UNIVERSITY OF MIAMI
ROSENSTIEL SCHOOL OF MARINE AND ATMOSPHERIC SCIENCE

A SINGLE ANTIBODY ENZYME-LINKED IMMUNOASSAY FOR THE DETECTION OF FLORIDA RED TIDE BREVETOXINS

By

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AN INTERNSHIP REPORT

Submitted to the Faculty of the University of Miami
in partial fulfillment of the requirements
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A single antibody enzyme-linked immunoassay for the detection of Florida Red Tide brevetoxins

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A Single Antibody Enzyme-linked Assay for the Detection of Florida Red Tide Brevetoxins.

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Abstract of an internship report at the University of Miami, School of Marine and Atmospheric Science supervised by Dr. Daniel Baden.

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Marine biotoxins cause significant health, managerial, and economic problems. The present situation demands the prompt implementation of the recently conceived management plan which recognizes the absolute need for scientific research to elucidate the intricacies of the toxic agents and the toxins they produce. Most importantly, the need for a quick, reliable, field-worthy, easy to operate means of toxin detection has been emphasized. After reviewing available literature, and practically comparing detection assays, it was concluded that an immunoassay, based on the use of toxin-specific antibodies, provided the greatest benefit. This research modified an existing enzyme-linked immunosorbent assay (ELISA), in order to expedite reaction times, and to optimize by diminishing the number of steps involved. The modified assay utilizes only one enzyme labeled antibody. The conjugation of this antibody to the indicator enzyme, horseradish peroxidase (HRP), was achieved at concentrations ranging from 50 to 163 μg/ml. Affinity chromatography purification of the antibody necessary for conjugation, from multi-species anti-serum to brevetoxin specific IgG, were also successfully

performed, and can be routinely employed as means of antibody preparation for conjugates and labels much in demand for current biotoxin research. Presently, the single antibody ELISA approach to biotoxin detection suffers from a high cost of implementation. Research continues seeking cost effective means and variations to enhance signal and improve recognition, in order to make this convenient assay a routine monitoring and field tool.

ACKNOWLEDGMENTS

This internship report is not just the product of my effort but also of those who have provided insight, labor and support to see it to its completion. I would like to thank Dr. Baden for introducing me to a most exciting area of research and for showing to me the extreme importance of the marine affairs approach to this scientific work. I am also indebted to him for his patience and support throughout our five years of collaborative efforts. I would like to thank Dr. Sarah Meltzoff for her continuos advice on opportunity and for a most refreshing and rewarding approach to academic life. Dr. Fernando Moreno deserves my gratitude for providing the guidance, enthusiasm and the confidence which inspired me to continue this work even during the most discouraging of times. I would like to thank Mr. Marty Healy for getting me interested in this graduate program to begin with; he certainly is the one of the very best spokespersons the MAP division has today. Additionally, I would like to express my gratitude to all laboratory researchers, technicians and postdocs for their help. Special thanks to Dr. K. Rein for taking the time to remind me how to draw my molecules straight.

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I would like to thank my sisters and brother. The hardest things really can be accomplished when you know your family is behind you. For all their love and support, thanks. Most importantly, to my mother and father I owe my greatest

vote of gratitude. Their efforts and sacrifices have seen me to this accomplishment; without their love, patience and wisdom I would have truly been lost.

Finally, I would like to express my extreme satisfaction with this graduate program and would like to thank again all those who have made it possible. Owing to their help and creativity I am now in a position of enhanced flexibility, able to dedicate my efforts and apply useful skills to various research, managerial and regulatory areas of wide interest. Again thanks.

A Jesus y Pastora

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LIST OF ABBREVIATIONS

ASP:	Amnesic Shellfish Poisoning
CDC:	Centers for Disease Control
CFP:	Ciguatera Fish Poisoning
DSP:	Diarrhetic Shellfish Poisoning
ELISA:	Enzyme-linked Immunosorbent Assay
FDA:	Food and Drug Administration
FLADNR:	Florida Department of Natural Resources
HACCP:	Hazard Analysis and Critical Control Point
HPLC:	High Pressure Liquid Chromatography
ICSSL:	Interstate Certified Shellfish Shippers List
Ig:	Immunoglobulin (antibody)
IgG:	Immunoglobulin-G
IRA:	Immunoradioassay
NACMCF:	National Advisory Committee on Microbiological Criteria for Foods
NIST:	National Institute of Standards and Technology
NSP:	Neurotoxic Shellfish Poisoning
OA:	Okadaic Acid
PbTx:	Ptychodiscus brevis toxin
PbTxIgG-HRP:	Horseradish peroxidase conjugated brevetoxin specific antibody.
PbTxIgG:	Brevetoxin specific antibody
PSP:	Paralytic Shellfish Poisoning
SSCA:	State Shellfish Control Authority
STX:	Saxitoxin
TLC:	Thin-layer Chromatography
TTX:	Tetrodotoxin

INTRODUCTION

Gymnodinium breve is the causative agent of the Florida Red Tide natural phenomenon, and producer of the neurotoxic molecules known as Brevetoxins (Steidinger *et al.*, 1973). The large number of fish mortalities associated with these red tide episodes together with their influence on the fishing industry and seafood safety make this dinoflagellate and the biotoxins it produces urgent topic of study throughout the scientific community. Brevetoxins are known to act as depolarizers upon the sodium channels of the nerve cells (Huang *et al.*, 1984; Baden *et al.*, 1983). Once the ion balance has been disrupted, muscle movement failure occurs and in the case of fish, the subsequent asphyxia and cardiac arrest lead to the death of the affected organism. Fish swimming within a red tide are exposed to the toxins through their respiratory and gas exchanging surfaces. Additionally, aerosol contact with the intoxicant material is possible via spraying action of waves breaking against the shore (Woodcock, 1948; Ingle, 1954; Pierce *et al.*, 1990). Humans in the beach area of an affected shore have experienced bronchoconstriction and some of the primary symptoms of brevetoxin intoxication which include numbness of lips and tongue, and tingling of finger tips. In severe intoxication cases, diarrhea as well as temperature reversal effects have been reported. Brevetoxins comprise a specific class out of several types of biotoxins; saxitoxin, domoic acid, ciguatoxin, okadaic acid, maitotoxin, and 35-methyl okadaic acid (DTX-1). These toxins generate neurotoxic, ciguatoxic, diarrhetic, amnesic, and paralytic syndromes. The need for efficient management of the biotoxin dilemma concerns scientific, commercial, as well as administrative and legislative agencies. In order to efficiently manage a naturally recurring event it is necessary to understand the fundamentals of its existence. Scientific background information upon which to make administrative and legislative decisions is

essential. There is a need for a cost and time efficient scientific means of biotoxin detection. The ability to determine toxin presence will allow for the establishment of appropriate international safety standards which will contribute to this effective management of marine biotoxins worldwide. Safety standards will facilitate trade among countries, establish universal quality control requirements, and diminish intoxication episodes caused by consumption of exposed shellfish or finfish stock. It has also been recognized that, in addition to cost and time efficiency, the appropriate test should be based on relevant toxin characteristics, in order to increase assay specificity.

The objective of this research study was to modify an existing enzyme linked immunosorbent assay (ELISA) developed by Trainer and Baden (1991), to detect the presence of brevetoxins and to contrast its suitability vs. biochemical assays for use in routine monitoring. The available ELISA assay employs a secondary antibody, conjugated with horseradish peroxidase (HRP) , linked to a primary antibody which is bound to the toxic antigen. The proposed research will create a similar assay which will employ a single, primary antibody, HRP conjugated and linked directly to the toxic antigen. This new assay will take less time to perform, and its sensitivity should be comparable to that of the existing one (0.04 picomolar; Trainer and Baden, 1991).

PART I

BIOTOXIN RESEARCH AND MANAGEMENT BACKGROUND

In the United States, marine biotoxins cause severe economic, health and logistic dislocations. Scientific research involving these biotoxins has been sketchy at best and, though significant advances have been made in recent years, there is great unawareness regarding the precise modes of action of each toxin and there is a need for efficient, reliable means of detection. Additionally, programs for monitoring, prevention and management of toxic bloom episodes, as well as plans for intervention upon intoxications by consumption of toxic seafood demand improvement and more effective coordination.

Marine biotoxins have been catalogued according to the syndromes associated with their toxicoses. These syndromes are: paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), ciguatera fish poisoning (CFP), amnesic shellfish poisoning (ASP), and diarrhetic shellfish poisoning (DSP). Scombroid fish poisoning syndrome has also been recognized as a major problem but it is different in origin, cause and management, than those previously mentioned.

Paralytic Shellfish Poisoning (PSP).

In the United States, the causative agents for PSP syndrome have been identified as *Gonyaulax tamarensis* and *G. catenella*. (Taylor, 1988). These dinoflagellates are producers of a complex family of molecules known as saxitoxins (STX) (fig. 1). PSP is caused by ingestion of bivalves such as mussels, clams, oysters or scallops, exposed to waters contaminated by these dinoflagellates. Bivalves derive their nourishment from plankton filtered from the

surrounding water. By feeding upon toxigenic plankton, the organisms retain and concentrate the toxins produced by the dinoflagellates. PSP is a serious condition

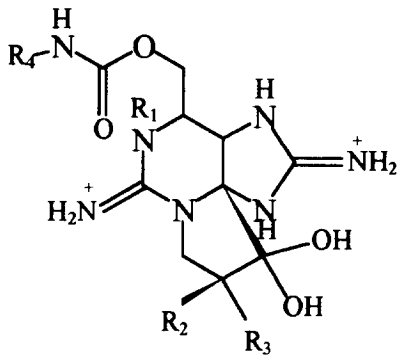


Fig. 1. Basic saxitoxin molecular structure. Various STX types are derived from this molecule; from Baden, 1983.

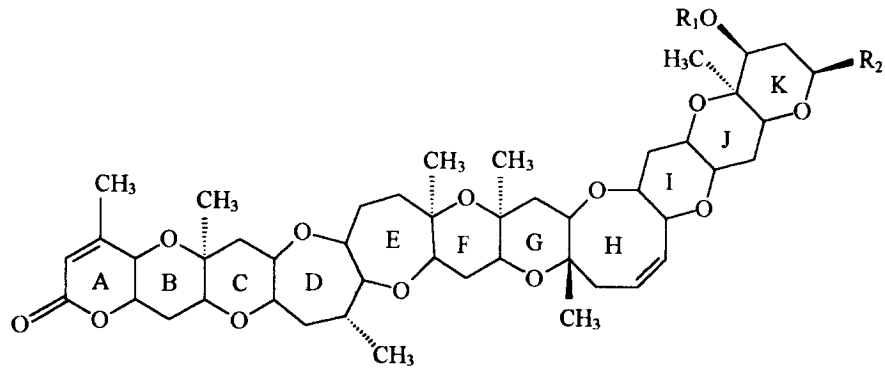
for which no antidote is known. It can be life threatening. Initial symptoms include drowsiness, incoherence, fever, rash, aphasia, tingling and numbness of lips and digits, burning of the perioral area, dry throat and dry skin. Respiratory failure and death occur in more severe cases, all within 24 hr. of intoxication (Ahmed, 1991). In mild cases, symptoms subside after several days and the surviving subjects usually recover fully experiencing no further effects (Anderson *et al.*, 1993). Presently the only available therapy is primarily supportive since, as previously mentioned, there is no antidote for PSP. Shellfish consumers are protected by established monitoring programs coordinated locally. Saxitoxin concentration in clam, mussel oyster and scallop tissues is periodically determined by state labs via FDA sanctioned bioassays. Concentrations greater than 80 μg /100 g of shellfish tissue are sufficient to prompt rapid closure of shellfish beds and suspension of harvesting activities. Beach closures and sampling collection for further analysis is carried out by county agencies (Ahmed, 1991). The structure of saxitoxin has been elucidated and up to 12 different varieties of related toxins

identified (Shantz *et al.*, 1975; Boyer *et al.*, 1978; Shimizu and Hsu, 1981). Potency has been found to vary among these different toxin types (Hall and Reichardt, 1984). Saxitoxins are water soluble molecules, molecular weight 299.30 (Budavari *et al.*, 1989) which act upon the sodium channels of the nerve cell membrane blocking ion passage, effectively depolarizing the cell; transmission of nerve impulses is impeded and muscle movement prevented. Among the species affected figure the California sea mussel *Mytilus californianus*, and the Alaskan butterclam *Saxidomus giganteu* and, unlike with other bivalve species, this latter species has been recognized to be permanently toxic (Ahmed, 1991). PSP affects areas of the Pacific northwest from Alaska to California and the New England areas of the north Atlantic coast.

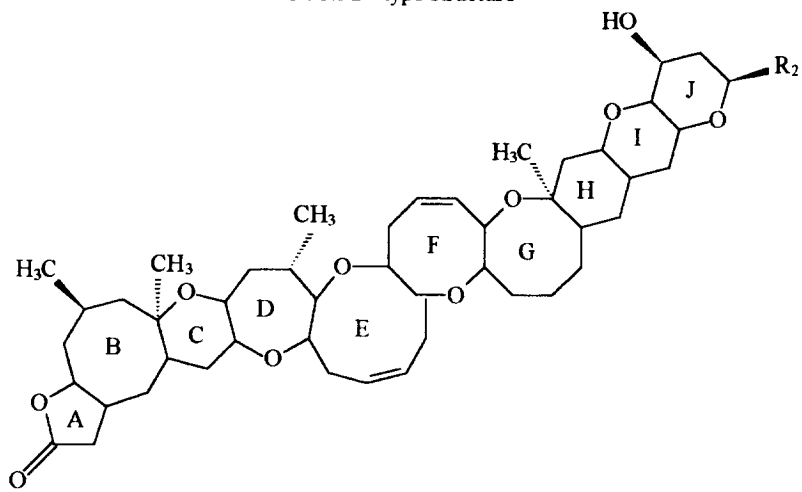
Neurotoxic Shellfish Poisoning (NSP)

NSP is caused by the dinoflagellate *Gymnodinium breve* and primarily affects coasts of the Gulf of Mexico and Florida (Baden *et al.*, 1984). The concentration of these organisms during bloom episodes alters the coloration of seawater to a reddish tone which has elicited the name Red Tide to describe the event. This coloration along with the dramatic spectacle of massive fish fatalities and numerous carcasses littering affected shores provide an evident method of bloom detection (Steidinger *et al.*, 1973). Ingestion of contaminated fin or shellfish affects humans and marine life alike. Toxins produced by *G. breve*, known as brevetoxins, are suspect in episodes of marine mammal intoxication and mortalities (Gerasi, 1989). Brevetoxins are derived from any one of two similar structural backbones formed primarily by as series of 10 or 11 polyether rings (fig. 2). Nine different brevetoxins, based on one of these backbone structures are known to date (Shimizu *et al.*, 1986). Unlike PSP where symptoms are almost

exclusively neurological in nature, NSP presents gastrointestinal symptoms as well. These include muscular aches, tingling and numbness of the lips, tongue, and digits, diarrhea, abdominal pain, temperature reversal sensation, dizziness, anxiety, and sweating (Anderson *et al.*, 1993; Ahmed, 1991). Additionally, bronchoconstriction and generalized asthmatic symptoms are present as a result of the exposure of respiratory membranes to toxic aerosols of the shore area (Music *et al.*, 1973; Pierce *et al.*, 1990).



a. PbTx-2 - type structure



b. PbTx-1 - structure type.

Fig 2. Brevetoxin basic structures. a) PbTx-2 type & b) PbTx-1 type. (from Baden and Trainer, 1994)

Brevetoxins act as depolarizers of the sodium channels on the cell membrane. It has been determined that these neurotoxins reversibly open channels, reducing the effective number of active channels and allowing uncontrolled entry of Na^+ ions into the cell (Strichartz and Castle, 1990; Trainer, 1990; Baden *et al.*, 1993). The net effect of brevetoxin upon cell membrane sodium channels, similarly to saxitoxin and ciguatoxin, leads to nerve impulse transmission failure and it is manifested by muscular paralysis, and respiratory as well as cardiac arrests. Nanomolar and even picomolar concentrations of brevetoxins have been documented as producing discernible effects in vitro (Baden *et al.*, 1993). NSP is reportedly a non-fatal syndrome symptoms of which subside within a few days with complete subject recovery (Ahmed, 1991; McFarren *et al.*, 1965). Currently, NSP management methods are primarily dependent on routine monitoring programs, and *G. breve* cell concentration assessment in the water column of the concerned area. These methods appear to have been sufficient to date in light of the few number of NSP cases reported to the Centers for Disease Control (CDC.) i.e., no cases form 1978 to 1986, and only 5 from 1973 to 1974. During the 1987-1988, North Carolina bloom incidents however, reported cases increased to 48 (Ahmed, 1991). Research efforts are being focused on the development of immunoassay methods of brevetoxin detection. An enzyme based immunoassay has been reported, capable of detecting 0.04-0.4 pM concentrations of brevetoxin in cells and contaminated tissue (Trainer *et al.*, 1991). This test employs antibodies produced against brevetoxins via animal vaccination, and linked to detector enzymes which provide rapid, visible, and measurable results to account for toxin concentration. It is hoped that similar methods, employing radioisotopes or indicator enzymes be made available for routine biotoxin testing, and to all research, management and commercial agencies. Additionally, similarity of

behavior among certain biotoxins such as between brevetoxins and ciguatoxins, may prove this testing methodology applicable to various syndrome detection programs.

Ciguatera Fish Poisoning (CFP).

Ciguatera is a syndrome caused by the ingestion of contaminated fish and differently than NSP the onset of an episode cannot be determined by evident signs such as water discoloration. CFP is closely associated with intricate marine trophic levels. The origin of the toxin, known as ciguatoxin, is believed to be the dinoflagellate *Gambierdiscus toxicus* (Adachi *et al.*, 1977; Bagnis *et al.*, 1980). These phytoplankters grow in association with corals of tropical reefs. The toxin is acquired by fish feeding upon coral tissue and in turn, on toxic dinoflagellates. Ciguatoxin (fig. 3) is carried up the food chain as these fish are consumed by carnivorous predators of the reef ecosystem. Humans become intoxicated by eating these dominant reef fish; grouper, snapper, amberjack, goatfish, jack, barracuda, although not in every reef, may prove to be ciguatoxic. Transport through the food chain magnifies the effects of ciguatoxin and renders the larger, older fish, the most toxic (Ahmed, 1991). Considering that these dominant reef predators have had toxic fish as a part of their diet, it may be assumed that the longer they live, the larger the number of toxic fish consumed and hence the greater the concentration of toxin contained in their bodies. Equating body size with old age, it has become customary to avoid large members of suspect fish species if not altogether (although the commercial sale of barracuda in banned in Dade County Florida, sports fishers of Cuban origin are known to customarily fish and consume only the small sized fish, anecdotally reporting no symptoms or related maladies). Presently there are no means of preventive detection of ciguatoxic fish tissue. It is

only after symptom onset that the suspect tissue has been analyzed and toxin evidence encountered.

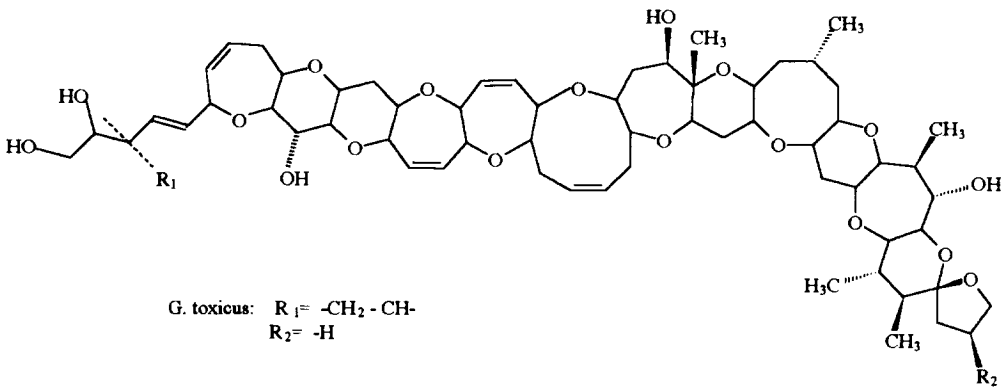


Fig. 3. Ciguatoxin. From G. toxicus, R_1= -CH_2 - CH - & R_2= -H from Baden and Trainer, 1994.

Avoidance practices such as described above are a primary contribution to the preventive management of the problem. CFP developed as a localized syndrome but it has evolved beyond its tropical origins. Aided by increased consumer travel to areas of ciguatera incidence, international trade and lack of widely implemented detection assays, CFP has transcended its endemic tropical reef and island water scenario (mainly Pacific and Caribbean) to the inland and non-tropical communities from where many cases have been reported. Between 1978 and 1987, ciguatera accounted for almost half of all seafood-borne disease reports presented by the CDC; several of these reported cases originated from Washington, Vermont, Louisiana, California, in addition to those from more tropical location such as Florida, Guam, Hawaii, Virgin Islands and Puerto Rico (Ahmed, 1991). Similarly to NSP, CFP symptoms are neurological as well as gastrointestinal. These include abdominal pain, diarrhea, nausea and vomiting shortly after intoxication. Muscle ache, temperature reversal effects, dizziness, itching, sweating, numbness of digits, tongue and lips, follow along with paralysis,

temporary blindness, dry mouth, anxiety, slow irregular pulse and chills. CFP may be lethal. In extreme cases, symptoms culminate in paralysis and death (Anderson *et al.*, 1993; Ahmed, 1991; Hokama, 1988). Symptoms may last from a few days, months, to even years. Cases of long term persistence along with temporary subsidence have been known. CFP symptoms have been reported to reappear apparently brought about by the effects of alcohol, heat or fish consumption. It can be theorized that a previously CFP affected individual, possessing an already low tolerance threshold to ciguatoxin, may have this tolerance lowered further by even small concentrations of the toxin possibly contained in fishfood. This diminished tolerance threshold would elicit the reappearance of symptoms. Given that assays for routine determination of ciguatoxin concentrations are not readily available, consumer information regarding locally known ciguatoxic fish species has been invaluable in the preventive management of CFP. Significant research progress has been made towards the development of a reliable means of ciguatoxin detection. These approaches vary from fish tissue extract preparations to be tested on animal subjects, to immunologically based, enzyme or radioisotope linked assays. The principal obstacle encountered has been cost effectiveness. Efficient assays must be cheap and tough as well as accurate and reliable. A simplified "stick test" has been produced by research teams in Hawaii which has provided successful screening of fish catches (Hokama, 1985). However, this stick test is not cost effective and a high implementation cost renders it impractical; regardless of its degree of effectiveness. As previously mentioned, assays are being developed which, employing immunological principles, allow for the rapid detection of approximately 0.01-0.05 ng. of ciguatoxin on fish tissue (Hokama *et al.*, 1983). Optimization of available testing means is still demanded in order to account for previously non-toxic species, to detect biotoxins exhibiting similar modes of

action and to be made available for the use of various research and monitoring agencies worldwide.

Amnesic Shellfish Poisoning (ASP)

ASP is a syndrome produced by a compound known as domoic acid (fig. 4). This syndrome is considered lethal and active management and research efforts are being implemented to control its incidence and learn more about its forms of action. Previously unknown, ASP has affected areas of Prince Edward Island, Canada, where four people reportedly died after consuming toxic mussels in 1987 (Anderson *et al.*, 1993). Additionally, 103 cases were reported to Canadian authorities during November and December 1988 (Ahmed, 1991). Differently than other biotoxic syndromes which are caused by dinoflagellates, ASP is believed to be caused by a diatom known as *Nitzchia pungens* (Ahmed, 1991).

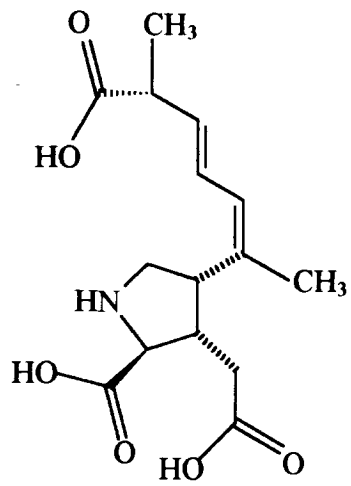


Fig. 4. Domoic Acid structure (Hampson and Wenthold, 1988).

Symptoms include vomiting, diarrhea, abdominal cramps, disorientation, seizures, respiratory problems, short-term memory loss and in severe cases, coma and death (Ahmed 1991; Anderson *et al.*, 1993; Perl *et al.*., 1988 and Teitelbaum *et al.*., 1990). In the majority of the symptoms, ASP behaves not unlike any one of the known biotoxic syndromes; short-term memory loss, persisting over a year in some cases, is however, uniquely characteristic of this affliction. Although presently affecting Canada, ASP should be considered as a potential problem in the United States. Fishery proximity to US. waters as well as water currents, migratory and bloom patterns make ASP a potential threat. Currently, the Canadian government monitors mussel and clam beds for the presence of domoic acid; concentrations of 20 μg/g are sufficient to cause the closure of the beds (Gilgan *et al.*., 1989). However, the presence of domoic acid has been confirmed in previously unsuspected species such as fish and crab viscera (Anderson *et al.*, 1993). Thus, the extent and potential threat to human health at the fishery industry at large has yet to be clearly determined.

Diarrhetic Shellfish Poisoning (DSP).

As its name implies, DSP in a syndrome producing primarily gastrointestinal complications. The symptoms manifest rapidly, from 30 minutes to a few hours after consumption of toxic shellfish (Yasumoto and Murata, 1990; Yasumoto *et al.*., 1984) and may last up to three days. At this time the majority of the symptoms have subsided and the afflicted individual recovers without any presently known further complications (Anderson *et al.*., 1993). Toxic vectors for DSP are mussels, scallops and clams. These become poisonous after feeding in waters containing blooms of *Dinophysis fortii, D. acuminata or Prorocentrum sp.* (Edler and Hageltorn, 1990; Yasumoto and Murata, 1990). Several toxigenic agents for DSP have been identified; from these Okadaic acid (fig. 5) has been

most commonly found in afflicted areas of Europe and Japan (Yasumoto and Murata, 1990). Until now there have been no reported cases of DSP in the United States and only one in Canada in 1990. DSP symptomology can easily be confused with and treated as GI complications of various origins. Often, DSP does not demand medical intervention and cases may go unreported to the respective disease control organizations. An accurate assessment of the numbers of people afflicted by DSP is therefore difficult to obtain. Preventive measures extend thus far to the monitoring of imported goods from afflicted areas via memoranda of understanding (MOU); agreements by which high levels of quality control are met by compromising trading nations. Necessity demands however that the current methods for DSP detection (mouse bioassay) be optimized and improved, and that educational campaigns of consumers as well as medical personnel regarding DSP symptom recognition and reporting be implemented. Present situation and current needs of the biotoxin dilemma will be mentioned later in this chapter.

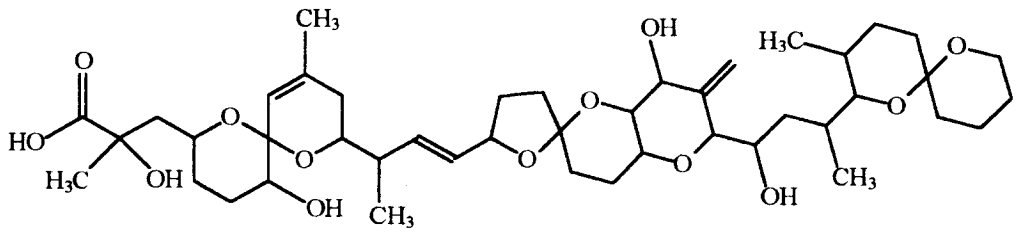


Fig. 5. Okadaic acid structure (Tachibana *et al.*, 1981).

Scombroid Fish Poisoning.

As previously mentioned, scombroid fish poisoning is different in origin, management and prevention than other known biotoxic syndromes. SFP or

histamine poisoning is caused by bacterial action due to the improper handling and preservation of harvested fish stock (Taylor 1986). Upon capture, fish must be refrigerated to prevent the development of bacterial colonies. When allowed to remain at temperatures greater that 15 °C for extended periods of time, bacterial cultures multiply producing histidine decarboxylase (Ahmed, 1991; FDA 1994). This enzyme breaks down histidine in the fish tissue and produces histamine which, in high concentrations, leads to toxic symptoms and allergenic reactions. Scombroid fish poisoning symptoms are generally mild and self resolving. Symptoms include nausea, diarrhea, vomiting, headache, swelling, cramping, palpitations, rash, itching, tingling and burning sensations. Subsidence occurs within a few hours and patients may additionally benefit from the relief provided by antihistamine drugs. Implicated bacteria are *Clostridium, Lactobacillus, Morganella morganii, Hafnia alvei, Klebsiella pneumoniae,* among others (Taylor 1986; Havelka, 1967; Kawabata *et al.*, 1956 and Taylor *et al.*, 1979). Determination of the quality or of the degree of spoilage of certain fish is possible by measuring the concentration of histamine in the fish tissue (Ahmed, 1991). This consideration varies from fish to fish according to the quantity of histidine naturally occurring in its flesh. The Food and Drug Administration has determined legal limits of spoilage for tuna at 20 mg histamine per 100 g of tissue and considers 50 mg/100 g hazardous (Federal Register, 1982). Management approach is based on education of the consumer as to avoidance of fish exhibiting signs of spoilage (fishy smell, cloudy eyes, discolored gills, slimy film), training of the harvesting operator (preservation of freshness by immediate storage of fish at temperatures below 5 °C or frozen) and of the medical practitioner, to consider the possibility of SFP by recognition and awareness of the signs.

REGULATORY AND MANAGEMENT EFFORTS.

The biotoxin dilemma affects all areas of seafood industry, health, natural resource management and regulation, and commercial as well as recreational fishing. This dilemma continues to increase. Clear understanding of the biology, ecology, potential dislocations to affected communities will provide the resources for efficient management both upon onset and preventive for dealing with these natural events. To that end, a marine biotoxin national plan has been developed based upon recommendations made by an expert panel convened in a workshop in Charleston, South Carolina, in the spring of 1992. The workshop brought together experts from involved areas of science, marine biotoxin and harmful algae, seafood safety, resource management, and public health. Dr. Daniel Baden, principal investigator of the laboratory where the research described herein was carried out, was a workshop participant. The national goal was described as the *"effective management of fisheries, public health, and ecosystem problems related to marine biotoxins and harmful algae"* (Anderson *et al.*, 1993). The workshop served to identify obstacles to the development of an action plan and impediments to the effective research and management of marine biotoxins. Lack of information regarding toxic bloom dynamics, species identification, physiology and biology, natural versus anthropogenic influences on the algae bloom, population size and bloom longevity, species interrelations, bloom composition, toxin production and its regulatory processes pertain to the harmful algae proper. Regarding the affected species, minimal information is available; from effects upon physiology, changes with growth stages, short as well as long term fisheries effects of toxin exposure, toxin modification upon incorporation by the fish or shellfish, effects on recruiting, and toxin movements through the food web, only to mention a few obstacles. Public health considerations regarding best consumer protection strategies, variations of these strategies according to toxic agent, sampling

programs and stock monitoring plans, education and information dissemination to medical and health personnel, managers, students, fishery workers and sports fishermen also need immediate attention. Finally, knowledge regarding the biotoxins themselves is limited. Molecular pharmacology needs further research and funding to enhance present understanding. Handling protocols must be implemented and those available modified to insure toxic sample preservation. Toxin depuration (elimination) processes need to better understood and laboratory cultures of toxigenic species demand more capital for better care and maximum toxin extraction potential. Several obstacles were found to be common to all areas of biotoxin management: lack of database distribution nets, lack of readily available, reliable standards, and sensitive, cost productive assays for shipboard or dock use. Assay development is a primary concern since the detection means will serve as tools for ease of implementation of a demanded Hazard Analysis and Critical Control Point (HACCP) action plan. This report documents research regarding the development or modification of a toxin assay possessing the demanded ease of operation, reliability, cost effectiveness and sensitivity which will greatly contribute to the effective management of marine biotoxins and harmful algae. Such a monitoring tool will allow for the implementation of sound management and preventive measures with a minimum degree of dislocation to those depending of the seas for subsistence. As previously mentioned, the development and adoption of international minimum safety standards will not only facilitate trade among nations, but will additionally provide a scientific basis of detection for implementing sanctions upon failure to meet agreements based on memoranda of understanding, and become an intricate part of the biotoxin management protocols following the suggested and recently made available as a FDA guide based on the Hazard Analysis and Critical Control Point (HACCP)

protocols. These plans provide means of management and regulation compliance by detailing how to proceed when dealing with potentially marine biotoxic foods.

Hazard Analysis and Critical Control Point (HACCP).

Originally pioneered by the Pillsbury food Company in the early 1960's as an effort to provide contaminant-free food for the space program, the concept of hazard analysis and critical control points was first introduced during the Conference on Food Protection in 1971 and has served as a preventive management system for food safety. Since then, modifications and expansions to include biological, chemical and physical hazards have been made. The original format has been changed based upon the recommendations of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) and published as a pamphlet in 1989. Modifications made included considerations regarding HACCP principles, definitions and expanded hazards as mentioned previously. In 1991, HACCP principles and applications were revised by the Codex Committee on Food Hygiene HACCP Drafting Group, and the revised document was approved on March 20, 1992 by NACMCF. The present HACCP format is based on the following seven applicability principles:

1. *Hazard analysis* concerns primarily identification of the hazard presented by a product, moment of hazard onset, and conditions for hazard development

2. *Critical Control Points* serve to locate the step(s) where the product processing system may allow for development of a hazardous condition. Identification of this critical control point provides the possibility of hazard mitigation and preventive management.

3. *Critical Limits* are developed as guideline standards beyond which the hazard condition is prevalent and processor control measures must be executed. Critical limits thus serve to gauge the efficiency of the control points and allow to determine if there is a need for their modification.

4. *Monitoring* is deemed necessary to ensure the control of the potentially hazardous situation. This may be achieved by routinely verifying the system's compliance with the critical limits. Monitoring should be performed as often as demanded by the nature of the perceived hazard.

5. *Corrective Action* is presented as the necessary series of steps to follow in order to safely manage potentially hazardous foods. These actions are customized to and vary depending on the specifics of the foods and the existing circumstances.

6. *Record keeping* is necessary to document compliance with the established plans and to provide a source of control of the critical points. Additionally, specific records, varying as the food type demands it, allow for quality assurance at the provider, processor and distributor levels.

7. *Verification* of the efficiency and applicability of the HACCP plans are needed to allow for the demanded flexibility of the food industry. A feedback system based on four steps reviews critical limits, verifying adequacy of control; (1) facility's HACCP plan effective implementation, including (2) timely compliance with the plan's procedures; (3) revalidation of the HACCP plan to account for developing needs as a result of the business growth and modifications; and finally, (4) government supervision, insuring regulatory compliance and system modification as merited.

The HACCP system has proven versatile enough to accept demanding modifications originated from the changing needs of the food industry and the

continuing scientific and technological advancements. The present situation regarding marine biotoxins is a clear example.

Hazards and Controls.

On February 16, 1994, the Food and Drug Administration presented to the public its *Fish and Fishery Products Hazards and Control Guide* for revision and commentary until April 28, 1994. The prepared guide resulted from the proposed regulations introduced in the Federal Register on January 28, of the same year. The Federal Government calls for the implementation of HACCP regulations for fish and fishery products by January 28, 1995 and HACCP-based programs in place in all fishery product processing facilities by January 28, 1996. FDA identified natural toxins as a species-related hazard and in this guide, proposed a plan for product handling based on the HACCP format. In accordance with the 7 principles of HACCP, the FDA guide presents a hazard statement identifying marine biotoxins as a threat to health when humans consume the shellfish or finfish. The critical control point when dealing with seafood and biotoxins is at the point of receiving the product shipment. At this moment it is imperative to determine the source of the available product and whether the product is finfish or shellfish. Four different options for management are presented then depending on this knowledge. Categorization has become the best approach when dealing with seafood. Presently, the primary consideration for segregation is the type of fish involved. The product is separated as shellfish or finfish; if molluscan shellfish, whether 1) received shucked or 2) not shucked; if finfish, 3) whether received directly from the fisher or supplier who has credible knowledge of the harvest areas or 4) received from someone other than the supplier or fisher who has credible knowledge of the harvest area. In any case, knowledge of the harvest area,

whether open for harvest or closed by Federal, State, foreign or local government agency due to biotoxin occurrence, is most important. The separation between fin and shellfish serves to facilitate management, since there are syndromes thus far perceived only in finfish as is the case with ciguatera fish poisoning.

Molluscan shellfish received in shell need to be identified as to area of origin from the harvester's tagged containers for each lot or batch. Upon receipt also, the harvester's commercial fishing license number must be checked or the dealer's certification number compared against the latest edition of the Interstate Certified Shellfish Shippers List (ICSSL) to verify certification. If this is not available, or not listed, then the certification is checked against the State Shellfish Control Authority (SSCA). If delivered by a fisher, the condition of the harvest area must be verified to determine that it is not closed due to natural toxins. This information may be obtained from the SSCA. Fish originating from a closed area must be rejected. Rejection is also mandated if no information can be ascertained as to the condition of the harvest area (improperly tagged containers), lack of dealer's certification or commercial fishing license. These verifications must be made for each lot or batch when determining origin, licenses and certifications, and harvest area closure. Further, the recipient is encouraged to check for harvest area closure as often as necessary to assure accuracy. Shellfish is to be rejected each time the lot or batch does not meet the critical limits. The critical limits determine that shellfish be rejected if originating from an area closed by federal, foreign, state or local health authorities due to natural toxins occurrence. Shellfish shall also be turned down if provided by an unlicensed or uncertified processor or harvester. Additionally, if shellfish containers are improperly tagged, the FDA recommends they be refused as well. A proper tag should include at least the date and location of harvest by State and site, type and quantity of fish. As to the harvester, the tag should include the name, address and assigned SSCA number.

For bulk shipments, where individual container tags may not be available, the previous information should be contained in the bill of lading. All this information can serve as records of the proper receipt of inshell shellfish. Control action for product exceeding the critical control limits warrants its destruction or recall.

Molluscan shellfish received shucked are controlled by determination of the name, address and certification number of the last processor of the product. This may be obtained from the properly tagged containers. A valid shipper's certification must be presented and compared to the list from the ICSSL. Again, if not listed, it is acceptable to check the certification from the SSCA. Failure to accurately produce the required information will result in the rejection of the shipment. Certification number and processor certification must be checked for every batch or lot; each lot not certified is to be rejected if not meeting the critical limits. Limits are determined from the certification validity. No molluscan shellfish shall be accepted if delivered from an uncertified processor, or if packed in improperly tagged containers, i.e., those without the last processor's name, address or certification number. The action to be taken in rejection case is to destroy or recall the product not meeting the critical control limits. Records of properly received shucked shellfish may be obtained from a proper tag.

Fish product, other than molluscan, received from the fisher or supplier directly, and possessing a clear knowledge regarding the harvesting area, or aquacultured fish are the third option for control considerations. As a control measure the receiver must determine the location of each lot or batch, determine whether this area was open or closed to harvesting due to natural biotoxins. As before, product originating from a closed area is to be rejected. Puffer fish, previously banned for import, are now to be rejected if imported without FDA permission. Puffer fish have long been recognized as a source of tetrodotoxin (TTX), and are currently the topic if extensive scientific research. Source of

origin and whether closed or open to harvesting, is to be determined for every lot or batch. Frequency for rejecting fish is to be every one fish which does not meet with the critical control limits. These critical limits are the refusal of every fish originating for areas closed by foreign, federal, state or local agencies due to natural toxin occurrence and rejection of all puffer fish not imported with the sanction of the FDA. As with other options, corrective action is the destruction or recall of failed product. The receiving agent shall keep record for each lot or batch showing harvest area. Receipts and tags from the supplier or aquaculture outfit may be sufficient provided the open area of harvest is shown. For product obtained from the open ocean, record showing harvest area determined by latitude and longitude may be required.

When fish product, other than molluscan shellfish, is received from someone other than the fisher or supplier, and the harvest area condition is questionable, periodical monitoring of the incoming product is necessary to determine toxin presence. Monitoring is to be performed via approved standard tests and should include testing for the presence of STX, OA, DTX-1, PbTx, and DA. Additionally, sampling should cover all available suppliers at several intervals during the year. Product failing the critical limits are to be rejected unless convincing evidence can be provided that only open harvest areas were used. Monitoring of each supplier's product shall be performed three time per year, rejecting batch or lot not meeting with the critical control limits. The limits in this case are determined according to the nature of the biotoxin and based on available scientific information such as minimum hazardous dosage tests, toxin residence times and other physical and chemical characteristics. Each limit varies then depending on the potency, permanence, and toxin type. Limits have thus far been determined for the following biotoxins:

DSP = 0.2 ppm (parts per million) okadaic acid plus DTX-1

NSP = 0.8 ppm PbTx-2 (brevetoxin 2).

PSP = 0.8 ppm saxitoxin

ASP = 30 ppm domoic acid in dungeness crab viscera and
20 ppm in other tissues and other species

Regarding tetrodotoxin, the critical control limit is determined by the FDA import approval. No limit reference is made with respect to toxin presence monitoring and, considering the high toxic effects of TTX, clarification regarding the criteria for import sanction should be made available. Monitoring should be performed for every batch or lot and test results may be retained as records of regulation compliance. As per the FDA guide, the corrective action once again, involves destruction or recall of the product failing the critical control limits. Ciguatera toxin evidence in any sample is to be controlled via notification in addition to sample recall or destruction. Presence determined via mouse bioassay is to be reported to the FDA's Office of Seafood in Washington, D.C. Consequently, local health authorities will be notified for mitigation efforts and proper action.

As additional corrective action in each critical limit excess case, the FDA's guide recommends HACCP or process evaluation to determine whether there is a need for modification, in order to reduce present and future risks and to provide appropriate mitigation action.

The FDA also considers histamine or scombroid fish poisoning a syndrome of different origin and demanding particularly specific management strategies. SFP is caused by the consumption of fish contaminated with bacteria capable of converting histidine into histamine via the enzyme histidine decarboxylase. These bacteria have been identified as *Morganella morganii, Clostridium perfringens, Klebsiella pneumonia, Vibrio alginlycticus* and *Hafnia alvei* as previously

mentioned. The decarboxylation of histidine may occur with or without organoleptic evidence of decomposition (FDA, 1994) thus, the implicated fish product must be inspected for presence of both. Temperatures greater than 15 °C, and conducive to the development of these bacterial cultures should be avoided in order to effectively manage the potential hazard of intoxication. As with other seafoods, the critical control point is upon receiving. When suspect species product is received directly from the harvest vessel the control measure consists on determination of the storage and handling temperatures for each lot or batch on board the vessel; records of the vessel's operation may be obtained to this end. These records must document the method of fishing and handling practices once the product is onboard. When these records are not available, it becomes necessary to assay a representative sample from every lot for histamine content. Additionally, a representative sample from each lot is organoleptically analyzed for signs of decomposition. According to the FDA guide, the sample must provide at least 95% confidence that the lot is not more than 2.5% spoiled (FDA, 1994). Further, if the total number of fish caught allows it, efforts should be made to sample every fish in the lot. As mentioned previously, all this applies upon receipt. Should the product arrive frozen, it may be necessary to delay inspection until the lot is thawed or until stored. Every individual exhibiting organoleptically detectable signs of decomposition shall be rejected.

When the product is received iced or frozen, internal temperature records must be obtained. A representative sample's internal temperature should be assessed using a National Institute of Standards and Technology (NIST) thermometer or equivalent. This thermometer is to be periodically calibrated to ensure accuracy; once when purchased and at least once per year thereafter. Every lot failing to meet internal temperature critical limits is rejected. Enough ice should be found as well, around, on top and underneath the fish. Product should be

rejected if lack of ice or other refrigeration means evidence non-compliance with the critical limits. Scombroid poisoning suspect fish product must be promptly brought to a temperature beneath 4.4 °C and maintained there until just before eating. The critical limits demand that every fish not immediately brought to and maintained at 4.4 °C or below be discarded. No fish possessing an internal temperature greater than 4.4 °C shall be accepted. Additionally, when ice is used as the only means for refrigeration, lots without ice around and above the fish shall be denied. Sample assays should reveal no greater than 2.5% decomposition and less than 5 mg histamine per 100 g of fish flesh or 20 mg histamine per 100 g of canned flesh (FDA, 1994). All fish failing to meet the critical limits will be rejected. Critical limits further demand that the thermometers used agree within 1 °C of the NIST calibrating thermometers. Fish processors are allowed to trim the products' portions found to be spoiled or decomposed as a corrective action. The trimmed portions are assayed for further signs of spoilage and to make certain they meet the critical limits. A representative sample from all product under suspicion of histamine development is collected and assayed for content. All samples exhibiting more than 2.5% organoleptically detected decomposition are collected and also analyzed for histamine content. Every sample presenting histamine concentrations greater than the critical limits are discarded. Samples exhibiting histamine content are to be immediately cooked, frozen or rejected. Cumulative exposure to temperatures greater than 4.4 °C shall not exceed 4 hours. If evidence exists to the contrary, the entire lot should be inspected or discarded. Finally, the thermometers used to determine internal product temperature are to be maintained and repaired as needed or discarded and replaced if irreparable.

Vessel temperature logs and refrigeration equipment maintenance records may serve as the processor's compliance records. These records may serve as control instead of product internal temperatures when a relationship between

cooling storage and internal temperatures of product have been established (FDA, 1994). Continued optimal technical and mechanical equipment performance, in accordance to manufacture's specifications, facilitates the process of management and control of this scombroid fishery problem. Additionally, histamine testing analytical results may be kept as record of compliance, thermometer calibration performance records and organoleptic test result forms may be retained for this purpose as well.

When the raw product is obtained from a supplier other than the harvest vessel and when the processor does not store the raw product it is necessary to examine each lot for decomposition. Similarly to the previous case, the sample assayed should provide greater that 95% certainty that the organoleptically assayed lot or batch is less than 2.5% decomposed. Same provisions for frozen raw material when received apply. It may be possible to test a sample when the lot is thawed or stored. Every lot or batch is assayed and every individual showing organoleptic signs of decomposition is discarded. The critical limits are determined as 2.5% decomposition; every lot showing decomposition greater than this value is discarded. Histamine as before is to be less than 5 mg/100 g flesh or 20 mg/100g canned flesh. Every fish failing to meet these limits may be trimmed to eliminate the decomposed portions and then be assayed again to make certain the limits are met. All lots exhibiting greater than 2.5% decomposition are assayed for histamine content via representative sample and all product in the lot is rejected if histamine concentrations are returned above 5 mg / 100 g flesh. All remaining product presenting signs of histamine are immediately cooked, frozen or rejected. Records of the sensory assays are retained as evidence of compliance as well as any documents about the corrective means and actions performed. These records are recommended to include final sensory analysis and histamine concentration results. As in the case of biotoxin HACCP management, every

departure from the critical control point demands that the system be inspected for improvement needs and that this be done in a routine manner to determine demanded follow up action.

PART II

BIOCHEMICAL ANALYSIS

Routine monitoring of a biotoxin prone area and assays of fish samples suspected to contain biotoxins must be performed in order to better manage and mitigate possible toxic bloom situations. Sampling methodology and assay procedure are critical for this efficient management. The applicable assays must not only specifically detect the toxin in question but must also be reliable, easy to perform, preferentially field worthy, quick, sufficiently sensitive and cost effective. Available mouse bioassay assays demand a laboratory setting and employ a large amount of precious toxic tissue. These assays allow for toxin detection as needed but carry a high price tag when used for routine monitoring purposes. Toxin presence can also be determined biochemically. By processing available tissue from suspect species and purifying the biotoxins from these tissues via various biochemical techniques it is possible to monitor toxin exposure or presence in the area of the sample's origin. Additionally, immunological tests are being developed and currently optimized for the routine sampling of biotoxins. Tests under development promise greater ease of operation, reliability, speed, resolution and field worthiness. Biochemical assays were compared against immunologically based ELISA methods during a portion of this internship. The purpose was to comparatively determine better candidate for routine monitoring tasks.

During 1993, 70 samples were received from MOTE Marine Laboratory, Sarasota, Florida and 19 samples from Florida Department of Natural Resources (FLDRN), St. Petersburg, Florida; partially as a collaborative effort in the monitoring of brevetoxin presence in the Gulf of Mexico. Samples included trout, mullet, menhaden, herring, sardines, clams, and oysters from specific areas of the gulf; all were recorded

and samples listed as received. All samples were processed according to the following protocol:

MATERIALS AND METHODS

I. Crude Extract Preparation.

a. Weigh in.

Shellfish samples were weighed whole, both with and without shell. Oysters were weighed in clumped samples of 10 individuals. Finfish were weighed whole and the entire organism used when small in size, e.g., thread herring and sardine. Large size individuals were dissected according to fig. 6, and the collected viscera and muscle samples processed. Several samples were received already eviscerated. In these cases the separate viscera containers belonging to a single individual were combined and processed.

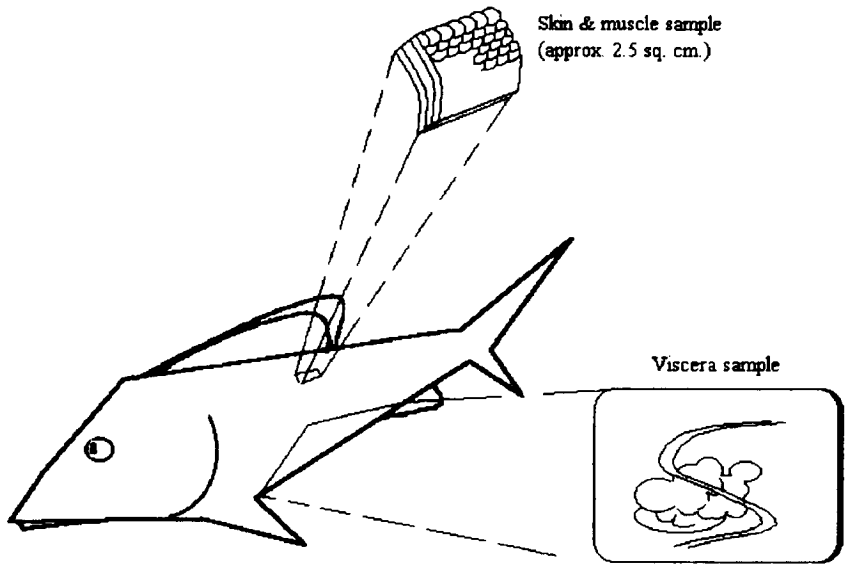


Fig. 6. Fish dissection pattern
for tissue sample collection

b. Soaking.

Samples were placed in a 250 ml beaker and allowed to soak completely submerged in acetone, for 24 hours.

c. Homogenizing and filtering.

Soaked tissues were blended using a Virtis, 45" tissue homogenizer (Virtis, Gardiner, N.Y., 12525) in the same acetone solution. They were then filtered through a #11, 1", Whatman filter paper, Buchner funnel and vacuum Erlenmeyer flask. The dried filtered cake was discarded and the filtrate retained for evaporation.

d. Sample Concentration and Crude Extract Final Preparation.

Filtrate was dried using a rotary evaporator and a water bath at a temperature no greater than 30 °C. The samples were dried until all traces of acetone were removed.

II. Crude Extract Bioassay.

Dried sample was brought up in 10 ml MeOH. Half a milliliter of this was added to a beaker containing 20 ml of water and 1 mosquito fish (*Gambusia affinnis*). Duplicates from each inoculation were run. Bioassays were allowed to proceed for 24 hours. Samples resulting in a negative bioassay for both duplicates were discarded.

III. Column and Thin-layer Chromatography.

Positive samples were completely absorbed in silica gel (ICN Adsorbentien, TSC, DCC, CCS, 60A., Si-gel., ICN Biochemicals, Inc., Costa Mesa, CA.). Enough gel was used to provide a soupy appearance to the solution (approx. 15-20 g.). The soaked

sample was loaded onto a glass column and developed using a mobile solution of 100:10:1, Cloroform:methanol:acetic acid. Collected fractions were each loaded onto a 1000 μm thin-layer chromatography plate (Analtech, Inc., Newark, N.J.) and developed once using 70:30, pet ether:acetone as developing solution. Developed plates were sectioned into 7 to 10 1 cm wide fraction bands and bioassayed.

a. Bioassay of TLC fractions.

A one cm^2 was scrapped from each fraction band, ground and added to a beaker containing 20 ml of water and 1 mosquito fish. As before, bioassays were performed in duplicate and allowed to proceed for 24 hours. Positive bioassay bands from each plate were scrapped, eluted using acetone and dried in a rotoevaporator as described before. TLC plates and accompanying bioassays were repeated as needed for samples which proved to be too oily for HPLC procedures.

IV. High Pressure Liquid Chromatography.

Dried positive samples were further purified via HPLC using a C-18, reverse phase column, 85%MeOH:15%dH_2O mobile phase and an ultraviolet detector at 215 nm wavelength. Purification was carried over a large volume preparatory column (0.5 ml. capacity) and then through an analytical column (0.1 ml volume capacity). Finally, toxin presence was determined by reference comparison to known brevetoxin standards, according to peak appearance and delay times.

RESULTS.

Florida DNR samples rendered no positive final bioassays. From all 19 sent, only 6 samples resulted positive after the initial bioassay. The positive samples, scaled

sardines, thread herring, mullet (x2), striped mullet, and trout, showed no positive bands after the first TLC. No brevetoxin was detected in the available samples as discernible by this assay.

MOTE Marine Laboratory's 70 samples produced 27 positive initial bioassays. From these, only 10 resulted positive after the first TLC bioassay. After assaying separate fractions there was only one sample (#22, menhaden) whose first TLC fraction 12 rendered 6 positive results. None of these last 6 fractions showed a brevetoxic peak when assayed by HPLC. A summary of the results is presented in the following table #1.

Table 1. Sample lists from a)Florida Department of Natural Resources and b)MOTE Marine Laboratory.

Florida Marine Research Institute Samples.

Sample #	Sample type	Collection area	Initial Bioassay	1st TLC Bioassay	2nd TLC Bioassay	HPLC Analysis
1	sardines	Unknown	(-)	----------	----------	----------
2	clams	N. Skyway rest area	(-)	----------	----------	----------
3	oysters	N. Skyway rest area	(-)	----------	----------	----------
4	scaled sardines	unknown	(+)	(-)	----------	----------
5	thread herring	unknown	(+)	(-)	----------	----------
6	mullet	Caloosahatchee	(+)	(-)	----------	----------
7	mullet	Charlotte Harbor	(-)	----------	----------	----------
8	clams	N. Skyway rest area	(-)	----------	----------	----------
9	oysters	N. Skyway rest area	(-)	----------	----------	----------
10	clams	N. Skyway rest area	(-)	----------	----------	----------
11	oysters	N. Skyway rest area	(-)	----------	----------	----------
12	stripped mullet	Riviera Bay	(+)	(-)	----------	----------
13	mullet	Manatee River	(-)	----------	----------	----------
14	trout	Cryptic	(+)	(-)	----------	----------
15	oysters	N. Skyway rest area	(-)	----------	----------	----------
16	clams	N. Skyway rest area	(-)	----------	----------	----------
17	mullet	Pine Island	(-)	----------	----------	----------
18	mullet	Coffee Pot Bayou	(-)	----------	----------	----------
19	stripped mullet	Manatee River	(+)	(-)	----------	----------

Mote Marine Laboratory Samples								
Sample #	Collection date	Sample weight (g.)	Sample type	Initial Bioassay	1st TLC Bioassay	Fraction Bioassay	2nd TLC Bioassay	HPLC Analysis
1	4/16/93	55.4	trout	(-)				
2	4/16/93	67.3	clam	(-)				
3	4/15/93	66.4	mullet	(-)				
4	8/3/92	63.5	mullet (visc.)	(-)				
5	8/3/92	104.6	trout (visc.)	(-)				
6	1/11/93	32.6	menhaden (visc.)	(+)	(-)			
7	1/11/93	93.1	trout (visc.)	(+)	(-)			
8	1/6/93	124.4	mullet (visc.)	(-)				
9	1/8/93	99.2	trout (speckled).	(-)				
10	1/8/93	112	menhaden	(+)	(-)			
11	11/13/92	96.4	trout (visc.)	(-)				
12	11/13/92	89.3	mullet	(+)	(-)			
13	11/13/92	75.9	mullet (visc.)	(+)	(+) fraction # 9	(-)		
14	11/9/92	77.1	calm	(-)				
15	11/9/92	32.2	jack	(-)				
16	11/6/93	34.8	menhaden (visc.)	(-)				
17	11/6/92	65.6	mullet	(+)	(-)			
18	12/7/93	102.3	mullet (visc.)	(-)				
19	12/7/92	127	mullet	(+)	(-)			
20	9/11/92	61.5	menhaden (visc.)	(+)	(-)			
21	9/11/92	25.7	menhaden	(+)	(-)			
22	11/6/92	30.2	menhaden	(+)	(+) fraction # 12	(+)	(+) fraction # 3,4,6,7,8,9	undetectable
23	9/24/92	49.8	calm	(-)				
24	9/24/92	66.1	mullet (visc.)	(+)	(-)			
25	9/24/92	47.3	mullet	(+)	(+) fraction # 11	(-)		
26	9/24/92	37.7	menhaden (visc.)	(-)				
27	9/24/92	27.6	menhaden	(+)	(-)			
28	9/18/92	68.6	calm	(-)				
29	9/15/92	107.6	trout (visc.)	(-)				
30	9/15/92	34.8	menhaden (visc.)	(+)	(-)			
31	9/15/92	98.2	mullet	(+)	(+) fraction # 2	(-)		
32	9/15/92	34	menhaden	(+)	(-)			

Table #1. Continued.

Mote Marine Laboratory Samples					(cont'd.)			
Sample #	Collection date	Sample weight (g.)	Sample type	Initial Bioassay	1st TLC Bioassay	Fraction Bioassay	2nd TLC Bioassay	HPLC Analysis
33	9/15/92	67.9	trout	(-)				
34	9/10/92	88.5	mullet	(+)	(+) fraction # 5,6	(-)		
35	9/10/92	45.7	mullet (visc.)	(-)				
36	9/9/92	36.8	mullet (visc.)	(+)	(-)			
37	9/9/92	79.3	mullet	(+)	(+) fraction # 8	(-)		
38	9/8/92	45.7	clam	(-)				
39	9/1/92	45.3	mullet (visc.)	(-)				
40	9/1/92	32.1	menhaden (visc.)	(-)				
41	9/1/92	77.8	mullet	(+)	(-)			
42	9/1/92	48.9	menhaden	(+)	(-)			
43	10/14/92	57.9	menhaden (visc.)	(-)				
44	10/14/92	30.3	menhaden	(+)	(+) fraction # 7	(-)		
45	10/14/92	37.6	trout	(+)	(-)			
46	10/14/92	78	trout (visc.)	(-)				
47	10/6/92	50.1	clam	(-)				
48	10/6/92	55.8	mullet	(+)	(-)			
49	8/6/92	47.4	menhaden (visc.)	(-)				
50	8/6/92	43.1	trout	(-)				
51	8/6/92	21.3	menhaden	(-)				
52	8/4/92	56.7	clam	(-)				
53	6/15/92	17.9	menhaden (visc.)	(-)				
54	6/4/92	1.1	trout (visc.)	(-)				
55	6/4/92	4.22	mullet (visc.)	(+)	(+) fraction # 7	(-)		
56	6/4/92	47.2	mullet	(-)				
57	6/4/92	50.2	trout	(-)				
58	6/4/92	71.6	clam	(-)				
59	8/3/92	52.6	mullet	(+)	(+) fraction # 4,5	(-)		
60	8/3/92	53.4	trout	(-)				
61	7/2/92	26.3	menhaden	(-)				
62	7/2/92	49.9	jack (visc.)	(-)				
63	7/2/92	169	trout (visc.)	(-)				
64	7/2/92	44.1	trout	(-)				

Table #1. Continued.

Mote Marine Laboratory Samples					(cont'd.)			
Sample #	Collection date	Sample weight (g.)	Sample type	Initial Bioassay	1st TLC Bioassay	Fraction Bioassay	2nd TLC Bioassay	HPLC Analysis
65	7/2/92	27.7	menhaden	(+)	(+) fraction # 3	(-)		
66	7/2/92	80.8	menhaden (visc.)	(-)				
67	7/1/92	118.5	trout (visc.)	(-)				
68	7/1/92	106	mullet (visc.)	(-)				
69	7/1/92	86.3	clam	(-)				
70	7/1/92	52.5	mullet	(-)				

Table #1. Continued.

DISCUSSION AND CONCLUSION.

The pharmacological analysis for the presence of brevetoxins although efficient, is time consuming, demands the use of costly equipment and is compromised by an exclusive laboratory setting. This type of analysis cannot be performed at sea or by the dockside. The time cost of the assay is also great. In order to process all 89 samples it was necessary to spend more that 3 months of constant laboratory work, demanding the expertise of 2 technical specialists. Assay time demanded by one sample alone was greater than 4 days (1 day soaking, 2 or more days in bioassays, at least 1/2 day of HPLC depending on positive fractions available and 1/2 day more in other technical and preparatory work). By reasons of time and expense alone the immunological approach is superior to this assay method not mentioning its demand for small amount of sample tissue, potential to be optimized to a field worthy assay, high sensitivity and comparative lower cost of implementation. An immunological microtiter assay such as ELISA can be performed by one operator, demanding little technical experience when compared to the biochemical analysis approach or even to the radioisotope-based IRA. Further efforts to its optimization and to the development of similarly based alternate approaches should be funded and encouraged.

PART III.

IMMUNOLOGICAL ANALYSIS

A. MATERIALS AND METHODS

I. Toxigenic Organisms

Gymmnodinium breve cells were obtained from cultures maintained in the laboratory under continuos artificial illumination (4000 lux intensity, cool- white, fluorescent studio lights), and grown in artificial media prepared similarly to the Wilson NH-15 protocol as follows:

For 10 liter batches, salinity was adjusted to 34 ppt. by addition of , 291.75 grams of NaCl; 7.37 grams of KCl; 52.25 grams of $MgCl_2$; 62.50 grams of $MgSO_4$ and 9.5 grams of $CaCl_2$. Ten milliliters of potassium phosphate, potassium nitrate, EDTA, vitamin B-12, biotin, vitamin-8, and adenine sulfate 1.0 % solutions were then added. Forty milliliters of 10 % Tris (pH 8.3), 50 ml. of sulfide stock solution and 50 ml. of trace metals preparation were finally included. Each 10 liter carbouy was then half emptied into a second and all were autoclaved using an AMSCO General Purpose High Speed Autoclave at a temperature greater than 121 ^{o}C for 1 hour. Ten liters of existing culture were then diluted into each of two, 5 liter (half-filled) media carboys (1:2 dilution).

Dinoflagellates were then grown in 10 liter carboys over a period of 4 weeks, producing a variety of Brevetoxins as previously reported by Roszell *et al.*, 1990.

II. Antibody Preparation and Purification

Development of a brevetoxin specific antibody was reported by Baden *et al.*, 1984. The material employed in this research was identical to that reported in preparation, handling and preliminary purification.

A. Immunogen construction.

a.1) PbTx-2 reduction.

Brevetoxin-2 was purified from laboratory cultures and was chemically reduced to brevetoxin-3 using cerium trichloride and sodium borohydride as follows:

A 0.01M sodium borohydride in N,N-Dimethylformamide (DMF) stock solution was prepared; for this 0.0038 g. of sodium borohydride were diluted in 10.0 ml of DMF and allowed to completely dissolve (approximately 30 minutes).

The cerium chloride was prepared as a 0.4M solution in methanol (MeOH) --0.986 g. per 10 ml. of MeOH-- and heated. The solution was gravity filtered through fluted filter paper and the filtrate was stored under nitrogen (N_2) for later use.

PbTx-2 (1.714 mg) was allowed to react with 147 μl cerium chloride (CeCl) in a conical micro-reaction vial while stirring. An equal volume of MeOH (147 μl) was then added.

Sodium borohydride was introduced into the reaction in intervals according to the timetable below:

time interval (min.)	volume added (μl).
t0	8
t1	8
t2	1.1
WAIT 5 MINUTES	
t7	8
t8	8
t9	1.1
WAIT 20 MINUTES	
t29	8
t30	8
t31	1.1
WAIT 20 MINUTES	
t51	8
t52	8
t53	1.1
WAIT 20 MINUTES	
t73	8
t74	8
t75	1.1
WAIT 30 MINUTES	
t105	8
t106	8
t107	1.1
ALLOW TO STIR UNTIL t120 (2 HR.)	
t120	8
t212	8
t122	1.1
ALLOW TO STIR UNTIL t135 (2 HR., 15 MIN.)	

The reaction was quenched by addition of distilled water and allowed to stir for a few seconds. The contents were transferred to a separatory funnel, extracted 3 times with ethyl ether, retaining the ether portion each time. Ether extracts were then dried under N_2, purified via HPLC and weighed using a CAHN C-28 electrobalance (CAHN Instruments, Inc. Cerritos, CA).

a.2) Conjugation to Antigenic carrier.

PbTx-3 was succinylated via succinic anhydride (SA) as follows:

PbTx3 and a trace amount of tritiated brevetoxin ([^{3}H]PbTx-3) were combined in a conical vial and dried down overnight in a desiccator using phosphorous pentoxide (P_2O_5). Then, 10-fold molar excess of SA to 1.0 ml of pyridine was added. A 1:1 ratio was employed considering that SA MW= 100 and that PbTx MW= 895. Certainty was made that the crystals of SA appeared long, glittering and white, not powdery. This evidences the anhydrous quality necessary for the reaction to succeed.

The solution was stirred for 2 hr. at 85 °C. A temperature bath was set up using a beaker containing mineral oil; the reaction vial was submerged in the oil and the temperature was closely monitored. The mixture was then allowed to cool and stir overnight.

Flash evaporation followed and the pyridine was removed by rinsing with MeOH. The dried reaction solution was suspended in MeOH, streaked on a 500 μm silica thin layer chromatography (TLC) plate and developed once using 70:30 ethyl acetate: petroleum ether as the developing mixture. The TLC plate was dried and covered with aluminum foil such as to leave one inch of the plate's edge exposed. This area was sprayed with bromocresol green to detect SA portions of the plate by turning them yellow.

The bromocresol green dye was prepared by adding a few crystals in 5 ml 99.5% ethanol (EtOH), 0.1 N Sodium hydroxide (NaOH) until the color just turns blue-green and 20 ml of acetone just prior to use. The solution was the transferred to a spraying can for use on the plate.

One cm square sections of the TLC plate were scraped to detect the [^{3}H]PbTx-3. Succinylated brevetoxin was found within the yellow plate fractions which exhibited high counts per minute on a scintillation counter (LS 1801, Beckman Instruments, Inc., Fullerton, CA.).

Succinylated toxin was scraped and eluted from the plate using MeOH. It was then dried, weighed and stored at -20 °C.

Ethyl-dimethylaminopropyl carboimide (EDC) was made up to 1 ml. solution of 200 mg/ml. in 50 % pyridine. Ten μl of this solution were added to the succinylated toxin previously dissolved in a minimal volume of 50 % pyridine. The solution was incubated while stirring, at room temperature (25 °C) for 2 hr.

Weight of the carrier protein was determined. Since it was desired to conjugate as many toxin molecules to the carrier molecule as possible, keyhole limpet hemocyanin (KLH) was employed. This choice of carrier molecule afforded coupling stoichiometries ranging from 75 to 100 toxin molecules per molecule of KLH as compared to only 10 to 13.5 when using bovine serum albumin (BSA), (Baden and Schultz, 1984 and, Trainer and Baden, 1991). KLH was added to succinylated toxin in small aliquots over 1 hr.

The mixture was allowed to stir at room temperature for 1 to 2 days, then it was dialyzed in 12,000MW tubing against several changes of phosphate buffered saline (PBS), pH 7.4. PBS was prepared to 4 L by combining 5.568 g potassium phosphate (K_2HPO_4), 1.104 g sodium phosphate (NaH_2PO_4), 35.08 g sodium chloride (NaCl), and 4.0 g (0.1 %) azide.

Buffer changes were counted for radioactive emission and the dialysis allowed to proceed until the counts inside and out of the tubing remained constant (approximately 48 hr.). The solution was removed, aliquoted in glass vials and stored at -70 °C until needed.

a.3) Immunizations.

Immunization and serum manipulations were performed exactly as previously described by Baden *et al.*, (1984). A single, castrated male goat was inoculated with the prepared KLH conjugate on a biweekly basis. Complete conjugate containing 0.33 mg of toxin equivalents was employed. Complete Freund's adjuvant (Difco Labs, Detroit, MI) was administered in the initial inoculation and incomplete adjuvant was employed for subsequent boosts. The animal was bled, for serum on alternate weeks, following five weeks after immunization. The plasmaphoresed and packed cells were reinjected dissolved in sterile saline one day after the bleeding. The yields obtained following this method were of 300-350 ml serum per bleeding.

B. Antibody Purification

b.1) Ammonium Sulfate Purification.

The obtained antiserum was treated with an equal volume of saturated neutral ammonium sulfate, placed in the refrigerator overnight and centrifuged at 3000 x ***g*** for 30 minutes. Having discarded the pellet, the supernatant portion was then combined with another equal volume of ammonium sulfate.

The resulting precipitate was separated via centrifugation at 5000 x ***g*** for 1 hour. The pellet was dissolved in 0.3 volumes of PBS, pH 7.4, dialyzed against 3 changes of PBS overnight, and saved at -20 °C until needed.

b.2) Centrifugation and filtration.

One hundred milliliters of the ammonium sulfate purified antiserum were allowed to thaw out in a water bath. The solution was aliquoted in centrifuge bottles (410 ml capacity) and were centrifuged using a SORVAL RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instruments) at 4000 rpm for 10 minutes. The supernatant was saved and the pellet discarded. The supernatant was vacuumed filtered through a 0.45 μm pore size, 47 mm diameter, nylon-66 filter (Rainin Instrument Co., Inc.) using a solvent filtration apparatus (Rainin Instrument Co., Inc.) . Only 10 ml could be filtered at a time due to membrane fouling complications. Increasing the centrifugation time to 20 minutes alleviated the problem allowing up to 20 ml of antiserum to be filtered at once. The filtrate was then prepared for protein-G column chromatography.

b.3) Protein-G purification.

A Genex Gammabind Plus radial preparative protein - G column (binding capacity 1.1 g IgG and 50 ml bed volume) was installed onto a BioRad 700 series High Resolution Liquid Chromatograph. Wash and Elution buffers were prepared as follows:

Wash buffer (pH 7.0): 0.01 M Sodium Phosphate
0.15 M Sodium Chloride
0.01 M EDTA.

Elution buffer (pH 3.0): 0.5 M Acetic acid. Both buffer solutions were degassed through the filtration apparatus described earlier.

The column was equilibrated with sufficient wash buffer volume to achieve a baseline (approximately 250 ml.), the flow rate was adjusted to 5 ml/min. and the pressure was closely monitored never to exceed 3.0 Kg/cm^2. One hundred ml. of

serum was adsorbed onto the column using wash buffer and rinsed with 10 column volumes of the same solution. The IgG was detached from the column using elution buffer (approximately 1 column volume) , collected and immediately neutralized with 1 M Tris base. Antibody was dialyzed (Spectra/por 4 molecularporous membrane 12,000-14,000 MWCO tubing, Baxter Scientific Products, McGraw Park, IL.), against several changes of PBS overnight, lyophilized (Virtis Bench top 3 series freeze dryer, Virtis Co., Inc., Gardiner, NY.) and aliquoted for storage at -20 °C until further needed.

b.4) Brevetoxin Affinity Chromatography.

b.4.1) Column preparation.

Succinylated PbTx-3 was attached to a aminohexyl-Sepharose solid support as described in Trainer and Baden, 1991. Succinylation of PbTx-3 was performed as described in the conjugation to the antigenic carrier section. The solid support was prepared by washing 5 g aminohexyl-Sepharose with 3 x 50 ml of dH_2O, followed by 3 x 50 ml rinses wit2h 50% pyridine. Five ml of the slurry obtained were added to 300 μmoles of EDC in 0.5 ml of 50% pyridine. The mixture was allowed to swirl for 2 hr. at room temperature. PbTx-3 -succinate was added as a solution containing 9.9 mg in 1 ml 50% pyridine, and swirled at room temperature overnight, without using a stir bar. The slurry was packed into a 25 ml glass column, and fitted with flow adapters (BioRad Laboratories, Hercules, CA). Finally, the column was rinsed sequentially with 1 column volume each of 50% pyridine, dH_2O, and PBS, pH 7.4 and stored at 4 °C. Azide was avoided in the preparation of the column. Solid support diagram shown in fig. 7 below.

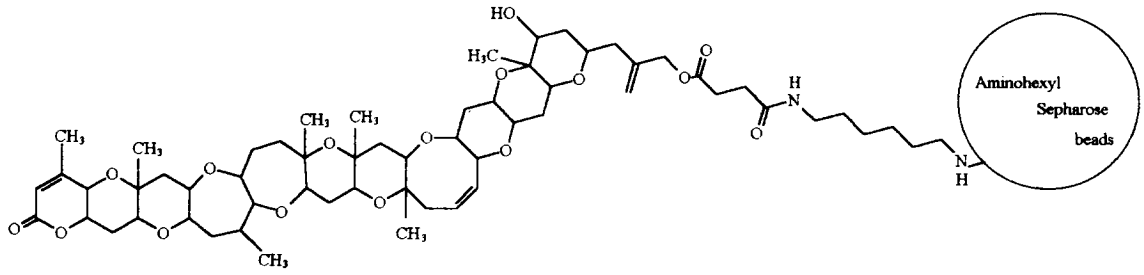


Fig. 7. Brevetoxin 3 succinilated to Aminohexyl sepharose beads as solid support. Calculated coupling efficiency 1-3 μmoles toxin per ml. (Trainer *et al*, 1991).

b.4.2) Affinity purification.

The affinity column was equilibrated by washing with 10 column volumes of each of 10 mM Tris, pH 7.5; 10 mM glycine, pH 2.5; 10 mM Tris, pH 8.8 and again with 10 mM Tris, pH 7.5. Protein concentration for the immunoglobulin (IgG) was determined using a Ready to use Dye available from BioRad Laboratories and based on Bradford, M., Anal. Biochemistry., **72**:248, 1976. The solution was loaded onto the column in 10 mM Tris, pH 7.5, was collected and recirculated 3 times to enhance adsorption, then rinsed with the same buffer until no more protein was eluted. A fraction collector (BioRad Model 2110, BioRad Laboratories, Hercules, CA.) was added to the chromatography array and programmed to collect 1.0 ml fractions. The fractions were monitored for protein concentration using the above mentioned assay, and described later in this chapter. The brevetoxin specific IgG (PbTx-IgG) was eluted using 10 column volumes of 0.1 M glycine, pH 2.5, and neutralized using 1 M Tris base protein elution was monitored via the BioRad assay. The solution was dialyzed against 3 changes of PBS overnight, vialed in 1 ml aliquots and stored at -70 °C. The affinity column was regenerated by repeating the initial washing series and stored in dH_2O containing 0.2% thimerosal.

b.4.3) Protein Concentration Determination.

The assay is available as a concentrated dye solution which must be stored refrigerated (BioRad Labs., Hercules, CA.). The working reagent was prepared by diluting the concentrate 1:4 in dH_2O. The solution was vacuum-filtered through fluted filter paper and placed in an opaque bottle for storage at room temperature. This working reagent remained viable for 2 weeks. The provided standard protocol was used to determine protein concentrations of 50-500 μg/ml.

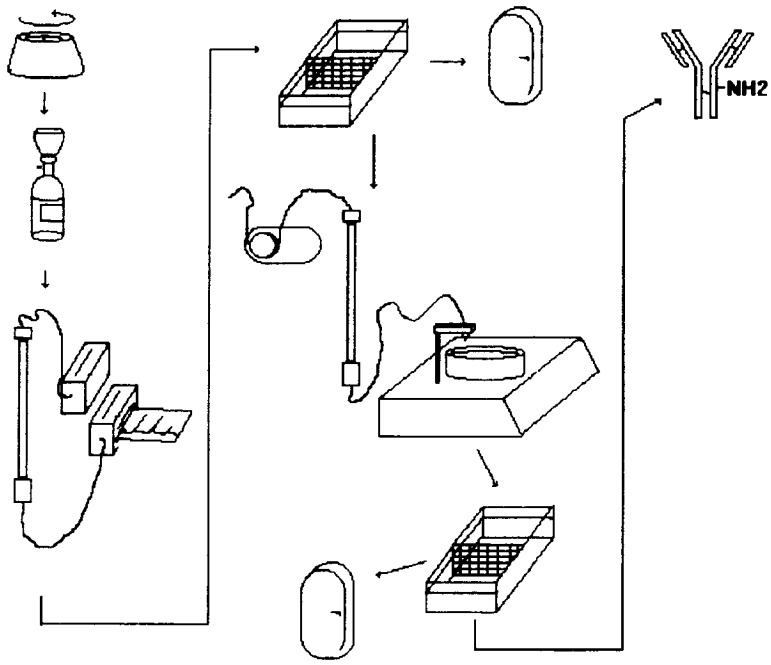


Diagram #1. Brevetoxin specific antibody purification scheme. T to B: centrifugation, filtration, protein-G affinity chromatography, dialysis and portion storage at -20 °C, PbTx affinity chromatography, dialysis and portion storage as above.

Standards were prepared as known BSA concentrations dissolved in dH_2O, aliquoted in 100 μl vials and individually stored at 4 °C. Standards remained viable for up to 6 months. Concentrations ranged from 0.2 to 2.0 μg/ml in 0.2 μg increments. Ten μl sample volumes and 10 μl BSA standards (0.2 - 2.0 mg/ml) were independently combined with 200 μl working reagent using a 96-well microtiter plate (Immulon 4, Dynatech Laboratories, Inc., Chattily, VA). Distilled water was used to obtain blank values. After 5 minutes of incubation at room temperature, the plate was read at 595 nm using an EL-309 Microplate Autoreader (Bio-Tek Instruments, Winooski, VT.).

Blank corrected absorbance values were plotted against known standard concentrations to obtain a standard curve. Individual sample protein concentration were determined by linear regression analysis using Microsoft EXCEL™ version 4.0, 1992, software package and personal regression analysis programs (courtesy of Ms. Paula Mazzer).

C. Conjugation to Activated Horseradish Peroxidase.

One mg lyophilized activated horseradish peroxidase (HRP) was reconstituted in 100 μl dH_2O and, using a conical micro-reaction vial, was added to 300 μg of PbTx-IgG previously dissolved in 100 μl of the provided conjugation buffer (0.1 M $NaHCO_3$, 0.9% NaCl, pH 9.5). Activated HRP was acquired as an Immunopure^R Activated Peroxidase conjugation kit from Pierce Chemical Co., Rockford, IL. Conjugation dynamics and procedure are shown in diagram #2 below. The mixture was allowed to incubate overnight at 4 °C, without stirring; stirring would foam up the antibody solution and lead to titer loss. Forty mg lysine were added to quench the reaction and allowed to incubate for 2 hr. at room temperature.

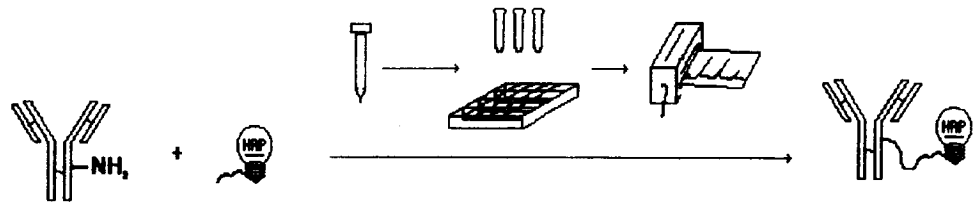


Diagram # 2. Conjugation of brevetoxin specific antibody to HRP. Antibody is incubated with activated HRP (1:3) at 4°C, overnight. Non-conjugated HRP is washed out using a protein A/G column, eluted conjugate fractions are reacted with HRP-substrate for 15 min. in the dark and read in a microtiter plate reader at 405 nm.

Meanwhile, the supplied protein A/G affinity column was equilibrated with 10 column volumes of the Immunopure^R Gentle Binding Buffer from the kit. The reaction was mixed 1:1 with the Immunopure^R Gentle Binding Buffer and applied to the protein A/G column. The column was washed with 15 ml binding buffer and 1 ml fractions were manually collected and monitored for HRP presence via HRP-substrate reaction. The HRP-substrate was prepared by combining 20 ml 0.1 M sodium citrate, 1 ml 40 mM ABTS (2,2'-AZINO-bis(3-ETHYLBENZTHIAZOLINE-6-SULFONIC ACID), and 20 μl 30% hydrogen peroxide (H_2O_2). This substrate has a limited viability and was therefore prepared fresh every time. After a baseline was achieved, the conjugate was eluted using the Immunopure^R Gentle Elution buffer and monitored by HRP-substrate. A 5 μl aliquot from each collected fraction was combined with 100 μl substrate and incubated in the dark for 15 minutes (HRP and substrate are light sensitive so dark incubations were necessary). Absorbencies were read using previously mentioned microtiter-plate reader at 405 nm. Positive conjugate fractions were

combined, dialyzed overnight against 3 changes of 50 mM Tris buffer containing 0.9% NaCl, 0.2% thimerosal (as a preservative), pH 7.5.

Protein concentration was determined using a Bicinchoninic Acid (BCA) assay (range 10-1200 μg/ml). The BCA protein assay kit was available from Pierce Chemical Co., Rockford, IL. Ten μl samples were combined with 200 μl working reagent (50 parts reagent A to 1 part reagent B), mixed for 30 seconds and incubated in the dark, at 37 °C for 30 minutes. Absorbance values were obtained using a microtiter-plate reader and the blank corrected absorbance values were plotted against known concentration standard values. Standard concentrations varied from 50 to 250 μg/ml in 50 μg increments and from 250 to 1200 μg/ml in 100 μg increments. The sample concentrations were determined from the standard curve using regression analysis as before. A modified BioRad assay was also employed to determine conjugate protein concentration. Fifty μl of sample and serially diluted standard volumes were individually mixed 1:1 with working reagent dye. The rest of the protocol remained unchanged. Sensitivity was enhanced by this modified assay and concentrations of 50 μg/ml were easily discerned. BSA was added as a stabilizer to the solution to a 1% concentration and the conjugate was vialed in 1 ml aliquots for storage at -20 °C. until needed.

The protein A/G column was regenerated with 5 ml 0.1 M citric acid buffer, pH 3.0, and stored in dH_2O containing 0.2% sodium azide.

III. Gymnodinium breve cell manipulation.

Cells were obtained from healthy, 3 week-old laboratory cultures and employed whole. Cell concentration per ml was determined by using a microscope 10X magnification and a 0.1 mm deep, improved Neubauer ruling haemacytometer

(A. O. Scientific Instruments, Buffalo, NY) (fig. 8). Cell counting protocols followed guidelines provided by R. Guillard, 1978.

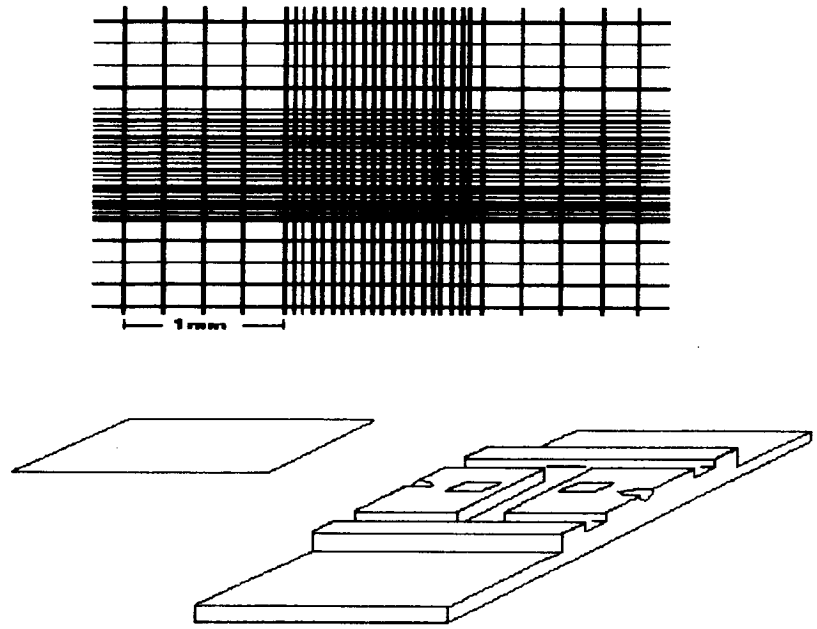


Fig. 8. Haemocytometer and detail of improved Neubauer ruling. Each ruled area contains 0.0018 ml of liquid.

Ten ml were drawn from a 10 liter culture carbouy and made available for counting. Using a disposable Pasteur pipette, both haemacytometer chambers were filled and every organism counted in every square area. To account for free swimming cells, averages on the number of cells found on each of the nine square areas of the 2 chambers were made. This accounted for the number of cells on any square at any given time. Multiplied the number of cells in any square at any one time by the total number of squares in the haemacytometer (18) obtaining the total number of cells in both ruled areas. Considering that each ruled area was designed

to contain 0.0018 ml of liquid, cells concentration then was the averaged number of cells encountered per 0.0036 ml (Guillard, 1978). Several haemacytometer counts were made and their averaged cell number was reported as the cell concentration per milliliter. Known volumes from this counted culture aliquot were incubated as antigen in microtiter plates.

IV. Enzyme-linked Immunosorbent Assay Protocol (ELISA)

The ELISA employed here varied from the standard dual antibody protocol. In this traditional method the antigen (material to be detected) in bound to a solid support, i.e., the flat bottom of a micro titer plate well. The remaining surfaces of the well are coated with blocking agent to impede non-specific binding of the antibody. The primary antibody, specifically designed to recognize this desired antigen, is introduced and allowed to bind. After incubation, the unbound antibody is aspirated and the well is rinsed to remove further remove any excess. A secondary antibody is introduced. This secondary antibody has been labeled via conjugation to an enzyme, and it is specific in its recognition of the primary antibody. The plate again is allowed to incubate. The excess secondary antibody is aspirated and the well is rinsed. The antigen-antibody complex is visualized by exposure to the respective enzyme-substrate following incubation for 15 minutes in the dark (fig. 9). The absorbance values are read in a microtiter plate reader at 405 nm, and the protein concentration is determined by regression analysis versus a standard curve. Thus, antigen recognition is obtained and its concentration assessed indirectly through a two-antibody system.

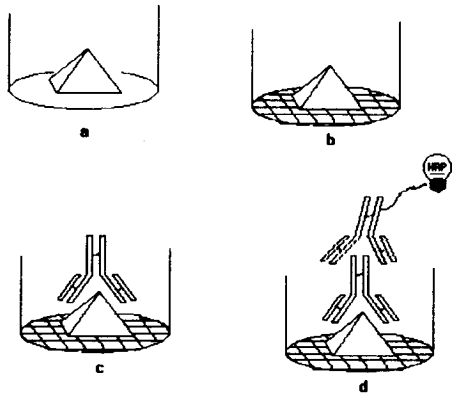


Fig. 9. ELISA protocol. a) binding to plate; b) blocking non-specific sites; c) binding primary antibody and d) binding secondary, labeled antibody.

In the modified ELISA assay presented here, the primary, toxin-specific, antibody was conjugated to a labeling enzyme (as detailed above). Having retained its ability to recognize the antigen, along with being labeled, this single antibody eliminated the need for a secondary antibody and reduced reaction time.

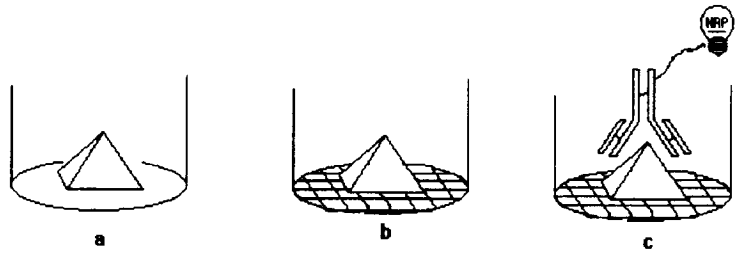


Fig. 10. Modified ELISA protocol. a) antigen bound to plate; b) blocking of non-specific binding sites; c) binding of HRP-labeled antibody to antigen. Response read on a microtiter plate reader at 405 nm.

G. brevis cell concentrations were serially diluted into the wells of a 96 well microtiter plate. Total volume per well remained constant at 100 μl of bicarbonate buffer (0.1 M $NaHCO_3$, pH 9.6) Cells solution was incubated in a

humid chamber, at 4 °C overnight and then aspirated leaving bound cells behind. The wells were rinsed three times with 200 μl wash buffer (0.5 M NaCl, 20 mM Na_2HPO_4 and 0.05% Tween). Blocking solution (PBS-Blotto) was prepared using 10 mM Na_2HPO_4, 0.15 M NaCl, and 40 % non-fat dry milk (NFDM). Non-specific binding was avoided by incubating cells for 1 hr. at room temperature with 100 μl PBS-blotto. Blotto solution was aspirated and the plate was rinsed three times with wash buffer. Brevetoxin-specific antibody conjugate (PbTx-IgG-HRP) was introduced by applying 100μl to each well, and allowed to incubate for 1 hr. The conjugate solution was vacuumed and rinsing was done as before. The toxin-antibody complex was visualized via HRP-substrate allowing the plate to develop in the dark, at room temperature for 15 minutes (fig. 10). Absorbance values were determined using a microtiter plate reader at 405 nm. Toxin concentrations were determined using regression analysis from a standard curve.

RESULTS AND DISCUSSION

I. Protein-G Purification

One hundred ml of antiserum were processed through the Gammabind protein-G column using Tris buffer pH 7.5. The resulting 120 ml (in elution buffer) were dialyzed against 3 changes of PBS overnight, and after immediate neutralization of pH using Tris-base. The reported protein concentration determined by Bradford assay was 14.588 mg/ml. Standard protein concentrations ranged from 0.2 to 2.0 as mentioned previously. The standard curve shown in fig. 11 represents these protein concentrations plotted versus their blank-corrected absorbance values.

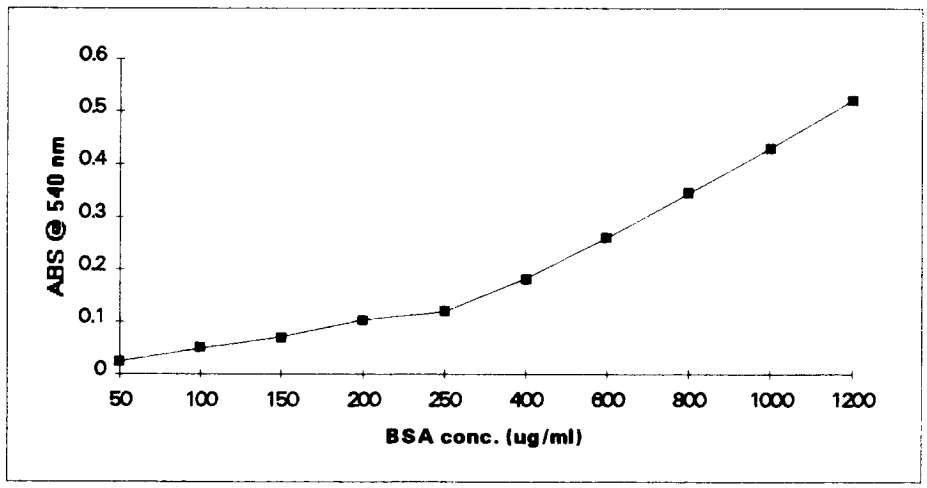


Fig. 11. Protein concentration standard curve for protein-G pure antibody solution.

Blank values were obtained from unknown sample diluent (5 μl PBS) reacted against 200 μl working reagent as per protocol.

II. Brevetoxin affinity chromatography.

Increased toxin detection efficiencies have been obtained through purification of IgG using this chromatography column (Trainer *et al.*, 1991). According to Trainer and Baden, brevetoxin specific IgG (PbTx-IgG) composes approximately 3.8 % of the total IgG pool. By providing only this IgG type for conjugation, the production of primary antibody to HRP conjugate is optimized. One ml of protein-G pure antibody was loaded onto the brevetoxin column using Tris 7.5 buffer as per protocol. Recirculation of the IgG solution through the column three times enhanced binding to the PbTx-3 succinate-aminohexyl-sepharose solid support. Protein assay monitored the adsorption of the IgG. Rinsing the bound material served to wash off the remaining IgG which showed no affinity for brevetoxin. Elimination of these non-specific IgG types contributed to eliminate interference with the antigen during ELISA runs. The rinsing was monitored for protein presence and the baseline obtained showed no more protein being released from the column. The elution profile is presented in fig. 12 as a plot of fraction absorbance @ 595 nm vs. 1 ml collected fractions. PbTx-IgG was contained within the first 2 fractions collected. After pH neutralization and dialysis, the concentration of the collected solution was determined to be 0.788 mg/ml, totaling 3.152 mg IgG via Bradford assay, and representing a yield of 2.8 %; fig. 13 below.

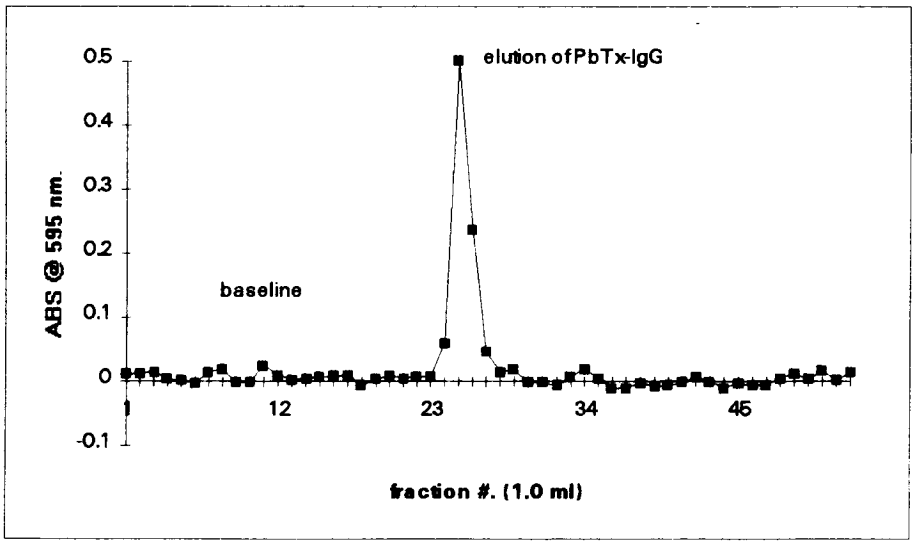


Fig. 12. Affinity chromatography purification.
Elution of brevetoxin specific antibody (PbTx-IgG).

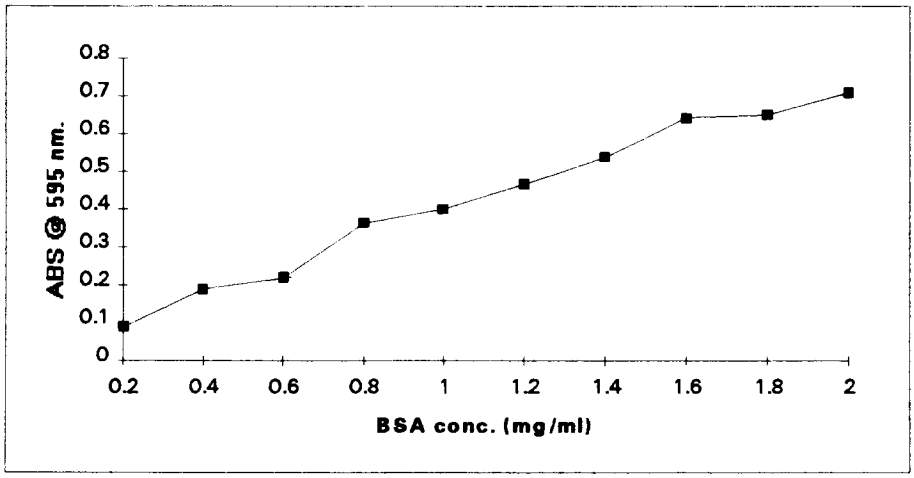


Fig. 13. Brevetoxin specific IgG standard curve.
Concentration determined at 3.152 mg/ml.

Brevetoxin specific IgG composed 2.8 % of the total IgG pool and it approximates Trainer's determination of 3.8 % yield.

III. Conjugate Preparation.

PbTx-IgG concentration was 0.788 mg/ml but less than 0.300 mg /100μl as demanded by the conjugation protocol. The total volume of available stock however was 4.0 ml. which represented a potential concentration of 3.152 mg/ml. Having been last dialyzed against conjugation buffer, the PbTx-IgG was ready for conjugation except for the concentration problem. The solution was dialyzed against dH_2O and lyophilized overnight to eliminate excess salts and concentrate it to the needed value. The lyophilized material was brought up in 1.0 ml of conjugation buffer which provided a concentration of 3.152 mg/ml. Only 95.238 μl of this solution were used and the final volume was adjusted to 100 μl with protein-free conjugation buffer. Similarly to the brevetoxin and protein-G columns, the conjugate material (PbTxIgG-HRP) was cleaned of the non-conjugated HRP by adsorption to an affinity chromatography column and then by rinsing off of the unbound material. In this instance the provided protein A/G column was employed. One ml fractions of both rinse and eluate were collected and assayed for HRP presence by exposure to HRP-substrate. The graphs of the baseline and elution profiles are shown in fig. 14 below. Conjugate concentration was 0.163 mg/ml as determined by the afore mentioned BCA assay and representing 5.17 % of the total available PbTxIgG (fig. 15).

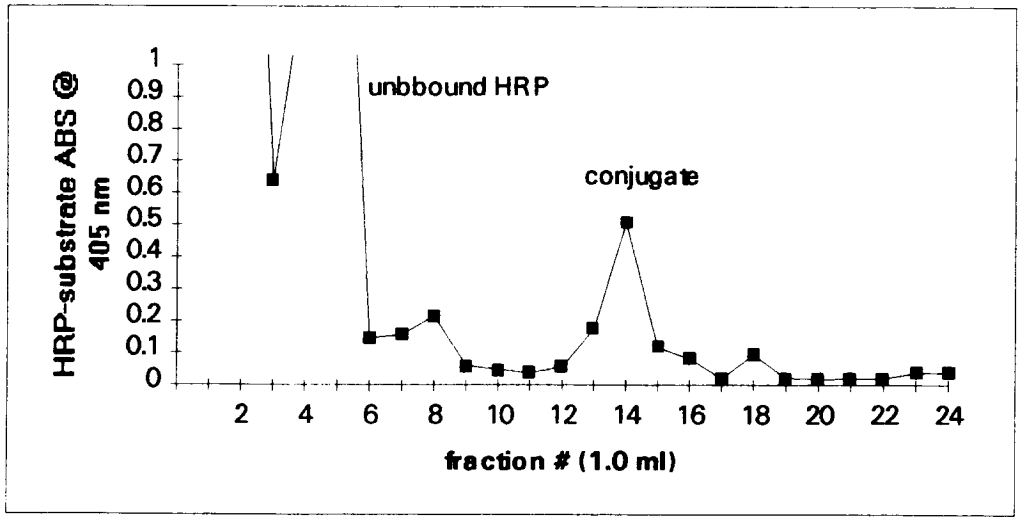


Fig. 14. HRP-IgG conjugate elution. Achieved baseline and following elution shown. Protein A/G column used to rinse out unbound HRP.

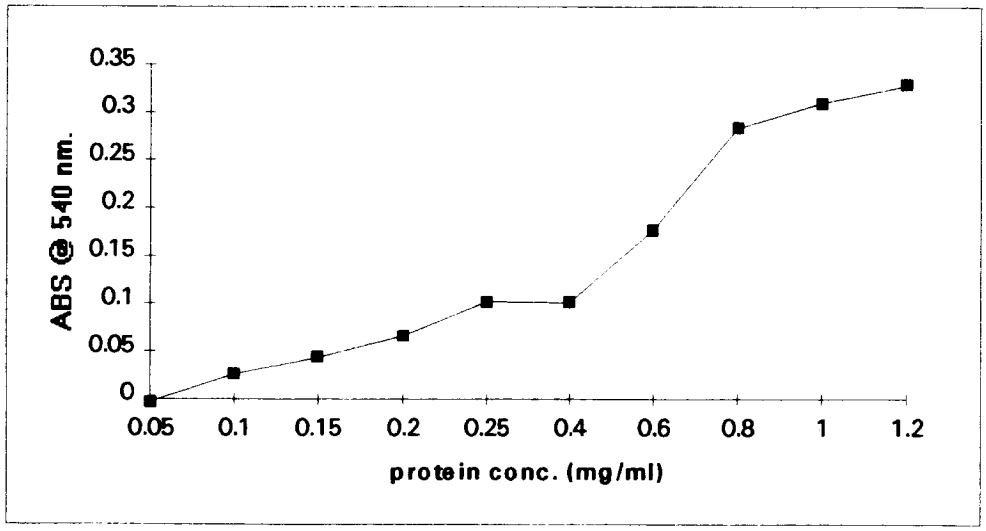


Fig. 15. Protein concentration standard curve for PbTx-IgG conjugate. Conjugate concentration calculated at 0.163 mg/ml.

IV. Cell counts

Gymnodinium brevis cells were found to be 16,000 cells/ml. Cell counts were obtained by averaging 6 haemacytometer readings to account for sample

variability and free swimming cells. Cooling cells 5 to 10 minutes at 4 °C has reportedly allowed for better counting by eliminating the free swimmer cell (Ellenberg, personal communication). However, since the ELISA used in this research provides toxin detection by using whole cells, it was not desirable to expose cells to potential lysing by exposure to adverse temperature conditions. Therefore, statistical manipulation of cell counts was preferred. However, due to the extreme cell concentration, a combination of both methods was necessary to accurately determine the number of cells.

V. Enzyme-linked Immunosorbent Assay (ELISA).

Serial dilutions of 100 μl from the original cell concentration solution provided 10 filled wells at 400, 200, 100, 50, and 25 cells respectively. Excepting cell binding incubations, all others were performed at 37 °C for the mandated times. Cells were incubated at 4 °C, in a humid chamber, overnight.

Toxic cells went unrecognized by the conjugate antibody and could not be detected within the mandated 15 minutes development time. Longer development times showed HRP-substrate indicator coloration i.e., a positive result. The samples were allowed to develop for 120 minutes and the results are presented in fig. 16 below.

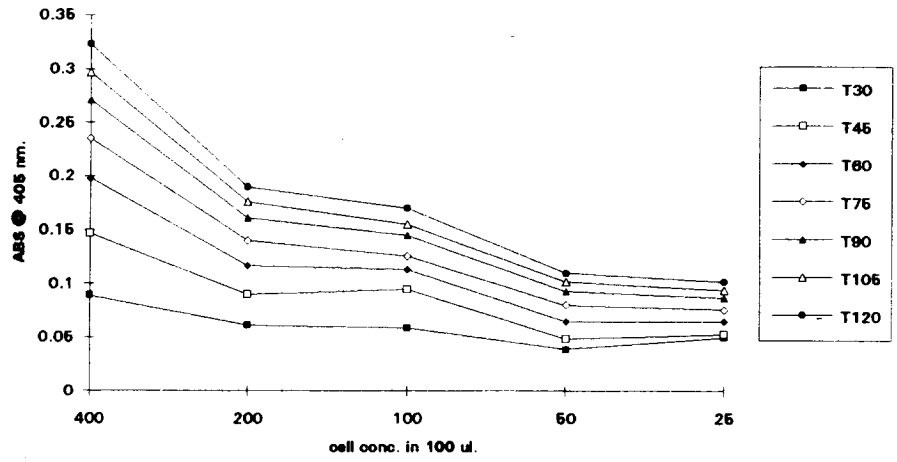


Fig. 16. ELISA of PbTx-IgG against P.b. cells over time. Absorbance values decreased with diminishing cell concentration. Values obtained for T30 to T120 minutes. Smaller cell concentrations could not be clearly resolved.

Conjugate complex did not resolve with the expected dilution capability of 1:1000. Previous conjugations provided protein concentrations of 50 μg/ml. Optimization of the conjugation protocol allowed for the increment of this concentration by a factor of three. Nevertheless, even increased concentrations of 160 - 170 μg/ml conjugate, are still not enough to account for the large number of assays to be performed and samples to be analyzed if this modified ELISA is to be used for routine monitoring purposes. Presently this modified assay is not cost-effective due primarily to the high price of the reagents employed. A high volume of these reagents must be used in order to provide enough conjugate to recognize the available toxins while at the same time produce a strong enough signal to be detectable by the monitoring devices within the expected timeframe.

CONCLUSIONS

The present biotoxin situation demands the immediate preparation of acceptable detection assays. Due to the special characteristics of quickness, ease of operation, field worthiness and high specificity for toxin recognition required by the regulating agencies, it is necessary that the research on immunological detection methods described herein be continued. Modification on the current approach to better bind the antigen to the plate can be made. Using a fixed antibody as a support upon which to link the toxin in question will insure antigen retention during washing steps and contribute to the production of an increased signal. As described herein, the modified ELISA can be implemented. The purification of the brevetoxin specific antibody solutions and following conjugations to HRP indicator enzymes were all successfully achieved. Further, when compared to biochemical purification procedures, the ELISA assay approach provides greater shorter implementation times, demand reduced numbers of technical personnel, greater flexibility for different situations (field, lab, dock) and practicality (in its speed and sensitivity). The primary shortcoming of the assay resides in the high cost of implementation. The processing cost of 96 samples (one microtiter plate) exceeds $665.00. Needed conjugate concentrations on the order of 5000 μg/ml would cost in excess of $1,270.00 and allow for the process of only 184 samples. Not nearly what routine monitoring demands. Further, improvements on the present assay design can only be performed if sufficient conjugate is available for numerous experiments. The present avenue of research has not been abandoned however; and, in light of the recent price breaks on needed supplies and reagents, new efforts for conjugation optimization are underway. Regardless of whether developed and employed exactly as described herein, biotoxin detection via ELISA present a reliable, easy, potentially cost effective means of routine monitoring.

PART IV.

HACCP PLAN GUIDE: DISCUSSION AND CONCLUSION.

The FDA suggests, through its Fish and Fishery Products Hazards and Controls guide, the implementation of HACCP principles based upon product arrival. The principal means of biotoxin presence determination is via assay of suspect species for specific toxins, employing available, FDA sanctioned laboratory methods. The main concern then is not solely the assay method or the species involved, but the sampling methodology. Biotoxin detection assays, as presently available, will determine the presence of the toxin of interest and aid to quantify its concentration per gram of available tissue. The species of seafood will help determine the type of possible biotoxin affliction, and the actions necessary to find potential toxin content. It is the sampling methodology however, which will reliably determine a degree of hazard to the consumer of seafood product. The FDA guide suggests a representative sample be obtained from the suspect lot. For various cases, the lot or batch is defined as the total volume capacity of the ship's hold and the sample is determined to be one which will afford a 95% or greater certainty that the product is not contaminated by greater than a given critical control limit (95% or greater certainty that 2.5% or less of the lot is free of histamine in the case of scombroid fish poisoning). However, what sampling methodology will provide detection of 50 toxic fish from a lot catch of 500,000? First, the sampling approach must consider what it is being looked for. The natures of the toxin and the causative agent must be known. Each toxin producing organism possesses specific biological characteristics which aid in the segregation of fish lots. Ciguatera as previously mentioned, is a toxic syndrome of tropical origin. The vectors for the transfer of this toxin are species living in close

association with tropic bound reef systems. Ciguatera is a coastal, shallow water syndrome. Fish products foreign in origin to this environment need not necessarily be assayed for ciguatera. Deep blue, nektonic species, such as tuna, may be discarded as ciguatera suspects since they do not inhabit potentially ciguatoxic areas. Further, checking for ciguatera in any lot originating from higher latitudes, i.e., alaskan waters, is similarly wasteful, ciguatera is a malady of tropical origin. The same zonality principle can be applied to PSP causing dinoflagellates which, by the effects of temperature are restricted to the northern coasts of the U.S. *G. catenatum*, cannot survive in the elevated water temperatures of the Gulf of Mexico or coastal Florida. It would not be cost effective to assay for PSP in these areas. The same extreme temperature cases could be applied for the reverse situation where *G. brevis*, NSP causative agent, is considered. This dinoflagellate is indigenous to warmer waters and would not thrive to bloom conditions in colder waters off New England. For areas of intermediate temperatures, where causative agents may thrive, it is necessary to increase sample size to include indigenous species. Thus, by knowing what is being sought and by discarding non-toxic species based on scientific knowledge regarding the toxins and their causative agents, it is possible to simplify the sampling problem, reduce the size of the lot and finally increase the confidence of the sampling methodology. Scientifically, segregation of non-contaminated tissues due to species origin, nature, and temperature zone, is the primary consideration.

Federal regulations call for the establishment of HACCP based plans in every facility and operation which deals with fish or shellfish products. These plans must be implemented by 1995 when the regulations become effective. Various firms have already complied with the government rules by developing or adopting such control systems in their operations. The response to the HACCP plans has been mixed. Several operators believe that HACCP is just another form

of government intervention in private enterprise, and the adoption of such systems will only contribute to the generation of more taxes and increased operation costs. Particularly for antiquated facilities and small operations, the cost of HACCP adoption can mount to several thousand dollars. These implementation costs may prove disastrous for small firms which, unable to cover the bill, would be forced out of business. HACCP may be referred to as a process line system. The design of the CCP methods allow for their implementation on the production line or flow of the seafood processing and distributing companies. HACCP does not apply to seafood retailers, food service or fishing vessels, unless these vessels are factory types which process their catch at sea (Evans et al., 1994). Streamlined operations suffer less from the adoption of HACCP plans than do older, non-lined, or re-configured plants. For firms such as John T. Handy Co., Crisfield, MD, implementation of HACCP plans have proved beneficial in the reduction of compliance costs by approximately one half (Evans et al., 1994). Cost benefits to a simple, streamlined plant can continue to increase every season. For some operations, the cost of implementing HACCP plans ($10,000 once) will prove to be more economical when compared to compliance cost to existing programs such as the Packed Under Federal Inspection ($25,000/year). Additionally, the initial disbursement may pay in the long run several times over. Benefits will take the form of increased consumer confidence in not only the product (representing more sales potential), but also in the processing company; in the seafood business, like in any other business arena, a good name goes a long way. Presently, several companies have developed training programs to acquaint processing personnel to HACCP plans and to the management practices demanded by its future adoption. Consulting firms have increased business as a result of the need to understand the HACCP plan format and update processing plants by 1995. Increasing numbers of seafood companies bill themselves as complying with future regulations today, in

order to attract customers by stimulating afore mentioned consumer confidence. As a liability insurance system, HACCP, via compliance records, may help determine proper handling, responsible practices at any moment on the part of the operators. The Federal government has left regulation up to the discretion of each fish and shellfish product companies. Since the quality control, sanitation, and safety needs of the industry vary greatly with each food type and company specialty, who better than the individual operations to accurately address the demands of the industry? The present Federal administration has made it possible to have local, private regulation with the necessary amount of government coordination. Some companies rather have policy implemented in this cooperative fashion than as an imposition of regulation by a Federal agency which may be deemed incompetent, unaware or inflexible, as it is believed to be the case of the local aquaculture industry. HACCP implementation costs also mean the elimination of the potentially dangerous, unsafe operators. Various companies believe that, unable to foot the bill, these food distributors will be forced out of business or be shut down by supervising agencies. Their disappearance will signify a renewed customer confidence in the industry as a whole and increased seafood consumer safety. Opposition to the plan's implementation contends that unscrupulous operators will always be able to find new ways to distribute their product, and that the HACCP plans will only contribute to increase the traffic of unsafe foods and augment poor processing practices. Frankly, loopholes can always be found in any type of regulatory system. In the case of a HACCP system, with such a positive influence upon the seafood industry, not just locally but globally as well, why go out of our way to look for these holes? Adoption of the plan is a better option than the present, unsafe and uncertain situation.

MARINE AFFAIRS INTERNSHIP ASSESSMENT.

The current format of the Marine Affairs M.A. degree affords the student the opportunity to design the class and employment curriculum according to personal needs and interests. This unique feature has been praised by students and internship hosts alike. Marine Affairs covers a vast spectrum of human activities as related to the oceans. Virtually all of what we now do at sea has an area of concern for which Marine Affairs has applicability. This internship has provided the opportunity to become a part of the biotoxin and harmful algae management effort; a current scientific, managerial and policy issue of national scope and importance. Further, combined with a primarily scientific background, the training received in the areas of policy, economics, management and law allows the understanding of the primary issues of concern to regulators, administrators and scientists alike. This is a unique position to be in since at this moment, lack of coordination and understanding among scientific, management and regulatory agencies and private organizations is at the root of the problem.

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