other sites appear to be toxin-free (Table 7.3), however, the transitory nature of fish behavior, even for groupers which tend to exhibit high site fidelity, makes it difficult to accurately predict ciguatoxicity based on location of catch alone. A recent tagging experiment showed that kahala tend to be highly migratory, with several individuals traveling hundreds of miles over the course of the study (Tagawa and Tam 2006), implying that feeding sites and thus potential for acquiring toxicity may change significantly over the life cycle of the individual fish. Spatial and temporal variations of the dinoflagellate *G. toxicus* at specific reef or bank sites may also contribute to the difficulty in predicting toxicity based on specific location, although general trends may be noted (Anderson et al. 1983). According to the results, roi from the Kona coast were at least three times as likely to harbor CTX than roi from Oahu, and that kahala from the NWHI were significantly more likely to harbor CTX than those caught in the MHI, particularly near Penguin Banks.

In conclusion, the newly developed ELISA has reasonable specificity and sensitivity for an immunological screening assay, and statistical correlation with that of the neuroblastoma assay indicate that it is a valid tool for screening fish for CTX, although the assay may require refinement with respect to threshold values and purification of samples to improve sensitivity and specificity values. The results of the survey using both the ELISA and NB assays indicates that roi and kahala caught at various locations in the MHI and NWHI exhibit spatial variation in ciguatoxicity that is not reliably predictable by size or depth of catch, and that total toxicity may be lower than previously reported.

Table 7.1. ELISA and NB assay results for detecting ciguatoxin in roi. NS: no significant change in cell death when compared to the control; NS^: cell death occurred with respect to the control approaching levels of significance; and (*), (**), (***): significant increase in cell death when compared to the control (0.01<P<0.05, 0.001<P<0.01, P<0.001, respectively).

Kona Roi Sample ID	ELISA Result (average OD ± SD) n=3 Positive and Borderline above 0.200 ± 0.020	ELISA score	NB Control Value (average OD ± SD) n=6	NB Result (average OD ± SD) n=3	Statistical Score (ANOVA)
3	0.186 ± 0.025	Borderline	0.197 ± 0.015	0.170 ± 0.027	NS^
5	0.208 ± 0.031	Positive	0.197 ± 0.015	0.214 ± 0.011	NS
21	0.186 ± 0.007	Borderline	0.261 ± 0.017	0.126 ± 0.008	***
42	0.203 ± 0.016	Positive	0.261 ± 0.017	0.215 ± 0.019	NS^
61	0.206 ± 0.022	Positive	0.261 ± 0.017	0.188 ± 0.002	*
83	0.260 ± 0.011	Positive	0.261 ± 0.017	0.110 ± 0.002	***
93	0.185 ± 0.007	Borderline	0.261 ± 0.017	0.203 ± 0.021	NS^
103	0.184 ± 0.004	Borderline	0.183 ± 0.015	0.234 ± 0.028	NS
104	0.187 ± 0.014	Borderline	0.183 ± 0.015	0.198 ± 0.026	NS
107	0.224 ± 0.003	Positive	0.261 ± 0.017	0.181 ± 0.025	*
115	0.203 ± 0.010	Positive	0.235 ± 0.036	0.260 ± 0.005	NS
126	0.183 ± 0.007	Borderline	0.261 ± 0.017	0.118 ± 0.024	**
127	0.245 ± 0.015	Positive	0.261 ± 0.017	0.168 ± 0.003	*
138	0.226 ± 0.020	Positive	0.261 ± 0.017	0.185 ± 0.014	NS^
141	0.263 ± 0.019	Positive	0.261 ± 0.017	0.193 ± 0.028	NS^
143	0.263 ± 0.031	Positive	0.261 ± 0.017	0.215 ± 0.002	NS^
149	0.176 ± 0.004	Negative	0.261 ± 0.017	0.145 ± 0.008	***
172	0.203 ± 0.005	Positive	0.261 ± 0.017	0.126 ± 0.020	***
177	0.204 ± 0.032	Positive	0.261 ± 0.017	0.166 ± 0.022	**
182	0.243 ± 0.024	Positive	0.261 ± 0.017	0.142 ± 0.005	***
183	0.196 ± 0.030	Borderline	0.261 ± 0.017	0.159 ± 0.012	***
Oahu Roi					
60	0.185 ± 0.044	Borderline	0.194 ± 0.026	0.214 ± 0.004	NS
54	0.104 ± 0.006	Negative	0.145 ± 0.028	0.102 ± 0.008	*
135	0.203 ± 0.018	Positive	0.182 ± 0.008	0.108 ± 0.024	*
139	0.207 ± 0.012	Positive	0.198 ± 0.022	0.164 ± 0.018	*
146	0.181 ± 0.029	Borderline	0.198 ± 0.022	0.168 ± 0.003	NS^
155	0.189 ± 0.017	Borderline	0.204 ± 0.045	0.219 ± 0.013	NS

Table 7.2. ELISA and NB assay results for detecting ciguatoxin in kahala. NS: no significant change in cell death when compared to the control; NS^: cell death occurred with respect to the control approaching levels of significance; and (*), (**), (***): significant increase in cell death when compared to the control (0.01<P<0.05, 0.001<P<0.01, P<0.001, respectively).

Kahala MHI and NWHI Sample ID	ELISA Result (average OD ± SD) n=3 Positive and Borderline above 0.200 ± 0.020	ELISA Score	NB Control (average OD ± SD) n=6	NB Result (average OD ± SD) n=3	Statistical Score (ANOVA)
MHI 8.3	0.189 ± 0.013	Borderline	0.173 ± 0.046	0.162 ± 0.008	NS
MHI 8.4	0.247 ± 0.022	Positive	0.173 ± 0.046	0.207 ± 0.049	NS
NWHI 3	0.205 ± 0.004	Positive	0.261 ± 0.017	0.138 ± 0.017	**
NWHI 5	0.208 ± 0.018	Positive	0.261 ± 0.017	0.181 ± 0.019	*
NWHI 6	0.229 ± 0.013	Positive	0.261 ± 0.017	0.175 ± 0.001	*
NWHI 7	0.271 ± 0.017	Positive	0.261 ± 0.017	0.164 ± 0.034	**
NWHI 11	0.200 ± 0.005	Positive	0.248 ± 0.047	0.235 ± 0.012	NS
NWHI L22	0.058 ± 0.011	Negative	0.249 ± 0.048	0.178 ± 0.008	NS^
NWHI KP 14	0.114 ± 0.021	Negative	0.156 ± 0.036	0.065 ± 0.008	NS^

Table 7.3. Number of fish scored as positive for CTX using the neuroblastoma assay and the total number of fish caught per sampling site.

Kona Roi *Catch Location*	Total CTX Positive/Total Tested from Location	% Positives from Location
Mahukona	1/3	33.3%
Puako	2/2	100%
Golden Arches	1/1	100%
Kaiwi Point	2/16	12.5%
South Kiholo	1/23	4.3%
Kaloko Arches	2/7	2.9%
Lone Tree Arches	3/3	100%
Honaunau	1/11	9.1%
Red Hill	0/13	0.0%
Kona Paradise	4/12	33.3%
Alakaha	0/2	0.0%
Oahu Roi		
Sand Island	0/4	0.0%
Kaneohe 1	0/15	0.0%
Kaneohe 2	1/20	5.0%
Kaneohe 3	3/31	9.7%
Ko Olina	0/12	0.0%
MHI Kahala		
Penguin Banks, Main Hawaiian Islands	0/81	0.0%
NWHI Kahala		
Gardner Pinnacles	4/22	18.2%
Maro Reef	1/26	3.8%
Raita Bank	0/5	0.0%
Pioneer Bank	0/3	0.0%
Laysan (Northhampton, West)	0/3	0.0%
Laysan (Northhampton, East)	0/7	0.0%
Nihoa(Twin Banks, West)	1/10	9.1%
Nihoa (Twin Banks, East)	0/5	0.0%

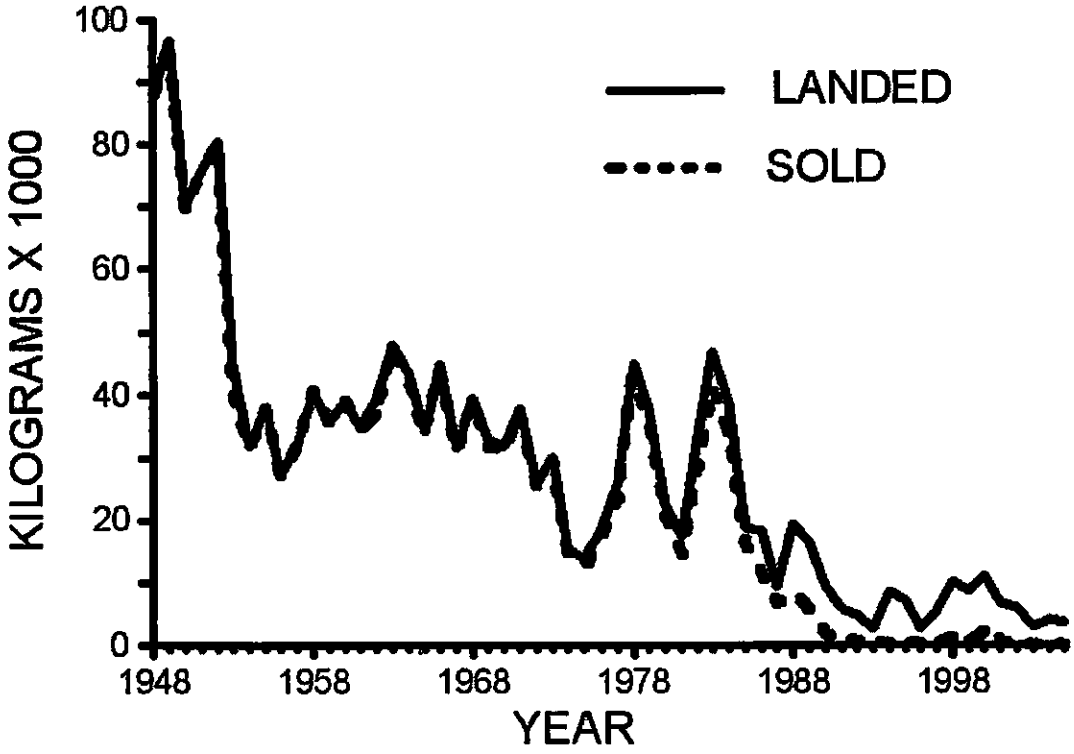


Figure 7.1. Historical data for kahala caught and sold in Hawai'i from 1948-2002 (Source: Division of Aquatic Resources).

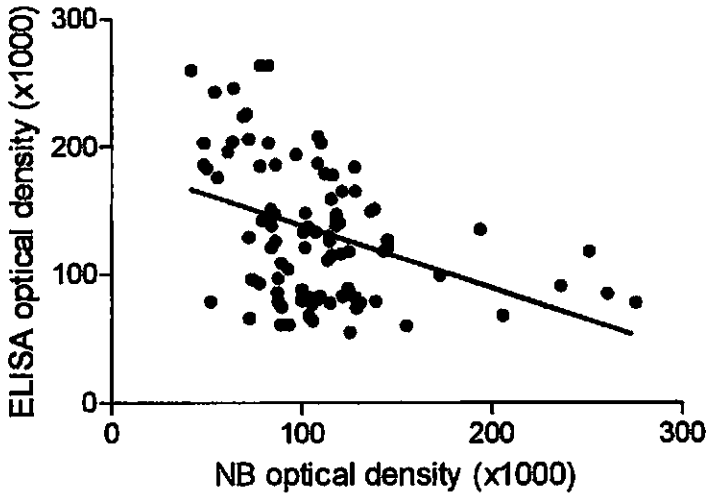


Figure 7.2. Correlation analysis comparing ELISA and NB results (R = -0.39, P<0.001).

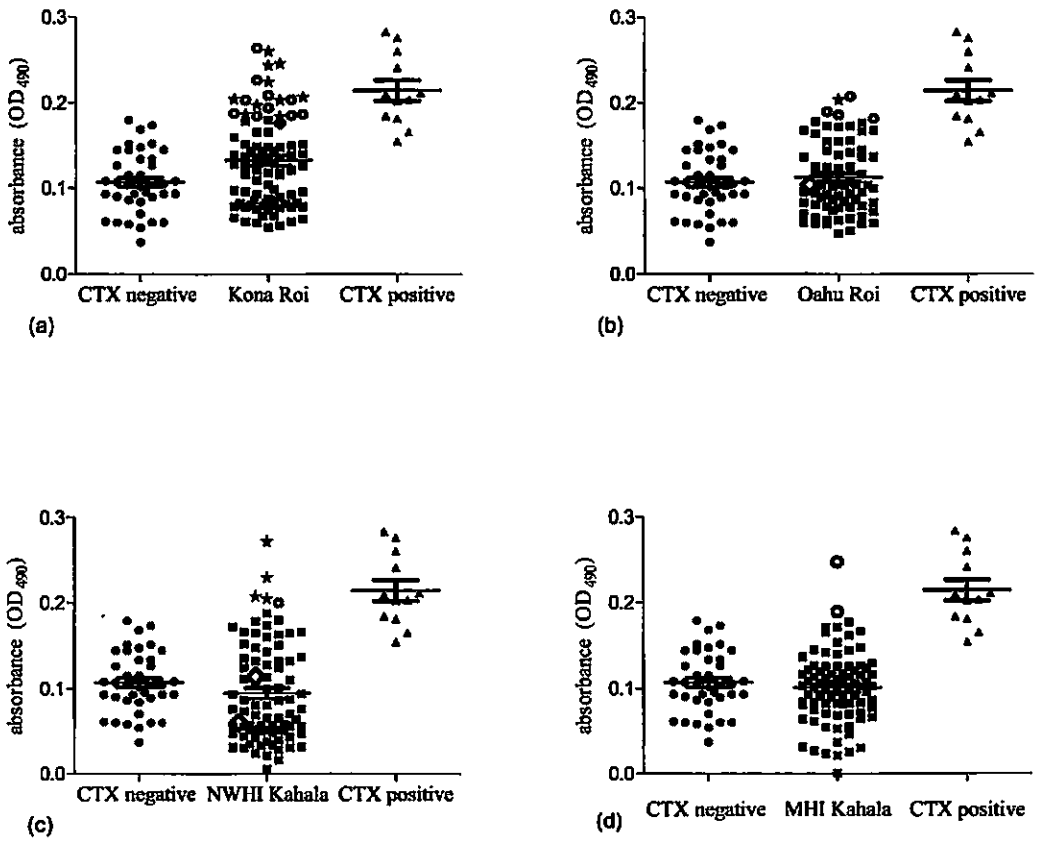


Figure 7.3. Distribution of ELISA and neuroblastoma assay results in roi and kahala samples. Roi from (a) Kona (n=93), (c) Oahu (n=82), and kahala from (c) NWHI (n=81) and (d) Penguin Banks (n=83) are shown compared to the CTX non-toxic extract control and CTX positive fish extract (0.016 mg/ml). Stars indicate results that were positive using both the ELISA and NB, open circles indicate false positive ELISA results, and data points that indicated cell death approaching significance using the neuroblastoma assay but were negative using the ELISA are shown as open diamonds.

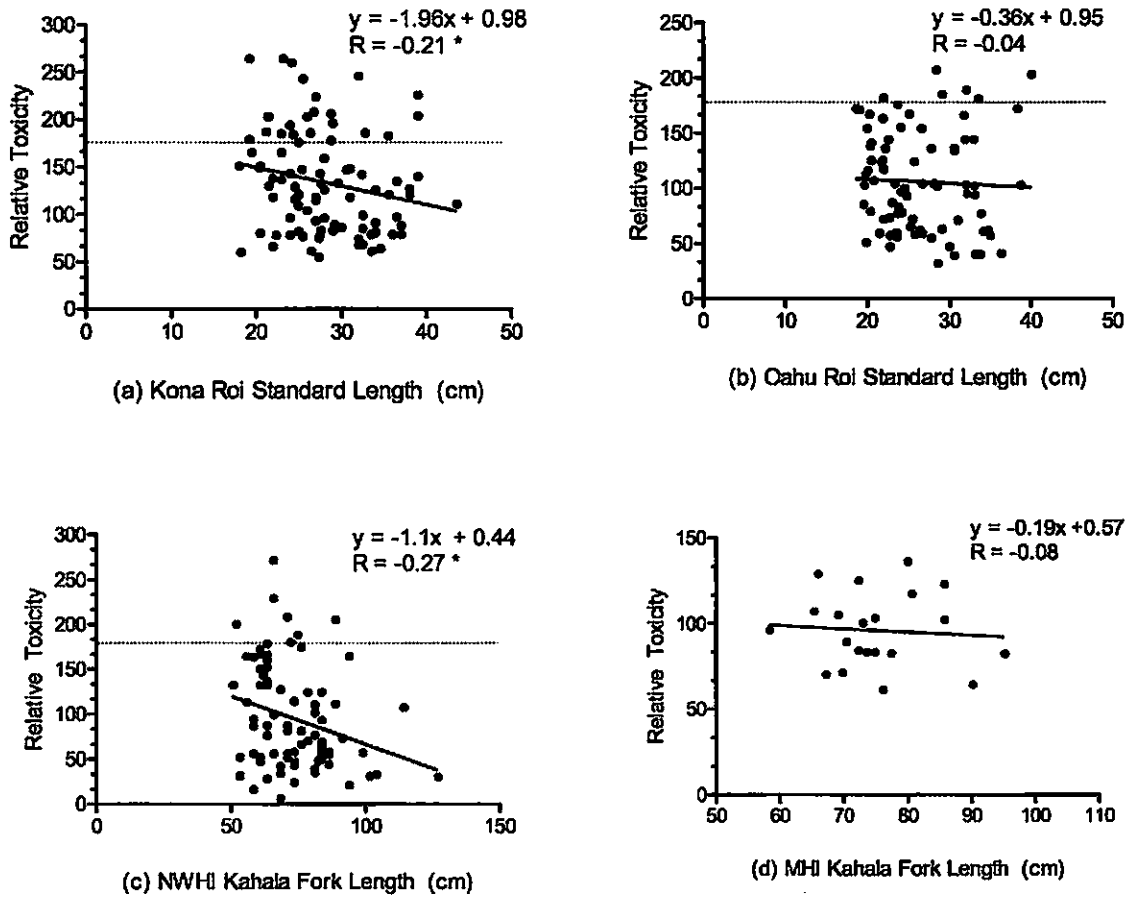


Figure 7.4. Correlation analysis between standard and fork lengths of roi and kahala and relative CTX toxicity where data points above 180 are considered positive.

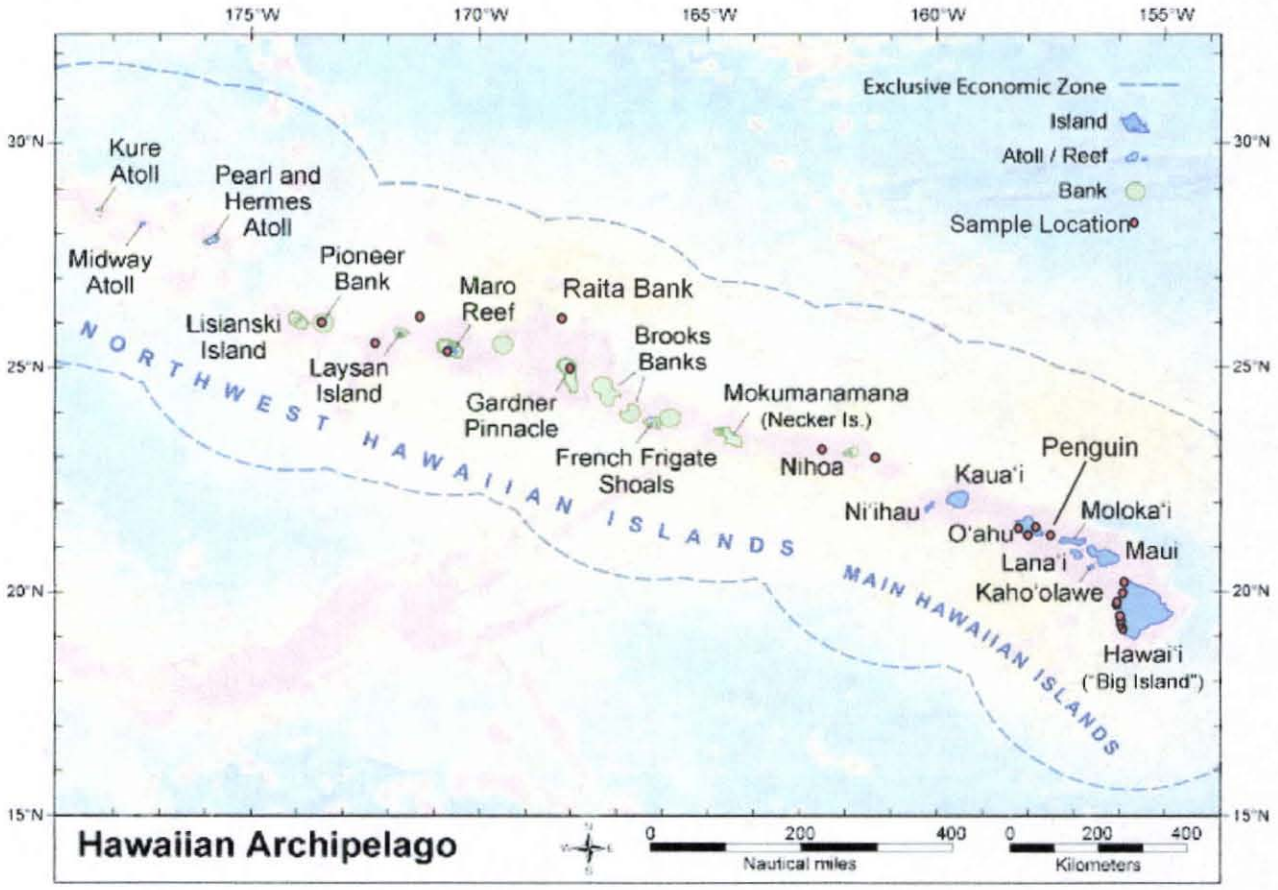


Figure 7.5. Map of the NWHI and MHI showing sampling locations of kahala and roi (Source: adapted from Pacific Islands Benthic Habitat Mapping Center).

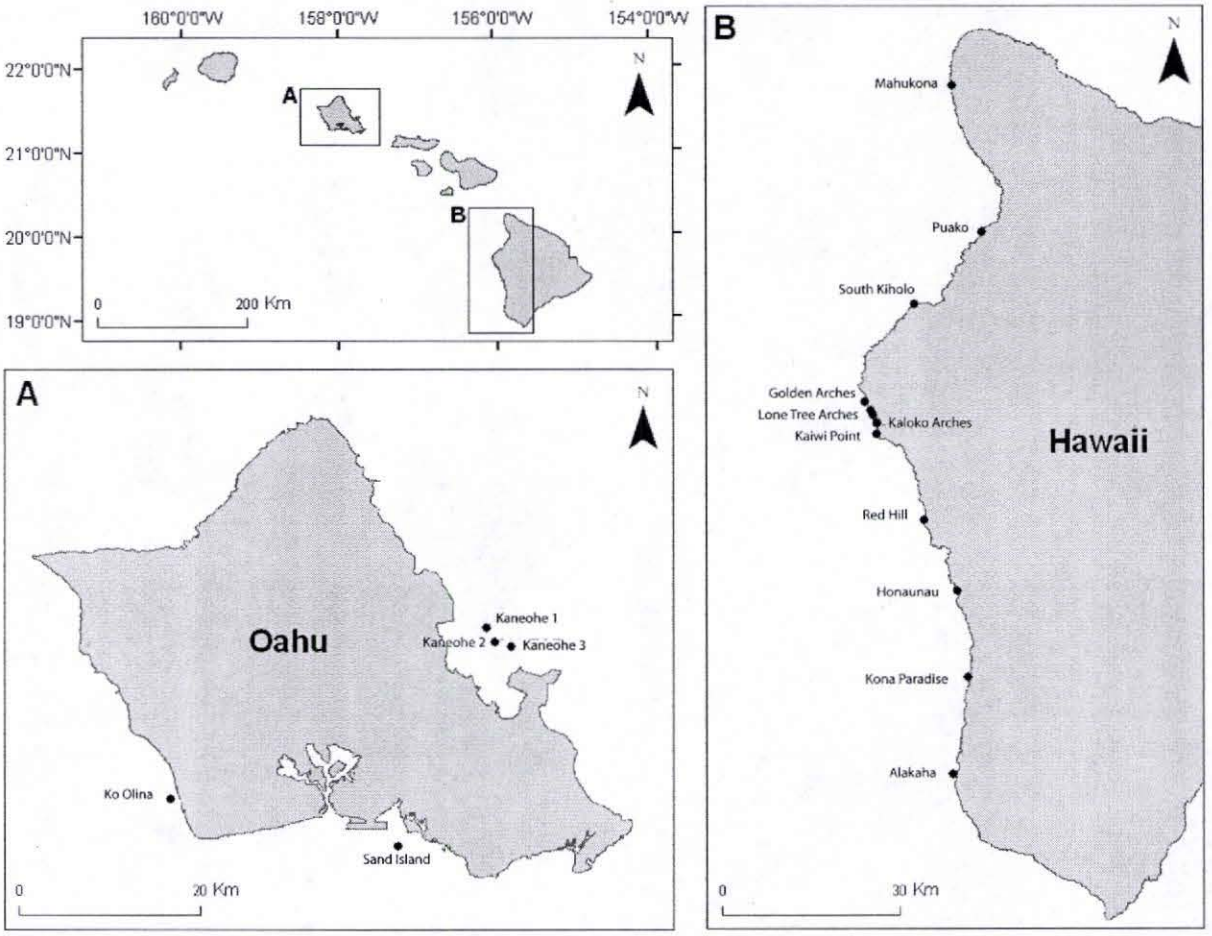


Figure 7.6. Map of Oahu, Hawaii and the Kona coast on the Big Island of Hawaii showing sampling locations of roi (Source: adapted from Dierking 2007).

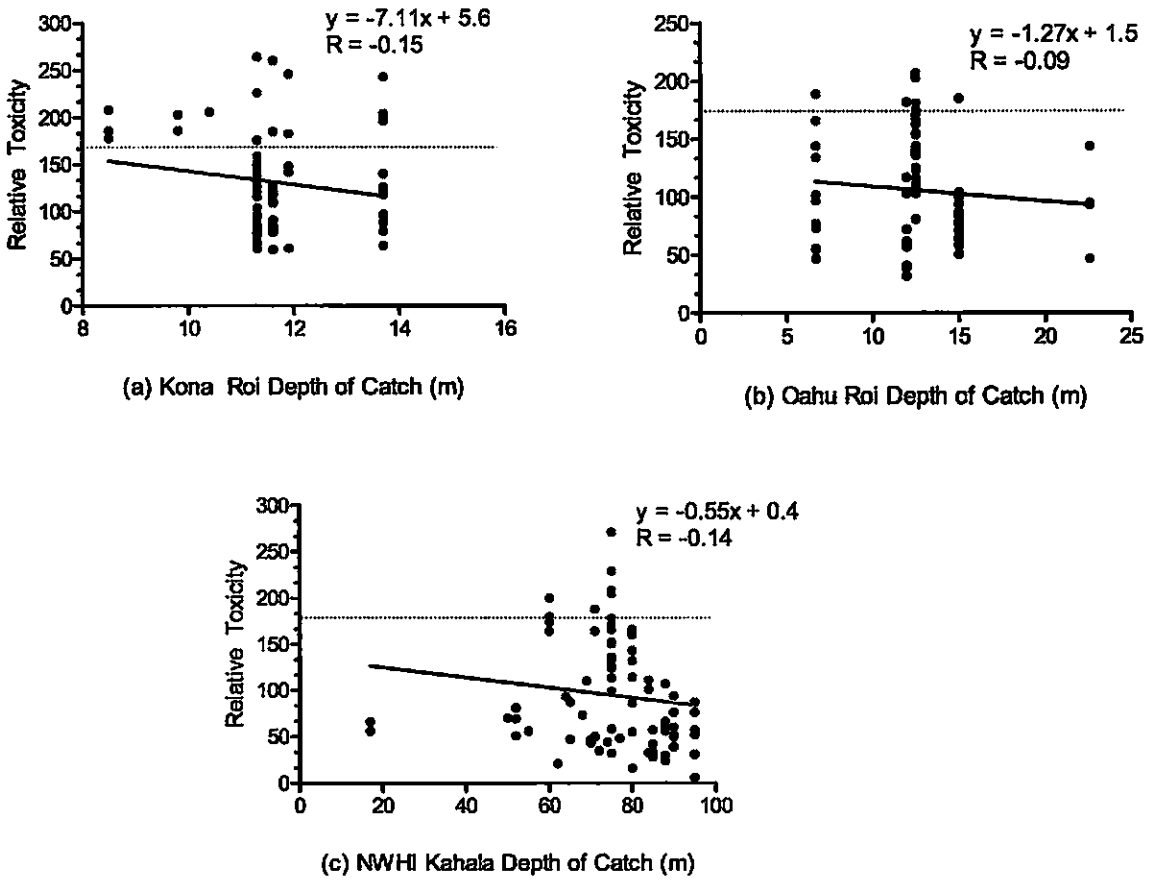


Figure 7.7. Correlation analysis between depth of catch of roi and kahala and CTX toxicity where data points above 180 are considered positive.

Chapter 8

Summary and Conclusions

This dissertation addresses ciguatera fish poisoning as an important food safety issue by developing and validating a sensitive and specific enzyme linked immunoassay (ELISA) capable of detecting picogram quantities of ciguatoxin in fish flesh, detailed in chapter five. The assay, which is the first of its kind in ciguatoxin research to use chicken IgY as an immunoanalytic tool and the first sandwich ELISA to date shown to accurately detect the toxin in fish tissues at sub-clinical levels, is a vast improvement to the current methods available to screen of fish for ciguatoxin. The promising versatility of the assay has the potential to increase consumer health and safety and to increase sales for commercial fisheries. The third chapter of this dissertation sets the stage for the development of the more refined ELISA by demonstrating that the antibody used in the assay is specific to purified ciguatoxin with limited cross reactivity with other marine toxins, and while there has been speculation as to the functions of each sphere of ciguatoxin, chapter four provides new evidence that the AB region of the CTX molecule is responsible for reacting with the receptor site in sodium channels in tissue culture experiments.

Chapters six and seven detail the results of testing over 300 fish (two species of kahala, *Seriola spp.*, and the blue-spotted grouper, *Cephalopholis argus*) commonly implicated in ciguatera fish poisoning from Hawaiian waters using both the ELISA and a secondary bioassay to validate results, and lead to the following significant conclusions: a) the ELISA developed during the course of this study is a valid screening tool for evaluation of presence or absence of ciguatoxin in fish tissues with reasonable sensitivity

and specificity values; b) the fish farmed in open ocean aquaculture cages in Hawai'i are not likely to be susceptible to ciguatoxin bioaccumulation despite the discovery of *G. toxicus* in the cages; c) CTX is present in three fish species commonly implicated in ciguatera fish poisoning at overall rates lower than previously reported; and d) the prevalence of ciguatoxin in tested wild-caught fish cannot reliably be correlated to the size of the fish or the depth at which it was caught, and that while geographic location of catch may provide some indicator of toxicity risk, the migratory nature of fish and their feeding behavior precludes it as a potential screen for ciguatoxicity.

Collectively, these findings are a significant contribution in the field of marine toxin research, and serve a practical purpose with respect to public health and the safety of consuming fish purchased from aquaculture and open ocean commercial fisheries.

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