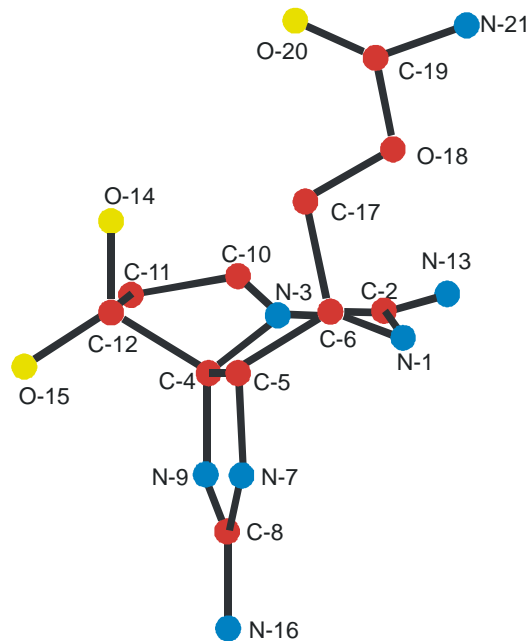


# Receptor Binding Assay Technique for Harmful Algal Bloom Toxins Quantification

## Reference Manual



*UNDP/IAEA/RCA/ Subproject 2.4 Application of Nuclear Techniques to  
Address Specific Harmful Algal Bloom Concerns*

**REGIONAL RESOURCE UNIT**  
**Philippine Nuclear Research Institute**  
PNRI, Quezon City, Philippines, 2000

Receptor Binding Assay Technique  
for  
Harmful Algal Bloom Toxins Quantification

**Reference Manual**

*UNDP/IAEA/RCA/ Subproject 2.4 Application of Nuclear Techniques to  
Address Specific Harmful Algal Bloom Concerns*

REGIONAL RESOURCE UNIT  
**Philippine Nuclear Research Institute**  
PNRI, Quezon City, Philippines, 2000



ACTIVITY OUTPUT  
OF  
**COMPONENT 4: APPLICATION OF NUCLEAR TECHNIQUES TO ADDRESS  
SPECIFIC RED TIDE (HARMFUL ALGAL BLOOM) CONCERNS**

[Sub-Project2: *Marine Coastal Environment and Its Pollution* of RAS/97/030  
*Better Management of the Environment, Natural Resources and Industrial  
Growth Through Isotope and Radiation Technology*]

UNDER THE AUSPICES  
OF THE  
Joint UNDP/IAEA/REGIONAL COOPERATIVE AGREEMENT FOR ASIA AND THE PACIFIC

Participating Member States  
(for this activity component)

**China**  
**Indonesia**  
**Malaysia**  
**Pakistan**  
**Philippines**  
**Thailand**  
**Vietnam**

In-kind Contribution  
of the  
Philippine Nuclear Research Institute  
**Regional Resource Unit**

Editor

Elvira Z. Sombrito  
Philippine Nuclear Research Institute  
Commonwealth Avenue  
Diliman, Quezon City  
Philippines  
Email: [ezsombrito@pnri.dost.gov.ph](mailto:ezsombrito@pnri.dost.gov.ph)

## FOREWORD

With the overall objective of contributing to the sustainable development and management of the marine coastal environment through the application of isotope techniques, seven RCA Member States namely **China, Indonesia, Malaysia, Pakistan, Thailand, Vietnam** and the **Philippines** formulated the project “*Application of Nuclear Techniques to Address Harmful Algal Bloom Concerns*” on November 30 to December 4, 1998 in Manila, Philippines. This project becomes Component 4 of Sub-project 2 Marine Coastal Environment and its Pollution under the joint UNDP/IAEA/RCA Project for Asia and the Pacific (RCA) *Better Management of the Environment, Natural Resources and Industrial Growth Through Isotope and Radiation* (RAS/97/030).

Two of the main objectives of the project are the transfer of the receptor assay technology to interested Member States and the establishment of the receptor assay method in these laboratories. These objectives address the need for a rapid and sensitive assay to complement and eventually replace the current live mouse bioassay in order to enhance shellfish toxicity monitoring program and improve the management of harmful algal blooms (HABs). The receptor binding assay that has been developed recently is recognized as the most viable alternative to the mouse assay.

Towards these objectives, a regional training workshop on receptor assay was conducted to demonstrate the theories and practice of receptor assays. The Regional (RCA) Training Workshop on Receptor Binding Assay Techniques For Harmful Algal Bloom Toxins Quantification Workshop was conducted at the Philippine Nuclear Research Institute from 29 November – 04 December 1999. It aimed to train the RCA Member States representatives in neuroreceptor binding assays for PSP toxins as an alternative method to mouse bioassay. Related to this activity is the production of a reference manual of procedures for distribution to laboratories engaged in shellfish toxicity research and monitoring activities. This manual is a Philippine contribution to the regional project. It was funded by the Philippine Department of Science and Technology and produced by the Philippine Nuclear Research Institute.

Special thanks is due to Dr. Frances M. Van Dolah of the NOAA National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, Charleston, South Carolina, USA, who contributed the laboratory protocols in this manual. Dr. Van Dolah served as the International Atomic Energy Agency expert for this activity. Appreciation is also expressed to all who provided the lecture materials and those who allowed the reprinting of articles included herein.



# CONTENTS

## General Introduction

Microplate Receptor Assays: Tools for Monitoring Seafood Toxins <i>Frances M. Van Dolah</i> .....	3
Marine Algal Toxins: Origins, Health Effects, and Their Increased Occurrence <i>Frances M. Van Dolah</i> .....	9
Impacts of Harmful Algae on Sea farming in the Asia-Pacific Area <i>Rhodora A. Corrales and Jay L. Maclean</i> .....	31
The ASEAN-CANADA Red Tide Network: 1993-1998 <i>Rhodora V. Azanza</i> .....	49
Receptors and Receptor Assay Principles <i>Lourdes J. Cruz</i> .....	57

## Laboratory Procedures

Receptor Binding Assay for Saxitoxin .....	65
Data Analysis .....	69
Assay Quality Control .....	70
Shellfish Extraction Protocol for Saxitoxin .....	73
Rat Brain Membrane Preparation .....	75
Microplate Protein Assay .....	77

## Appendices

Radioisotopes – Characteristics, Handling, Measurement, Calculations <i>Estelita G. Cabalfin</i> .....	81
Items Required for Receptor Assay .....	85

Saxitoxin Receptor Binding Assay Worksheet .....	89
Saxitoxin Dihydrochloride Standard .....	90
Commercially Available Tritium-labeled Saxitoxin .....	91
Analyzing and Graphing Radioligand Data.....	93

# GENERAL INTRODUCTION



## Microplate Receptor Assays: Tools for Monitoring Seafood Toxins

Frances M. Van Dolah

Marine Biotoxins Program, NOAA National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, Charleston  
South Carolina USA

In response to a global increase in toxic algal blooms and the resultant increase in the incidence of human illness, many seafood monitoring programs have been established worldwide to determine the presence of algal toxins (Table 1). The standard method of detection for algal toxins in monitoring programs worldwide is the mouse bioassay. However, increasing pressure to replace animal testing with reliable, specific detection methods has led to the development of a number of *in vitro* assay approaches, including antibody-based assays and functional assays. Functional assays include enzyme inhibition assays, cell assays and receptor assays. The primary difference between antibody based assays and functional assays is that antibody based assays are based on recognition of the toxin structure, to give a measure of toxin content, whereas functional assays are based on the interactions of the toxins

with their pharmacological receptors and give a measure of toxic potency.

Receptor assays are appropriate for those toxin classes that interact with membrane receptors (Table 1), including the voltage dependent sodium channel (PSP, NSP, and CFP) and the glutamate receptor (ASP), and are the method of choice when a rapid, high throughput measure of total toxic potency is desired.

Most classes of algal toxins in seafood occur as suites of related congeners that possess different toxic potencies and are present in varying proportions in contaminated seafood; thus for regulatory purposes, the assay of total toxic potency is more relevant than analysis of the content of individual toxin congeners. Receptor assays are particularly applicable for measuring total toxic potency since, within a toxin class, all congeners bind to the same receptor and relative binding affinities correlate well with their relative toxic potencies.

Table 1.

Human Toxic Syndrome	Toxins	Molecular Target
Paralytic shellfish poisoning	Saxitoxins	voltage dependent sodium channel
Neurotoxic shellfish poisoning	Brevetoxins	voltage dependent sodium channel
Diarrhetic shellfish poisoning	Dinophysis toxins Okadaic acid	protein phosphatases
Amnesic shellfish poisoning	Domoic acid	glutamate receptor
Ciguatera fish poisoning	ciguatoxin	voltage dependent sodium channel

## GENERAL INTRODUCTION

Because the binding affinities of algal toxins for their receptors are in the nM range, the detection limits of receptor assays are at the pmol level.

### General Receptor Assay Methodology

For the sodium channel toxins, a convenient source of receptor for competitive binding assays is a crude membrane preparation from rat brain. For analysis of the ASP toxins, our laboratory uses a cloned glutamate receptor expressed in cultured cell membranes, which totally eliminates animals from the testing procedure.

A receptor assay is carried out by incubating the known receptor for the toxin (R), in the presence of the radiolabeled toxin analog (T\*) which together form a radiolabeled receptor-toxin complex (T\*R) (Figure 1a). With the addition of unlabeled toxin (T) to this incubation mixture, in the form of toxin standard or unknown sample, the unlabeled toxin competes with the radiolabeled toxin for the receptor, forming unlabeled complex (TR). The amount of radiolabeled complex formed in this mixture is quantified by liquid

scintillation counting. With increasing amounts of unlabeled toxin, the amount of radiolabeled complex (B) decreases relative to the amount of radiolabeled complex formed in the absence of unlabeled toxin (B<sub>0</sub>). This competition between labeled and unlabeled toxin for the receptor generates a competition curve which can then be used to quantify the amount of toxin present in an unknown sample (Figure 1b).

Radioreceptor assays have been used for more than two decades as a research tool for investigating the pharmacologic actions of compounds that interact with membrane receptors, including a number of neurotoxins that interact with ion channels. A major drawback to the regulatory application of receptor assays for marine biotoxins has been their time consuming and laborious nature. However, the modification of traditional protocols to microplate formats has eliminated the extensive manipulation of individual samples and reduces analysis time to 3 hours, making them suitable for high sample throughput required for regulatory purposes.

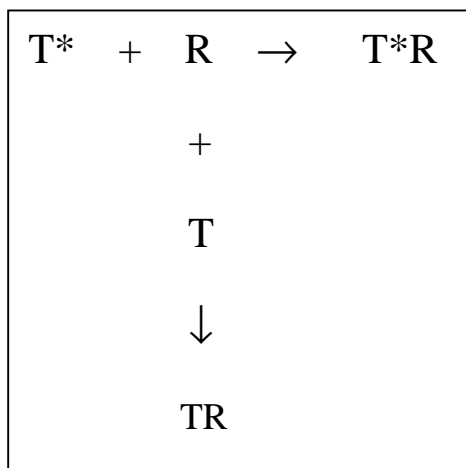


Figure 1a. Receptor- toxin complex

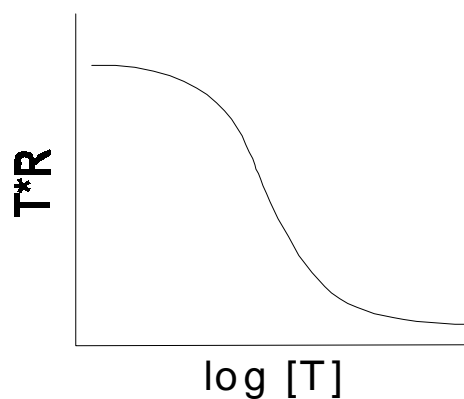


Figure 1b. Competition curve

**Microplate Formatting**

In the microplate format, receptor assays are generally carried out by incubating membranes containing the receptor of interest in the presence of radiolabeled toxin (with or without competing toxin standard or sample) in the wells of a 96-well microplate, until equilibrium binding is reached, generally 1 h at 4 °C (Figure 2). The toxin-receptor complexes are then transferred from the wells to a 96-place glass fiber filter mat, using a vacuum manifold, and rinsed twice with ice cold buffer. Upon filtration, toxin bound to the receptor remains attached to the filter, while unbound toxin is washed through the

filter. The filter mat is dried for 15 min at 40 °C, covered with a sheet of wax-like solid scintillant, and is then placed 1 min on a hot plate to melt the scintillant. Finally, the scintillant-saturated mat is placed in a cassette for scintillation counting. In cases involving the use of an isotopic label which is exchangeable upon heating (e.g., [<sup>3</sup>H] saxitoxin), a modification of this method is used which employs incubation in a microtitre filter plate (Multiscreen, Millipore), *in situ* filtration, and addition of liquid scintillant directly to the microplate prior to counting in the microplate scintillation counter.

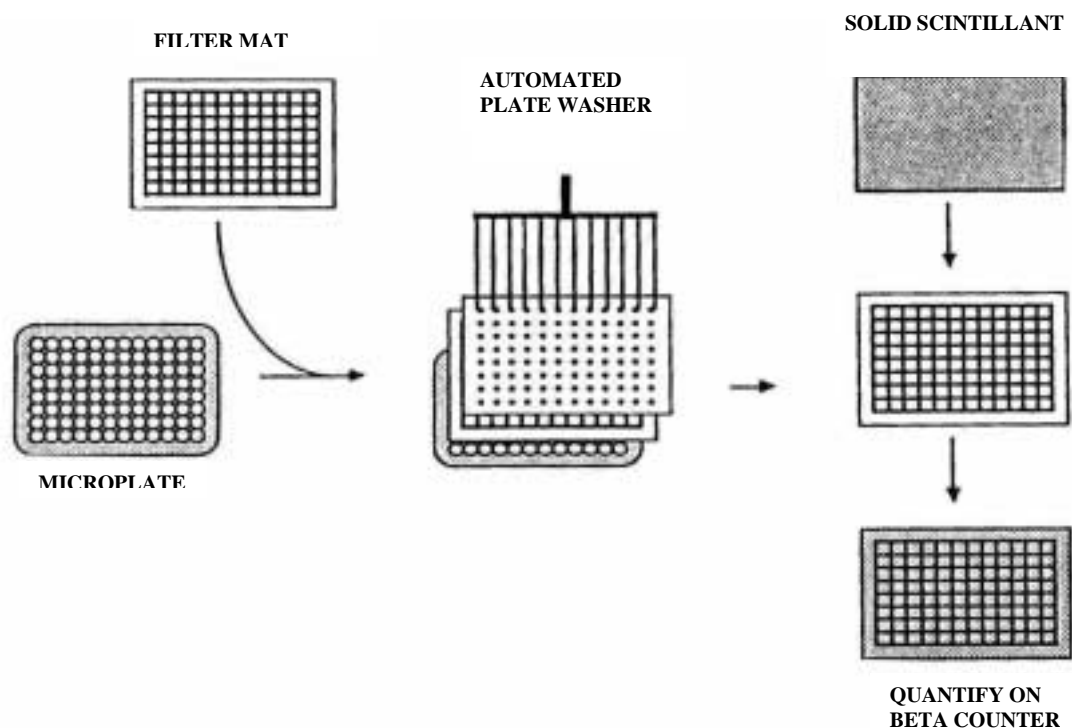


Fig. 2. Procedure for high capacity “drug discovery” format receptor binding assays. Radiolabeled toxin, unknown sample, or standard and membrane preparations are incubated in individual 0.3 ml wells of a 96-well cluster for 1 h at 4 °C. Membrane-bound toxin is separated from unbound toxin in each of 96 wells by filtration onto a 96-place filter mat using an automated cell harvester. The filter mat is then impregnated with solid scintillant and counted on the microplate scintillation counter. Assay time is less than 3h.

## GENERAL INTRODUCTION

Microplate scintillation counters are generally equipped with multiple detectors, which read either 2, 3 or 6 wells at a time, so that an entire 96-well plate may be read in about 20 min. In addition, microplate counters may be interfaced with automated receptor data analysis software which will carry out curve fitting routines and calculation of toxin concentration in unknown samples.

### Receptor Assays for PSP

Paralytic shellfish poisoning, which occurs in North and South America, Europe, Asia, and Australia, is caused by a suite of heterocyclic guanidinium toxins collectively termed saxitoxins, of which there are currently 21 known congeners. All congeners bind to site 1 on the voltage dependent sodium channel and inhibit neurotransmission. The microplate receptor assay for PSP toxins developed by our laboratory [1] is based on competition between [<sup>3</sup>H] STX and increasing concentrations of STX ( $10^{-11}$  to  $10^{-6}$  M) for binding to the voltage dependent sodium channel. Half-maximal inhibition ( $IC_{50}$ ) of [<sup>3</sup>H] STX binding is observed in the presence of  $5.0 \pm 0.63$  nM STX. Interassay variability is approximately 12%. The detection limit of the microplate assay is about 0.5  $\mu$ g/100 g mussel tissue, as compared to a limit of detection in the mouse bioassay of 40  $\mu$ g/100 g (regulatory limit = 80  $\mu$ g/100g, U.S.A.). The microplate assay has performed well in comparisons with the mouse bioassay and HPLC analysis of shellfish extracts, algae and seawater samples [2]. It has also proved useful for quantification of PSP toxins in serum and urine samples involved in a human disease outbreak [3].

### Receptor Assays for NSP and CFP

Neurotoxic shellfish poisoning is produced by the "red tide" algae which has historically occurred in Florida, but recently caused an NSP outbreak in New Zealand. NSP is responsible both for extensive fish kills and for shellfish poisoning in humans, as the result of consumption of contaminated shellfish and aerosols formed from the breakup of algal cells in the surf zone. NSP is caused by a suite of nine heterocyclic polyethers collectively called brevetoxins (PbTx). Ciguatera fish poisoning (CFP), a neurological syndrome associated with consumption of reef fishes that have accumulated toxins, affects over 50,000 people annually worldwide. Ciguatoxins (CTX), heterocyclic polyethers responsible for ciguatera fish poisoning, are structurally related to the brevetoxins, and similarly occur as a suite of structural congeners. Both brevetoxins and ciguatoxin bind to site 5 on the voltage sensitive sodium channel. Reference standards suitable for quantitative analysis of ciguatoxin currently remain unavailable, and radiolabeled CTX has not yet been produced. Thus, the quantitative assessment of PbTx or CTX is currently based on binding competition with [<sup>3</sup>H] PbTx-3 for site 5 on the voltage gated sodium channel. Two assay formats have been employed, a centrifugation assay [4] and a microplate filtration assay [5]. Both formats gave comparable quantitative results in an interlaboratory method comparison for determination of PbTx in oyster (Van Dolah and Poli, unpubl.) and linear correlation with the mouse bioassay (Steidinger and Baden, unpubl.). In the microplate assay, half maximal binding ( $IC_{50}$ ) of [<sup>3</sup>H] PbTx3 is observed in the presence of  $15.9 \pm 1.3$  nM PbTx3, with an interassay variability

of approximately 8%. The assay has also been used to measure PbTx in toxic algae and marine mammal tissues. Collaborative analysis of CTX in fish flesh by receptor assay, mouse bioassay, cytotoxicity, and mass spectrometry yielded good qualitative agreement between detection methods [6]. This method has also been used to confirm CTX as the cause of an outbreak of fish poisoning among soldiers in Haiti [7].

**ASP** Amnesic shellfish poisoning is caused by domoic acid, an analog of the neurotransmitter glutamate, which binds to the kainate glutamate receptor, causing excessive neuroexcitation and neuronal damage to several brain regions, including the hippocampus, a region of the brain involved in learning and memory processing. ASP first occurred in Canada in 1987, when approximately 100 people became ill after consuming mussels, and three people died. Because [<sup>3</sup>H] domoic acid is not commercially available, the receptor assay for domoic

acid and its isomers is based on binding competition with [<sup>3</sup>H]kainic acid, another neurotoxin which binds with high affinity to certain subtypes of glutamate receptor. A receptor assay for domoic acid was first published using frog brain synaptosomes [8], was more recently modified to utilize a cloned glutamate receptor (GLUR6), expressed in SF9 cells [9]. A competition curve is generated by incubating GLUR6-containing membrane preparation with increasing concentrations of domoic acid ( $10^{-11}$  to  $10^{-6}$  M). Domoic acid inhibits [<sup>3</sup>H] kainic binding with a half maximal effect ( $IC_{50}$ ) at  $2.8 \pm 0.33$  nM. Limit of detection of the assay is 1nM ( $IC_{80}$ ). The assay performs well when compared with HPLC analysis of algae, mussel extracts and extracts of crab hepatopancreas[8], and has also proved useful for the rapid screening of domoic acid in serum serum, urine and feces of marine mammals intoxicated by domoic acid [10].

## SUMMARY

Receptor assays have the demonstrated sensitivity and reliability to measure algal toxins in seawater, algae, seafoods, and human serum, and hold promise for applications in both research and regulatory settings. In addition to laboratory research, we have utilized microplate receptor assays for shipboard analysis of toxins from a red tide in the Gulf of Mexico. The microplate scintillation counter used in these studies is both small and rugged enough to be easily transported. We have found that

microplate scintillation technology substantially increases sample throughput, while reducing analysis time and manual sample handling. For regulatory purposes, detection methods must meet a number of criteria, including quantitative reliability, speed of analysis, high sample throughput, and cost effectiveness. The development of microplate scintillation technology has placed receptor assays among the methods meeting these criteria.

### References

1. Doucette, G.J., Logan, M.L., Van Dolah, F.M. and Ramsdell, J.S. (1997) Development and preliminary validation of a microtiter plate-based receptor assay for paralytic shellfish poison toxins. *Toxicon* **35**, 625-636.
2. Powell, C.L. and Doucette, G.J. (2000) A receptor binding assay for paralytic shellfish poisoning: recent advances and applications. *Nat. Toxins* (in press).
3. Gessner, B., Bell, P., Doucette, G.J., Moczydlowski, E., Poli, M.A., Van Dolah, F.M., and Hall, S. (1997) Hypertension and identification of toxin in human urine and serum following a cluster of mussel-associated paralytic shellfish poisoning outbreaks. *Toxicon* **35**, 711-722.
4. Poli, M.A., Mende, T.J., & Baden, D.G. (1986) *Mol. Pharmacol.* **30**, 129-135.
5. Van Dolah, F.M., Finley, E.L., Haynes, B.L., Doucette, G.J., Moeller, P.D., and Ramsdell, J.S. (1994) Development of rapid and sensitive high throughput assays for marine phycotoxins. *Nat. Toxins* **2**: 189-196.
6. Dickey, R.W., Manger, R.L., Poli, M.A., Van Dolah, F.M., Delgado, J.A., Leighfield, T.A., & Granade, H.R. (1995) Assessment of methods for the determination of ciguatoxins. Proc. Fifth Pan American Symposium of the International Society of Toxinology, Frederick, MD, August 1995, 117.
7. Poli, M.A., Musser, S.M., Dickey, R.W., Eilers, P.P., & Hall, S. (2000) *Toxicon* **38**, 981-993.
8. Van Dolah, F.M., Zevotek, N.A., Finley, E.L., Doucette, G.J., Moeller, P.D., and Ramsdell, J.S. (1995) A high throughput domoic acid receptor assay utilizing microplate scintillation technology. In, "Harmful Algal Blooms" [Lassus, P., Arzul, G., Erard, E., Gentien, P., and Marcaillou, C., Eds.] , Lavoisier Science Publ., Paris, 365-370.
9. Van Dolah, F.M., Leighfield, T.A., Haynes, B.L., Hampson, D.R., and Ramsdell, J.S. (1997) A microplate receptor assay for the amnesic shellfish poisoning toxin, domoic acid, utilizing a cloned glutamate receptor. *Anal. Biochem.* **245**, 102-105.
10. Scholin, CA, Gulland, F., Doucette, G.J., Benson, S., Busman, M., Chavez, F.P., Cordaro, J., De Long R., de Vogelaere, A., Harvey, J., Haulena, M., Lefebvre, K., Limscomb, T., Luscatoff, S., Lowenstine, L.J., Marin, R., Miller, P., McLellan, W.A., Moeller, P.D.R., Powell, C., Rowles, T., Silvagni, P., Silver, M., Spraker, T., Trainer, V., Van Dolah, F.M. (2000) *Nature* **403**, 80-83.

π

---

## Marine Algal Toxins: Origins, Health Effects, and Their Increased Occurrence

Frances M. Van Dolah

Marine Biotoxins Program, NOAA National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, Charleston  
South Carolina USA

### ABSTRACT

Certain marine algae produce potent toxins that impact human health through the consumption of contaminated shellfish and finfish and through water or aerosol exposure. Over the past three decades, the frequency and global distribution of toxic algal incidents appear to have increased, and human intoxications from novel algal sources have occurred. This increase is of particular concern, since it parallels recent evidence of large-scale ecologic disturbances that coincide with trends in global warming. The extent to which human activities have contributed to their increase therefore comes into question. This review summarizes the origins and health effects of marine algal toxins, as well as changes in their current global distribution, and examines possible causes for the recent increase in their occurrence. Key words: amnesic shellfish poisoning, diarrhetic shellfish poisoning, dinoflagellates, domoic acid, global climate change, harmful algal blooms, marine toxins, neurotoxic shellfish poisoning, okadaic acid, paralytic shellfish poisoning.— *Environ Health Perspect* 108(suppl1):133-141 (2000).

[http://ehpnetl.niehs.nih.gov/docs/2000/suppl-1/133-141\\_vandolah/abstract.html](http://ehpnetl.niehs.nih.gov/docs/2000/suppl-1/133-141_vandolah/abstract.html)

---

Marine algal toxins are responsible for an array of human illnesses associated with consumption of seafood and, in some cases, respiratory exposure to aerosolized toxins. Approximately 20% of all foodborne disease outbreaks in the United States result from the consumption of seafoods, with half of those resulting from naturally occurring algal toxins (1). On a worldwide basis, marine algal toxins are responsible for more than 60,000 intoxication incidents per year, with an overall mortality rate of 1.5%. In addition to their human health effects, algal toxins are responsible for extensive die-offs of fish and shellfish and have been implicated in the episodic mortalities of marine mammals, birds, and other animals dependent on the marine food web. The impacts of algal toxins are generally observed as acute intoxications, whereas the environmental health effects

of chronic exposure to low levels of algal toxins are only poorly documented and are an emerging issue (2-5).

The origins of marine algal toxins are unicellular algae that, in response to favorable conditions in their environment, may proliferate and/or aggregate to form dense concentrations of cells or "blooms." In many cases, toxic species are normally present in low concentrations, with no environmental or human health impacts; toxicity in general depends on their presence in high cell

---

Address correspondence to F.M. Van Dolah, Marine Biotoxins Program, NOAA National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research, 219 Fort Johnson Rd., Charleston, SC 29412. Telephone: (843) 762-8529, Fax: (843) 762-8700. E-mail: [fran.vandolah@noaa.gov](mailto:fran.vandolah@noaa.gov)

Received 6 October 1999; accepted 19 November 1999.  
(This article was originally published in *Environmental Health Perspective*, Vol. 108, supplement 1, March 2000 and reprinted here with the author's permission.)

## GENERAL INTRODUCTION

Table 1. Toxic syndromes associated with marine algal toxins.

Syndrome	Causative organism	Primary vector	Toxin	Pharmacologic target
PSP	<i>Alexandrium</i> spp. <i>Gymnodinium</i> spp. <i>Pyrodinium</i> spp.	Shellfish	Saxitoxins	Voltage-dependent sodium channel Site 1
NSP	<i>Gymnodinium breve</i>	Shellfish	Brevetoxins	Voltage-dependent sodium channel Site 5
CFP	<i>Gambierdiscus toxicus</i>	Reef Fish	Ciguatoxins	Voltage-dependent sodium channel Site 5
ASP	<i>Pseudo-nitzschia</i> spp.	Shellfish	Domoic acid	Glutamate receptors
DSP	<i>Dinophysis</i> spp. <i>Proracentrum</i> spp.	Shellfish	Dinophysis toxins, Okadaic acid	Ser/thr protein phosphatases
Estuary syndrome	<i>Pfiesteria piscicida</i>	Water	Unknown	Unknown

concentrations. Phytoplankton species that produce toxins, currently included under the broad term harmful algal blooms (HABs), previously were called red tides. Only about 2% (60-80 species) of the estimated 3,400-4,000 known phytoplankton taxa are harmful or toxic (6). Of these, members of two algal groups, the dinoflagellates and diatoms, produce toxins that impact humans. Filter-feeding shellfish, zooplankton, and herbivorous fishes ingest these algae and act as vectors to humans either directly (e.g., shellfish) or through further food web transfer of sequestered toxin to higher trophic levels. Consumption of seafood contaminated with algal toxins results in five seafood poisoning syndromes (Table 1): paralytic shellfish poisoning, neurotoxic shellfish poisoning, amnesic shellfish poisoning, diarrhetic shellfish poisoning, and ciguatera fish poisoning. Most of these toxins are neurotoxins and all are temperature stable, so cooking does not ameliorate toxicity in contaminated seafoods. In addition to foodborne poisonings, toxins

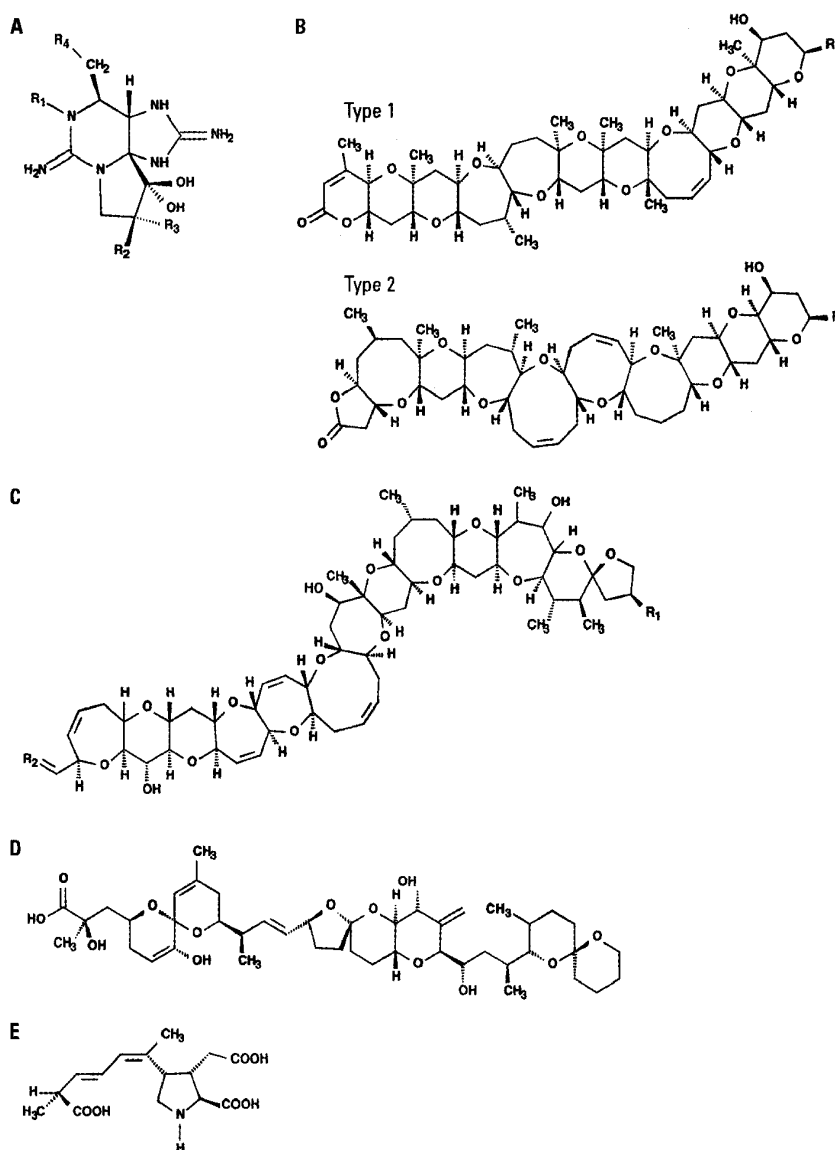
from two dinoflagellates sources are aerosolized (brevetoxins) or volatilized (a putative *Pfiesteria* toxin) to impact human health through the respiratory route.

Over the past three decades, the occurrence of harmful or toxic algal incidents has increased in many parts of the world, both in frequency and in geographic distribution (7-9). There are many contributing factors to this expansion, not the least of which is increased awareness of the issues and consequent establishment of research programs and surveillance systems, which in turn have helped identify problems not previously recognized. In certain instances, however, the expansion of toxic algal blooms to new geographic areas, resulting in human illness or environmental impacts, is well documented. There has been much speculation about the causes and significance of the observed expansion; however, few definitive studies have been carried out to date. Of particular concern is determining whether the apparent increase in harmful and toxic algal

blooms is a consequence of anthropogenic activities, which might therefore be modified to reverse the current trends. Human activities may contribute to the problem directly or indirectly through the introduction of non-indigenous species via ballast water transport or shellfish transplantation, local and regional environmental change caused by eutrophica-

tion or contaminant loading, large-scale climate fluctuations (anomalous weather events; El Niño), and global climate change mediated by the anthropogenic increase in greenhouse gases. In this review, we first summarize the origins, health effects! and changes in global distribution of each of the toxin classes of human health significance, and then

Figure 1. Marine algal toxins that impact human health. (A) Saxitoxin (B) brevetoxin (C) ciguatera toxin (D) okadaic acid (E) domoic acid



## GENERAL INTRODUCTION

consider the current status of our understanding of the causes and implications of their increased occurrences. The review does not attempt to address nuisance blooms or freshwater algal toxins, which can be found in previous reviews on the global increase in harmful algal blooms (8-13).

### Paralytic Shellfish Poisoning

Paralytic shellfish poisoning (PSP) is caused by the consumption of molluscan shellfish contaminated with a suite of heterocyclic guanidines collectively called saxitoxins (STXs) (Figure 1A). On a global basis, almost 2,000 cases of human poisonings are reported per year, with a 15% mortality rate (8). In addition to human intoxications, PSP has been implicated in deaths of birds (14) and humpback whales (15). An ecologic role for PSP toxins as a chemical defense is suggested by the sequestration of PSP toxins in the butter clam, modulation of feeding behavior in sea otters by toxic clams, and a distribution of Alaskan sea otters that coincides with nontoxic clam populations (16;17). STX elicits its effects by binding with high affinity ( $K_d \sim 2$  nM) to site 1 on the voltage-dependent sodium channel, inhibiting channel conductance and thereby causing blockade of neuronal activity. The primary site of STX action in humans is the peripheral nervous system, where its binding results in rapid onset of symptoms (less than 1 hr) that are classic for PSP: tingling and numbness of the perioral area and extremities, loss of motor control, drowsiness, incoherence, and in the case of high doses, respiratory paralysis. The lethal dose in humans is 1-4 mg STX equivalents (18). Since shellfish may on rare occasions contain greater than 10,000  $\mu\text{g}/100$  g STX equivalents, ingestion of

only a few shellfish meats may in these instances be fatal. In a study of a PSP out-break in Kodiak, Alaska, clearance of PSP toxins from the blood was complete in < 24 hr, even in patients who experienced respiratory paralysis (19). The primary route of clearance was the kidney, as has also been demonstrated in rats (20) and cats (21). There are currently over 21 known STX congeners that are produced in varying combinations and proportions by several gonyaulacoid and gymnodinioid dinoflagellate species in three genera: *Alexandrium*, *Gymnodinium*, and *pyrodinium*.

PSP-producing dinoflagellates occur in both temperate and tropical waters. In North America, PSP occurs seasonally both on the east coast (Newfoundland to Massachusetts) and on the west coast (Alaska to California). Anecdotal reports identify PSP in both regions well before this century. Prior to the 1970s PSP was known to be endemic only to North America, Europe, and Japan, whereas, currently, PSP outbreaks are also documented in South America, Australia, Southeast Asia, and India (8). The reasons for this expansion into the southern hemisphere, or in some cases whether these in fact represent expansions of geographic ranges of toxic organisms, are not entirely clear. The first confirmed outbreak of PSP in South America occurred in Chile in 1972 and was associated with a bloom of *Alexandrium catenella*. Subsequent outbreaks occurred in 1981 and 1989 (22). Since 1991, 21 fatalities and several hundred intoxications have occurred in Chile (23). A similar pattern of increased occurrence of PSP is reported in Argentina, where the first PSP outbreak, which occurred in 1980, was associated with a bloom of *Alexandrium tamarense*. Following

sporadic outbreaks during the 1980s, shellfish toxicity associated with PSP has been an annual occurrence throughout the 1990s along the Argentine coast (24). As in North America, however, anecdotal stories suggest that PSP occurred in both Chile (22) and Argentina (25) in the last century, an indication that the recent increase in reported PSP events may be attributed at least in part to the establishment of algal and shellfish monitoring programs.

A similar scenario occurred over the past two decades in the Indo-Pacific. The causative organism for Indo-Pacific PSP intoxications is *pyrodinium bahamense* var. *compressum*. The first reported outbreak of PSP in this region occurred in 1972 in Papua New Guinea and subsequently spread to Brunei beginning in 1976, central Philippines in 1983, northern Philippines in 1987, and Malaysia in 1988. In the Philippines alone, approximately 2,000 cases of PSP occurred between 1983 and 1998, with a mortality rate of 5.8%; most cases occurred among the young (< 20 years of age) (26). The expansion of *Pyrodinium* blooms in this region appears to be a range extension in at least some of these instances, as it is absent from phytoplankton records in the Philippines prior to the initial PSP outbreak in 1983 (27). There is compelling evidence to suggest that the expansion of toxic blooms of *Pyrodinium* in the tropical Pacific may correlate with the occurrence of El Niño-Southern Oscillation events (28).

Recurrent PSP events in Tasmania and southern Australia associated with the dinoflagellate *Gymnodinium catenatum* began in late 1985 (8). The absence of cysts from sediments before 1972 suggests that this species was introduced to the region around that time (29). In

this case, the route of introduction has been proposed to be ballast water transport from Japan or Europe, as viable cysts have been isolated from vessel ballast water arriving in Tasmania (30). However, genetic analysis of Tasmanian and Australian strains has not unequivocally identified their geographic origins (31).

### Neurotoxic Shellfish Poisoning

Neurotoxic shellfish poisoning (NSP) generally results from consumption of molluscan shellfish contaminated with brevetoxins (PbTx), a suite of nine structurally related ladderlike polycyclic ether toxins (Figure IB). Brevetoxin congeners are of two types based on backbone structure: brevetoxin B backbone (type 1; PbTx-2,3,5,6,8,9) and brevetoxin A backbone (type 2; PbTx-1,7,10).

Brevetoxins bind with high affinity ( $K_d$  1-50 nM) to site 5 on the voltage-dependent sodium channel (32). Binding to this site both alters the voltage sensitivity of the channel, resulting in inappropriate opening of the channel under conditions in which it is normally closed, and inhibits channel inactivation, resulting in persistent activation or prolonged channel opening. This complex action is believed to result from the intercalation of the brevetoxin polyether backbone between transmembrane domains of the sodium channel such that it interacts with both the voltage sensor near the outside of the channel and the inactivation gate on the intra-cellular side (33-37). Symptoms of NSP include nausea, tingling and numbness of the perioral area, loss of motor control, and severe muscular ache. Steidinger et al. (38) examine an unusually severe intoxication of young children that occurred because of consumption of

## GENERAL INTRODUCTION

contaminated clams (*Chione cancellata*) and resulted in seizures and unconsciousness. Unlike PSP, NSP has not been documented as a fatal intoxication in humans.

The occurrence of NSP has historically been limited to the west coast of Florida, where blooms of the dinoflagellate *Gymnodinium breve* initiate offshore and are subsequently carried inshore by wind and current conditions (39). Gulf of Mexico *G. breve* blooms are occasionally carried around the Florida peninsula by the Loop Current and northward by the Gulf Stream, resulting in red tides on the east coast of Florida and, in a single incident in 1987, as far north as North Carolina (40). The unprecedented red tide in North Carolina, which resulted in over 48 cases of shellfish poisoning, was supported by a shoreward intrusion of warm Gulf Stream water onto the narrow continental shelf which remained intact in nearshore waters for several weeks (41). Although the 1987 outbreak of *G. breve* is often cited as a range extension, low but consistent background concentrations of *G. breve* are routinely found in the Gulf Stream in the South Atlantic Bight (42). Therefore, the 1987 bloom in North Carolina may be the result of the concurrence of a large-scale sea surface temperature anomaly and local conditions (43) rather than being a true range extension of the organism. In 1993, an unprecedented outbreak of shellfish toxicity in New Zealand was identified as NSP. The source organisms of toxins in this outbreak were novel *Gymnodinium* species (referred to as *Gymnodinium* cf. *breve*) that produce NSP-like toxins (44).

Unlike most other dinoflagellates responsible for seafood poisonings, *G. breve* is an unarmored dinoflagellate that is easily lysed in turbulent water. *G.*

*breve* red tides therefore are frequently associated with massive fish kills. The extreme sensitivity of fish to the Florida red tide may result from lysis of cells passing through the gills, with direct transfer of toxin across the gill epithelium. An additional route of human exposure to brevetoxins is similarly through respiration, in this case of aerosolized toxin, which is the result of cells breaking due to wave action. A common symptom associated with exposure to aerosolized brevetoxin is irritation and burning of the throat and upper respiratory tract. In 1996 at least 149 manatees died during an unprecedented epizootic in Florida concurrent with a persistent red tide. Immuno-histochemical staining of tissues from affected animals revealed brevetoxin immuno-reactivity in lymphocytes and macrophages associated with inflammatory lesions of the respiratory tract and with lymphoid tissues (45). Molecular modeling studies have implicated brevetoxin as an inhibitor of a class of lysosomal proteases, the cysteine cathepsins, which are important in antigen presentation (46). The demonstration of brevetoxin immuno-reactivity in lymphoid tissue of the manatees raises the possibility of immunosuppression as a second mode by which brevetoxin exposure may affect human health, particularly in individuals with chronic exposure to aerosolized toxin during prolonged red tide incidents.

### Ciguatera Fish Poisoning

Ciguatera fish poisoning (CFP) is another seafood intoxication caused by ladderlike polyether toxins, primarily attributed to the dinoflagellate, *Gambierdistus toxicus* (47), which grows as an epiphyte on filamentous macroalgae associated with coral reefs and reef la-

goons. The lipophilic precursors to ciguatoxin produced by *G. toxicus* enter the food web when these algae are grazed upon by herbivorous fishes and invertebrates. These precursors are biotransformed to ciguatoxins (48) and bioaccumulated in the highest trophic levels. Large carnivorous fishes associated with coral reefs are a frequent source of ciguatera. Baracuda, snapper, grouper, and jacks are particularly notorious for their potential to carry high toxin loads; however, smaller herbivorous fishes may also be ciguatoxic, particularly when viscera are consumed. CFP is estimated to affect over 50,000 people annually and is no longer a disease limited to the tropics because of travel to the tropics and shipping of tropical fish species to markets elsewhere in the world (1). The symptoms of ciguatera vary somewhat geographically as well as between individuals and incidents and may also vary temporally within an area, but they generally include early onset (2-6 hr) gastrointestinal disturbance – nausea, vomiting, and diarrhea – and may be followed by a variety of later onset (18-hr) neurologic sequelae such as numbness of the perioral area and extremities, reversal of temperature sensation, muscle and joint aches, headache, itching, tachycardia, hypertension, blurred vision, and paralysis. Ciguatera on rare occasions can be fatal. A chronic phase may follow acute intoxication and can persist for weeks, months, or even years (49). Ciguatera symptoms in the Caribbean differ somewhat from those in the Pacific; gastrointestinal symptoms dominate in the former, whereas in the latter neurologic symptoms tend to dominate. This may reflect geographic differences in the toxin congeners involved (50).

The ciguatoxins (CTXs) (Figure IC) are structurally related to the brevetoxins and compete with brevetoxin for binding to site 5 on the voltage-dependent sodium channel with a high affinity ( $K_d \sim 0.04\text{-}4$  nM) (51). The minimum toxicity level to humans is estimated at 0.5 ng/g (52). Among the CTX congeners, binding affinity correlates well with toxic potency intraperitoneally in mice. However, the toxic potency of CTX in mice is several orders of magnitude greater than that of the brevetoxins relative to their binding affinities at the sodium channel (e.g., for CTX1 and PbTx3,  $LD_{50} = 0.25$   $\mu\text{g}/\text{kg}$  vs  $> 200$   $\mu\text{g}/\text{kg}$ , whereas  $K_d = 0.04$  nM vs 2 nM, respectively). This may be related to differences in the bioavailability of the toxins or to undefined toxic effects of ciguatoxin.

Although CFP occurs persistently at certain locations, outbreaks are sporadic and unpredictable at others. Reef disturbance due to storm damage or human activities frequently precedes ciguateric conditions (53,54). The overgrowth of corals by macroalgae because of coral bleaching, overfishing, or nutrient enrichment (55,56) may also promote ciguateric conditions by providing increased substrate for the epiphytic *G. toxicus*. Changes in sea surface temperature associated with El Niño events also appear to correlate with localized changes in the frequency of ciguatera cases in the Asian Pacific (57).

### Diarrhetic Shellfish Poisoning

Diarrhetic shellfish poisoning (DSP) is a comparatively milder seafood intoxication that consists of rapid onset (3 hr) gastrointestinal symptoms such as vomiting and diarrhea that generally resolve within 2-3 days. The diarrhetic shellfish toxins (DTX) are a class of acidic poly-

## GENERAL INTRODUCTION

ether toxins consisting of at least eight congeners including the parent compound, okadaic acid (Figure ID). The first incidence of human shellfish-related illness identified as DSP occurred in Japan in the late 1970s, where the dinoflagellate *Dinophysis fortii* was identified as the causative organism; the toxin responsible was termed dinophysin toxin (DTX-1) (58,59). DSP is widespread in its distribution, with essentially seasonal occurrences in Europe and Japan, but it has also been documented in South America, South Africa, New Zealand and Australia, and Thailand. The first confirmed incidence of DSP in North America occurred in 1990 in Nova Scotia, Canada. The causative organism in this outbreak was the benthic dinoflagellate, *Prorocentrum lima* (60,61), which was found in association with filamentous algae growing on raft cultures of mussels. Toxic *P. lima* was recently identified in Maine coastal waters, where the presence of DSP has been suspected (62). *P. lima* is a cosmopolitan dinoflagellate, with a range that extends from temperate waters to tropical reefs.

Okadaic acid, DTX-1, and DTX-2 are the primary congeners involved in shellfish poisoning, with the other congeners believed to be either precursors or shellfish metabolites of the active toxins. The DTXs are inhibitors of ser/thr protein phosphatases. Inhibitory activity against protein phosphatases is selective for classes PP2A (okadaic acid  $IC_{50} \sim 0.5$  nM) and PPI (okadaic acid  $IC_{50} \sim 50$  nM), with PP2B being inhibited only at high concentrations (okadaic acid  $IC_{50} > 1\mu M$ ) and PP2C being insensitive. The binding site for okadaic acid resides on the catalytic subunit of the protein phosphatase at the active site of the enzyme, as determined by X-ray crystal structures

(63), molecular modeling (64), and mutational analyses (65). Ser/thr protein phosphatases are critical components of signaling cascades in eukaryotic cells that regulate a diverse array of cellular processes involved in metabolism, ion balance, neurotransmission, and cell cycle regulation (66). Diarrhea associated with DSP is most likely due to the hyperphosphorylation of proteins, including ion channels, in the intestinal epithelia (67), resulting in impaired water balance and loss of fluids. The toxic potency of okadaic acid is much lower than that of the neurotoxin polyethers, with an  $LD_{50}$  of 192  $\mu g/kg$  (intraperitoneally) in mice (68). However, okadaic acid like polyether toxins have been identified as tumor promoters (69,70), thus raising the question of what effect low levels of chronic exposure to DSP toxins may have on humans as well as wildlife such as marine turtles (3).

### Amnesic Shellfish Poisoning

Amnesic shellfish poisoning (ASP) is the only shellfish intoxication caused by a diatom. The first recorded occurrence of ASP was in Prince Edward Island, Canada in 1987 when approximately 100 people became ill and several died after consuming contaminated mussels. None of the known shellfish toxins were found to be involved in the outbreak; rather the toxic agent was identified as domoic acid (71,72). The source of domoic acid was the diatom *Pseudo-nitzschia multiseries* (formerly known as *Nitzschia pungens fmultiseries*) (73,74). Domoic acid is a water-soluble tricarboxylic amino acid that acts as an analog of the neurotransmitter glutamate and is a potent glutamate receptor agonist. Domoic acid is related both structurally and functionally to the excitatory neurotoxin kainic

acid isolated from the red macroalga *Digenea simplex* (75). Seven congeners to domoic acid have been identified (Figure 1E). Of these, three geometrical isomers, isodomoic acids D, E, and F and the C5' diastereomer are found in addition to domoic acid in small amounts in both the diatom and in shellfish tissue (76,77).

The symptoms of ASP include gastrointestinal effects (e.g., nausea, vomiting, diarrhea) and neurologic effects such as dizziness, disorientation, lethargy, seizures, and permanent loss of short-term memory. Domoic acid binds with high affinity to both kainate ( $K_d \sim 5$  nM) and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ( $K_d \sim 9$  nM) subtypes of glutamate receptor (78,79). Persistent activation of the kainate glutamate receptor results in greatly elevated intracellular  $\text{Ca}^{2+}$  through cooperative interactions with N-methyl-D-aspartate and non-N-methyl-D-aspartate glutamate receptor subtypes followed by activation of voltage dependent calcium channels (80). Neurotoxicity due to domoic acid results from toxic levels of intracellular calcium, which leads to neuronal cell death and lesions in areas of the brain where glutaminergic pathways are heavily concentrated. The CA1 and CA3 regions of the hippocampus, an area responsible for learning and memory processing, are particularly susceptible (81). However, memory deficits occur at doses below those causing structural damage (82). In rats, the  $\text{LD}_{50}$  (intraperitoneally) for domoic acid is 4 mg/kg, whereas the oral potency is substantially lower (35-70 mg/kg) (83). In the 1987 outbreak, human toxicity occurred at 1-5 mg/kg, suggesting that susceptible individuals are more sensitive than rodent models to the oral toxicity of domoic

acid. Individuals found most susceptible were the elderly and those with impaired renal function resulting in poor toxin clearance. A predisposing factor of impaired renal clearance is consistent with results observed in studies on experimental animals (84).

No human outbreaks of ASP have occurred since the original 1987 incident in Canada. However, since the 1987 outbreak, domoic acid has been identified as the causative agent in the mass mortality of pelicans and cormorants in Monterey Bay, California, in 1991 (85,86) and in the extensive die-off of California sea lions in the same region in 1998 (87,88). In both instances the vector for toxin transfer was anchovy. The causative organism in both the 1991 and 1998 mortality events was identified as another member of the same diatom genus, *Pseudo-nitzschia australis*. At least seven species of *Pseudo-nitzschia* are now recognized as domoic acid producers. These toxin-producing *Pseudo-nitzschia* species have since been identified in widely inverse geographic areas around the world, including New Zealand, Denmark, Spain, Scotland, Japan, and Korea (89). The presence of *Pseudo-nitzschia* in New Zealand was recorded as early as 1959. However, domoic acid was not detected in New Zealand until after implementation of a comprehensive marine toxin monitoring program following the unprecedented NSP shellfish poisoning event in 1993. Domoic acid in New Zealand mussels and scallops occasionally reaches levels that result in shellfish bed closures (90).

#### **Pfiesteria and Estuary-Associated Syndrome**

*Pfiesteria piscicida*, a fish-killing dinoflagellate first identified in aquaculture

## GENERAL INTRODUCTION

tanks in North Carolina (91,92), has been linked to fish kills in the mid-Atlantic region of the United States and is characterized by the presence of open, ulcerative lesions (93). *Pfiesteria* has been termed an "ambush predator" because it is believed to release a toxin that narcotizes or kills fish and then phagocytizes the sloughed tissue from its prey (94). *Pfiesteria* has a complicated life cycle with numerous life cycle stages (94,95) including resting cysts, free-swimming flagellates (toxic and nontoxic), and amoeboid forms in which an amoeboid form is the dominant life-cycle stage. The cyst stage is viable under extreme conditions and is induced to excyst to form the toxic flagellate stage in the presence of fish. Subsequent depletion of its food supply results in rapid encystment of the flagellate. The identity of the putative chemical stimulus for *pfiesteria* excystment has not yet been elucidated.

In addition to its effects on fish, *pfiesteria* has been linked to a human intoxication syndrome, with symptoms that include fatigue, headache, respiratory irritation, skin lesions or burning sensations on contact, disorientation, and memory loss (96). An epidemiologic study of people exposed to *Pfiesteria* or *Pfjesteria*-associated water demonstrated severe impairment in cognitive functions compared to those of unexposed individuals from the same occupational, educational, and geographic area (97). Because of the remaining uncertainty regarding the causal role of *Pfiesteria* in these impairments, these symptoms have conservatively been termed estuary-associated syndrome (98). Experimental injection of rats with *Pfiesteria*-containing aquarium water resulted in neurocognitive impairment in the rats when they were tested using a radial-arm

maze (99). The toxins responsible for fish lethality or neurologic symptoms have not yet been identified. There is currently no evidence that toxicity is transferred through food.

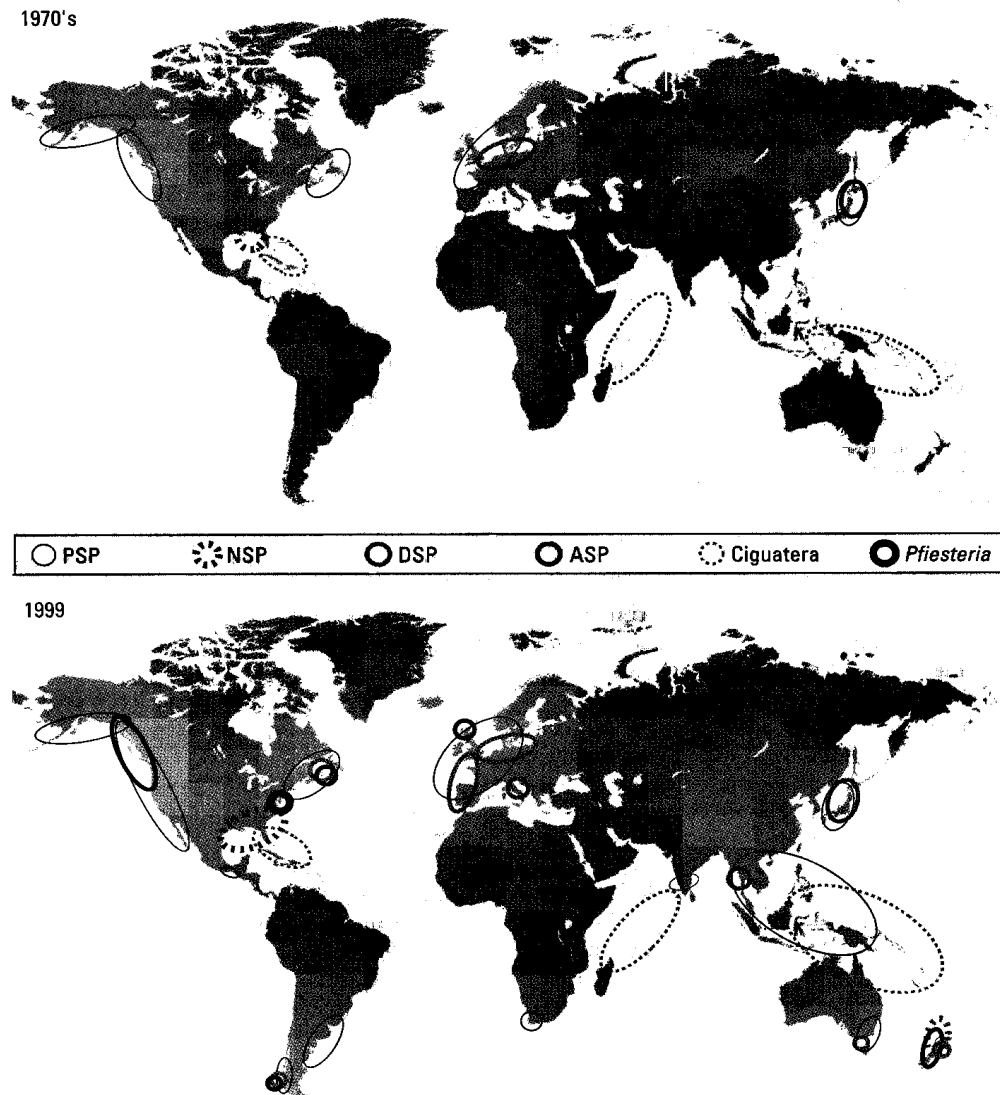
*Pfiesteria* differs from the previously discussed dinoflagellates in that it is a non-photosynthetic, heterotrophic dinoflagellate. Nonetheless, there is compelling evidence that its occurrence at toxic levels coincides with the eutrophication of coastal waters through intensive swine and poultry agriculture in areas of North Carolina and Maryland. Lagoon-stored and land-applied wastes from poultry and swine are a major source of nitrogen input from both runoff and atmospheric deposition and are suspected of supporting enhanced growth of autotrophic algae that in turn support the proliferation of *pfiesteria* (4, 100). The study of *pfiesteria piscida* has led to recognition of the widespread occurrence of heterotrophic dinoflagellates in estuarine waters, referred to as the *Pfiesteria*-like complex. *Pfiesteria*-like dinoflagellates have been documented on the east coast of the United States from Long Island to Florida. The historical distribution of these dinoflagellates is not known nor is their toxicity or their role(s) in fish kills firmly established at this time.

### **Global Increase in the Occurrence of Algal Toxins**

Figure 2 illustrates the change in global distribution of the occurrence of algal toxins of human health concern over the past three decades. Of particular significance is the occurrence of novel toxic algal syndromes not previously known as well as expansion of a number of toxin classes from the northern to the southern hemisphere during this time frame. It is important to note that this

distributional map is a composite picture that reflects both increases in reports due to increased monitoring for toxins as well as true geographic expansions in the occurrence of toxic outbreaks. Newly established or expanded research and ▼

following an unprecedented toxic outbreak in 1992 established a comprehensive monitoring program that has since identified four or the five major toxin classes in shellfish at levels sufficient to cause occasional closures of shellfish



**Figure 2.** Global increase in reported incidence of algal toxins. Encircled areas indicate where outbreaks have occurred or toxin has been detected at levels sufficient to impact human or environmental health.

monitoring programs for algal toxins have been significant in identifying the presence of toxic or potentially toxic organisms in places never before documented. For example, New Zealand had no reported algal toxins in the 1970s but

harvest. Improvement in toxin detection methods has further contributed to expanded ranges reported in Figure 2. For example, the occurrence of ASP was unknown until the 1987 outbreak in Canada. It has subsequently been impli-

## GENERAL INTRODUCTION

cated in bird (1991) and marine mammal (1998) mortalities on the west coast of North America. Although these mortality events raise significant alarm in light of the apparent increase in the global impacts of algal toxins, unexplained episodic marine mammal mortality events, some of which appear to have similarities to the ASP-associated events, have occurred every few years on the west coast of North America for the past several decades (87). Thus, retrospective analysis of tissues from earlier mortality events, where available, may provide insight into the historical impacts of toxic blooms of *Pseudonitzschia* in this region.

In other cases, the distributions shown in Figure 2 are expansions in the known geographic range of toxic algal species at concentrations sufficient to impact human or environmental health. The expansions of PSP in southeast Asia and South America are key examples. The causes of these expansions are not well defined. The lack of long-term phytoplankton data often hinders the ability to determine if novel outbreaks are the consequence of the introduction of organisms to new regions, an increase in the local abundance of a toxic organism because of changes in local or regional nutrient conditions, or range extensions due to large-scale climatic changes. The key issue is to what extent human impacts on the environment are responsible for these increases. The expansion of harmful and toxic algal blooms is seen by some as a bioindicator of large-scale marine ecologic disturbances (9,101,102). The four primary means by which human activities may contribute to the expansion in the distribution of toxic algae must therefore be viewed in the broader context of human impacts on marine ecosystem health: transport of

exotic species, eutrophication of coastal waters, anomalous weather events, and global climate change.

### Transport

A principal mechanism for the transfer of non indigenous and invasive species among marine ecosystems is through the movement of ships' ballast water (103). Ballast water is used to stabilize ships when they are not carrying cargo. Water is pumped into a ship's ballast tanks in the port of origin, then released upon arrival at its destination port prior to loading cargo. If conditions are conducive to growth, organisms carried in the released ballast water may establish populations in the receiving port. It is estimated that 10 billion tons of ballast water are transported each year, making it a major source of international pollution (104). Ballast water has been shown to be responsible for the transfer of pathogenic bacteria, protists, algae, zoo-plankton, benthic invertebrates, and fish. A number of these invasions have had significant ecologic and economic impacts (105) or human health effects (106). Transport of toxic algal cells and cysts in the ballast water of ships is a documented mode of transfer of toxic dinoflagellates and diatoms to previously unaffected regions (30,107,108) and a likely source of PSP in Australian waters. To address this issue, open ocean exchange of ballast water has been recommended by the International Maritime Organization (IMO), under current voluntary guidelines, as an effective means to minimize the introduction of exotic species (104). Further, a joint study group on ballast water and sediments by the International Council for Exploration of the Seas, the Intergovernmental Oceanographic Commission, and the IMO has since been

established to develop mandatory ballast water guidelines.

Perhaps the most direct human impact on the distribution of toxic algal species is through the transfer of molluscan shellfish from growing waters in an area endemic to toxic algal species to areas in which toxic blooms have not previously occurred. When shellfish are transported from a toxic area to clean waters, they may release cysts and/or motile algal cells that may seed a bloom of the toxic algae if environmental conditions in the receiving waters are conducive to growth (109). Thus, many countries have established regulations to prohibit placing mussels from potential PSP risk areas to other areas in an effort to control spreading of blooms (10).

### **Eutrophication**

Phytoplankton productivity in oceanic, estuarine, and coastal waters is primarily limited by the availability of nitrogen (12). Nitrogen loading has been implicated in accelerated phytoplankton production, or eutrophication, which is most notably manifested as algal blooms. A major source of anthropogenically introduced nitrogen is atmospheric deposition from agricultural, urban, and industrial sources (12). Atmospheric deposition by-passes estuarine processes that filter terrestrial sources of nitrogen, thereby directly affecting both coastal and oceanic environments. Coastal waters in developed countries have experienced a long-term increase in the loading of both nitrogen (N) and phosphorus (P), by more than a factor of four, compared with several decades ago (110). There is strong evidence that coastal eutrophication contributes to the increased incidence of certain harmful algal blooms (4,8,9,12,111). An often cited example is

the correlation between the 8-fold increase in frequency of algal blooms in Hong Kong Harbor from 1976 to 1986 and a 6-fold increase in population during that period; the latter was accompanied by a 2.5 times increase in nutrient loading (112). Although long-term data sets on phytoplankton assemblages are not available for many areas, changes in dinoflagellate cyst assemblages in surface sediments can be used to document eutrophication, as shown in Tokyo Bay (113). This approach was critical in identifying *Gymnodinium catenatum* as an introduced organism in Australian waters and is currently being employed to establish the history of PSP-producing *Pyrodinium* blooms in Southeast Asia. Not only is the total concentration of enhanced nutrients of significance, but the altered ratios of these nutrients relative to those of other nutrients such as silica are also relevant. Long-term data sets in northern Europe strongly support the hypothesis that decreases in Si:N and Si:P ratios favor the growth of flagellates over silica-dependent diatoms (9, 111,114). Similar observations have recently been made in conjunction with Mississippi River input to the Gulf of Mexico (115). However, the role of coastal eutrophication in the increased incidence of harmful algal blooms may not be generalized to all HAB species. For example, there currently is no strong evidence to suggest that eutrophication of coastal waters contributes to PSP (*Alexandrium tamarense*) or NSP (*Gymnodinium breve*) blooms that originate in oligotrophic waters. The frequency and persistence of red tides on the west coast of Florida, for example, do not appear to have changed over the last 120 years (Figure 3). Each of the major range expansions of these organisms in North America has coin-

## GENERAL INTRODUCTION

cided instead with unusual climatic events (11,43).

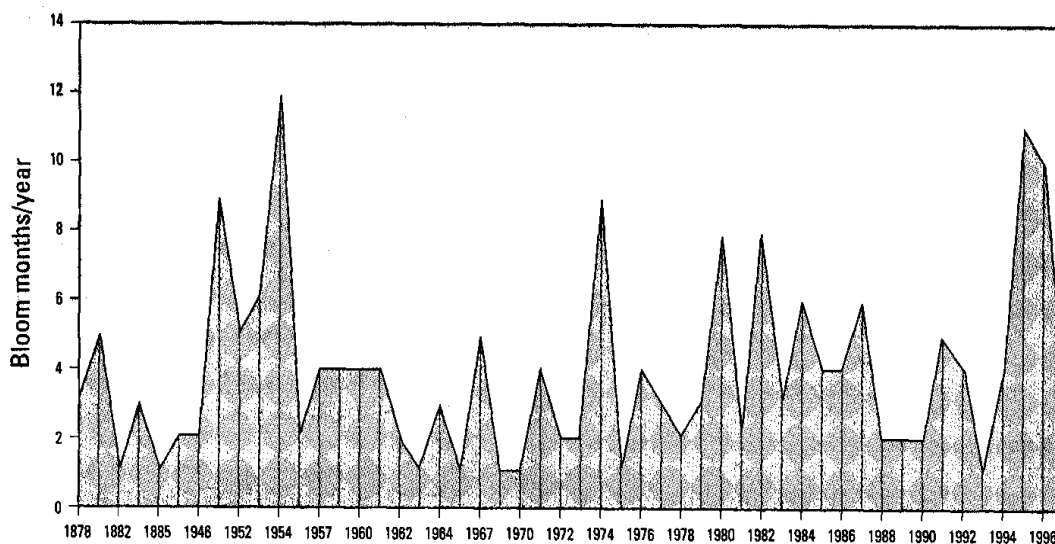
### Anomalous Weather Events

It is well established that many human disease outbreaks peak during unusual climatic events such as drought, storm events that produce heavy rainfall, and El Niño (57,106,116). Similarly, El Niño events are linked with the occurrence of diseases in marine species, including coral bleaching and shellfish diseases, and possibly marine mammal mortality events (102). El Niño occurs when prevailing easterly winds of the tropical Pacific fail. This ultimately suppresses upwelling of cold nutrient-rich water in the eastern Pacific and creates a pool of warm water in the west. Global precipitation changes as a consequence of El Niño are generally increased rainfall in Southeast Asia and drought in Australia, Africa, and India. The occurrence and/or expansion of human intoxication by PSP and CFP in the Indo-Pacific have been compellingly linked to the occurrence of El Niño conditions

(28,57). Historically, El Niño events have occurred at a frequency of one to two per decade. However, since the mid-1970s they have occurred more frequently and have persisted longer. The strongest recorded El Niño events (1982-1983 and 1997-1998) occurred during the past two decades, a period during which the sea surface temperatures rose and the incidence of HABs increased. The co-occurrence of these events late in this century has led to speculation that global warming may be an underlying cause (101,102).

### Global Climate Change

An observed increase in the global average temperature during the past century (0.8°C between 1889 and 1990) concurrent with industrialization suggests that the world may be entering a period of global warming to which human activities have contributed through production of greenhouse gases (117, 118). The oceans, because of their capacity as heat reservoirs, both influence and respond to



**Figure 3.** Occurrence of *Gymnodinium breve* red tides on the west coast of Florida (1878–1996), in months per year. Data from Steidinger (119).

global climate through thermohaline circulation, which recirculates nutrients, oxygen, and CO<sub>2</sub>. Long-term increases in sea surface temperature are predicted to modify the behavior of global oceanic circulation, which will further alter marine environmental regimes (46). The recent increases in frequency and emergence of novel diseases affecting marine organisms, from coral bleaching to shellfish pathogens, are believed to be linked to climate change (101,102). The role of global warming in the expansion of toxic algal blooms is difficult to test, however, because of the complexity of overlying issues. First, the issue of increased reporting of toxic algal incidents must be factored out. In addition, increased utilization of coastal regions must be taken into account. Currently, approximately 50% of the U.S. population resides within the coastal zone, and this figure is predicted to increase. Thus, in some instances, the increased human health impacts of toxic algal blooms are a result of increased human exposure to preexisting bloom activity rather than to increased bloom activity, as is the case for the rapidly developing west coast of Florida. Changes in local environment due to coastal development or eutrophication also must be factored out from large-scale climatic changes. Many areas of the world where toxic blooms have recently expanded are as used heavily for aquaculture. Aquaculture activity not only results in localized nutrient enrichment that may encourage growth of toxic species but places the vector for transmission of toxin to humans in this enriched environment. Finally, many areas of the world where toxic blooms have recently expanded lack long-term historical phytoplankton records with which to determine the origins of recent toxic algal

blooms. Many of the time series available that might be useful for quantifying changes in the frequency of toxic blooms are no more than a few decades in length, not a sufficiently long period of time to factor out interannual to decadal variability in climate and phytoplankton abundance (13). Therefore, until longer time series are available, it will be difficult to demonstrate definitively that the increases observed are true long-term increases as opposed to recent responses to interannual or decadal climate variability. The distribution of fossil cysts of certain toxic dinoflagellate species has yielded useful insight into their distribution during previous warmer climate regimes and long-term variability in abundance; thus, paleoecology may be a useful tool for developing potential distributional maps that might be encountered under warmer global conditions (8,46).

### Conclusions

Marine algal toxins impact human health through seafood consumption and respiratory routes. The apparent increase in their occurrence over the past three decades has raised alarm. A key issue is whether the increase in the occurrence of toxic algal blooms reflects large-scale marine ecologic disturbance due to human activities and whether these trends can be reversed. The environmental conditions that trigger toxic algal blooms are not well understood and differ between different algal species, making generalizations inappropriate. Thus, insight into physiology and bloom dynamics must be addressed for each toxic species. It is clear in some cases that human activities may directly contribute to the occurrence of favorable growing conditions for these microalgae. In other

## GENERAL INTRODUCTION

cases, the potential indirect role of human activities through large-scale changes in our global environment remains viable but is difficult to establish. Insight into the historical distributions of toxic species through cyst analysis and molecular genetics may help distinguish between changes in geographic range of organ-

isms and proliferation due to changes in local environmental conditions favoring bloom formation. Establishment of algal and toxin monitoring programs worldwide in the past two decades should assist in providing time series needed to assess inter-annual and long-term variability in algal and toxin occurrence.

## REFERENCES AND NOTES

1. Ahmed FE Seafood Safety Washington, DC:National Academy Press, 1991;432 pp.
2. Landsberg JH Neoplasia and biotoxins in bivalves: Is there a connection? *J Shellfish Res* 15:203-23 (1996).
3. Landsberg JH, Balazs GH, Steidinger KA, Baden DG, Work TM, Russel DJ, The potential role of natural tumor promoters in marine turtle fibropapillomatosis. *J Aquat Anim Health* 11:199-210 (1999).
4. Burkholder JM Implications of harmful microalgae and heterotrophic dinoflagellates in management of sustainable fisheries, *Ecol Appl* 8: S37-S62 (1998).
5. Edmunds, JSG, McCarthy RA, Ramsdell JS Ciguatoxin reduces larval survivability in finfish. *Toxicon* 37:1827-1832 (1999).
6. Smayda TJ Harmful algal blooms: their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnol Oceanogr* 42:1137-1153 (1997).
7. Anderson DM Toxic algal blooms and red tides a global perspective In: *Red Tides Biology, Environmental Science and Toxicology* (Okaichi T, Anderson DM, Nemoto T, eds). New York:Elsevier, 1989;11-16.
8. Hallegraeff GM A review of harmful algal blooms and their apparent global increase. *Phycologia* 32:79-99 (1993).
9. Smayda TJ. Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: *Toxic Marine Phytoplankton* (Graneli E, Sundstrom S, Edler L, Anderson DM, eds). New York:Elsevier, 1990;29-40
10. Shumway SE. A review of the effects of algal blooms on shellfish and aquaculture. *J World Aquaculture Soc* 21:65-1104 (1990).
11. Anderson DM, Red tides. *Sci Am* 271:52-58 (1994).
12. Pearl HW, Whitall DR Anthropogenically-driven atmospheric nitrogen deposition, marine eutrophication and harmful algal bloom expansion: is there a link? *Ambio* 28:307-311 (1999).
13. Richardson K. Harmful or exceptional phytoplankton blooms in the marine ecosystem. *Adv Mar Biol* 31:301-385 (1997).
14. Nisbet IC. Paralytic shellfish poisoning effects on breeding terns *Condor* 85:338-345 (1983).
15. Geraci JR, Anderson DM, Timperi RJ, St. Aubin DJ, Early GA, Prescott JH, Mayo CA. Humpback whales fatally poisoned by dinoflagellate toxin. *Can J Fish Res Aquat Sci* 46:1895-1898 (1989).
16. Kvitek RG, Beitler MK. A case for sequestering of paralytic shellfish toxins as a chemical defense. *J Shellfish Res* 7:629-636 (1988)
17. Kvitek RG, DeGange AR, Beitler MK. Paralytic shellfish poisoning toxins mediate feeding behavior in otters. *Limnol Oceanogr* 36:393-404 (1991)
18. Levin RE. Paralytic shellfish toxins: their origins, characteristics, and methods of detection a review. *J Food Biochem* 15:405-417 (1992).
19. Gessner BD, Bell P, Doucette GJ, Moczydlowski E, Poli MA, Van Dolah FM, Hall S. Hypertension and identification of toxin in human urine and serum following a cluster of mussel-associated paralytic shellfish poisoning outbreaks. *Toxicon* 35:711-722 (1997).
20. Hines HB, Naseem SM, Wannamacher RW Jr. <sup>3</sup>H-Saxitoxinol metabolism and elimination in the rat. *Toxicon* 31:905-908 (1993).
21. Andrinolo D, Michea LF, Lagos N. Toxic effects, pharmacokinetics and clearance of

- saxitoxin, a component of paralytic shellfish poison (PSP) in cats. *Toxicon* 37:447-464 (1999).
22. Benavides H, Prado L, Dial S, Carreto, JI. An exceptional bloom of *Alexandrium catenella* in the Beagle Channel, Argentina. In Harmful Algal Blooms (Lassus P, Arzul G, Erard E, Gentien P, Marcaillou C, eds) Paris: Lavoisier, 1995;113-119.
  23. Suarez-Isla BA. Personal communication
  24. Carreto JI, Molltoya NG, Cucchi Colleoni AD, Akselman R. *Alexandrium tamarense* blooms and shellfish toxicity in the Argentine Sea: a retrospective view, In: Harmful Algae (Reguera B, Blanco J, Fernandez M, Wyatt T, eds). Santiago del Compostella, Spain: Xunta de Galicia and Intergovernmental Oceanographic Commission, 1998; 131-134.
  25. Carreto JI, Elbusto C, Sancho H, Carignan M, Cucci Colleoni D, de Marco S, Fernandez A. An exploratory analysis of the Mar del Plata shellfish toxicity area (1980-1990). Toxic Phytoplankton Blooms in the Sea (Smayda TJ, Shimizu Y, eds). New York: Elsevier, 1993; 377-382.
  26. Azanza R, Cruz L, Personal communication.
  27. Furio EF, Fukuyo Y, Matsuoka K, Gonzales CL. The vertical distribution of resting cysts of PSP-producing dinoflagellate *Pyrodinium bahamense* var *compressum* in Manila Bay, Philippines. In: Harmful and Toxic Algal Blooms (Yasumoto T, Oshima Y, Fukuyo Y, eds). Paris: International Oceanographic Commission of UNESCO, 1996; 185-188.
  28. Maclean JL. Indo-Pacific red tides, 1985-1988 *Mar Pollut Bull* 20:304-310 (1989).
  29. McMinn A, Hallegraef GM, Thomson P, Jenkinson AV, Heijnis H. Cyst and radionucleotide evidence for the recent introduction of the toxic dinoflagellate *Gymnodinium catenatum* into Tasmanian waters, *Mar Ecol Prog Ser* 161:165-172 (1997).
  30. Hallegraef GM. Transport of toxic dinoflagellates via ships' ballast water bioeconomic risk assessment and efficacy of possible ballast water management strategies. *Mar Ecol Prog Ser* 168:297-309 (1998).
  31. Bolch CJS, Blackburn S, Hallegraef GM, Vaillancourt RE. Genetic variation among strains of the toxic dinoflagellate *Gymnodinium Catenatum* (Dinophyceae), *J Phycol* 35:356-367 (1999).
  32. Poli MA, Mende TJ, Baden DG. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Mol Pharmacol* 30:129-135 (1986).
  33. Trainer VL, Baden DG, Caterall WA. Identification of peptide segments of the brevetoxin receptor site of rat brain sodium channels, *J Biol Chem* 269:19904-19909 (1994).
  34. Rein K, Baden DG., Gawley RE. Conformational analysis of the sodium channel modulator, brevetoxin A, comparison with brevetoxin B conformations, and a hypothesis about the common pharmacophore of the site 5 toxins, *J Org Chem* 59:2102-2106 (1994).
  35. Rein K., Lylln B, Gawley R, Badell DG, Brevetoxin B chemical modifications, synaptosome binding, toxicity, and all unexpected conformational effect. *J Org Chem* 59:2107-2113 (1994).
  36. Gawley RE, Rein KS, Jeglitsch G, Adams DJ, Theodorakis EA, Tieves J, Nicolau KC, Baden DG. The relationship of brevetoxin 'length' and a-ring functionality to binding and activity in neuronal sodium channels. *Chem Biol Interact* 2:533-541(1995).
  37. Jeglitsch G, Rein K, Baden DG, Adams DJ. Brevetoxin 3 (PbTx-3) and its derivatives modulate single tetrodotoxin sensitive sodium channels in rat sensory neurons, *J Pharmacol Exp Therap* 284:516-525(1998).
  38. Steidinger KA, Carlson P, Baden D, Rodriguez C, Seagle J Neurotoxic shellfish poisoning due to toxin in the clam *Clione caninata*, In: Harmful Algae (Reguera B, Blanco J, Fernandez ML, Wyatt T, eds). Santiago del Compostella, Spain Xunta de Galicia and Intergovernmental Oceanographic Commission, 1998; 457-458 .
  39. Steidinger KA, Vargo GA, Tester PA, Tomas CR Bloom dynamics and physiology of *Gymnodinium breve* with emphasis on the Gulf of Mexico In: Physiological Ecology of Harmful Algal Blooms (Anderson DM, Cembella AD, Hallegraef GM, eds) Berlin: Springer-Verlag, 1998; 133-154.
  40. Tester PA, Steidinger KA *Gymnodinium breve* red tide blooms initiation, transport, and consequences of surface circulation *Limnol Oceanogr* 42:1039-1051 (1997).
  41. Tester PA, Stumpf RP, Vukovich FM, Fowler PK, Turner JT, An expatriate red tide bloom: transport, distribution, and persistence *Limnol Oceanogr* 36:1053-1061 (1991).

## GENERAL INTRODUCTION

42. Tester. PA, Geesey ME, Vukovich FM, *Gymnodinium breve* and global warming: what are the possibilities? In Toxic Phytoplankton Blooms in the Sea (Smayda TJ, Shimizu Y, eds) 1993;67-72.
43. Tester PA. Climate change and marine phytoplankton. *Ecosys Health* 2:191-197 (1996).
44. Haywood A, MacKenzie L, Garthwaite I, Towers N. *Gymnodinium breve* 'look-alikes'. three *Gymnodinium* Isolates from New Zealand In: Harmful and Toxic Algal Blooms (Yasumoto T, Oshima Y, Fukuyo Y, eds), Paris International Oceanographic Committee of UNESCO, 1996;227-230.
45. Bossart GD, Baden DG, Ewing RY, Roberts B, Wright SD. Brevetoxicosis in manatees (*Trichechus manatus latirostros*) from the 1996 epizootic gross, histologic, and immunochemical features. *Toxicol Pathol* 26:276-282(1998).
46. Sudarsanam S, Virca DG, March CJ, Srinivasan S An approach to computer-based modeling: application to cathepsin . *J Comput Aid Mol Des* 6:223,-233 (1992).
47. Yasumoto T, Nakajima I, Bagnis R, Adachi R. Finding of a dinoflagellate as a likely culprit of ciguatera. *Bull Jpn Soc Sci Fish* 43;1021-1026(1977)
48. Lewis RJ, Holmes MJ. Origin and transfer of toxins involved in ciguatera. *Comp Biochem Physiol* 106C;615-628(1993).
49. Freudenthal AR Public health aspects of ciguatera poisoning contracted on tropical vacations by North American tourists. In: Toxic Marine Phytoplankton (Graneli E, Sundstron B, Edler L, Anderson DM, eds) New York:Elsevier, 1990;463-468.
50. Vernoux, J-P, Lewis RL. Isolation and characterization of Caribbean ciguatoxins from the horse-eye jack (*Caranx latus*) *Toxicon* 35:889-900 (1997).
51. Dechraoui M.Y, Naar J, Pauillac S, Legrand A-M. Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. *Toxicon* 37:125-143(1999).
52. Legrand AM Ciguatera toxins: origin, transfer through food chain and toxicity to humans. In: Harmful Algae (Reguera B, Blanco J, Fernandez ML, Wyatt T, eds). Santiago del Compostella. Xunta de Galicia and Intergovernmental Oceanographic Commission, 1998;39-43.
53. Ruff TA. Ciguatera in the Pacific. a link with military activities. *Lancet*:201-205 (1989).
54. Kaly UL, Jones GP Test of the effect of disturbance on ciguatera in Tuvalu. *Mem Od Mus* 34:523-532 (1994).
55. Kohler ST, Kohler CC. Dead bleached coral provides new surfaces for dinoflagellates implicated in ciguatera fish poisonings. *Environ Biol Fishes* 35:413-416(1992).
56. Bagnis RS. Ciguatera fish poisoning an objective witness of coral reef stress In: Human Impacts on Coral Reefs. Facts and Recommendations ISAivat 8, edi French Polynesia Antenne Museum, 1987;241-253
57. Hales S, Weinstein P, Woodward A. Ciguatera (fish poisoning) El Niño, and Pacific sea surface temperatures. *Ecosys Health* 5:20-25 (1999).
58. Yasumoto T, Oshima Y, Yamaguchi M. Occurrence of a new type of shellfish poisoning in Japan and chemical properties of the toxin. In: Toxic Dinoflagellate Blooms (Taylor O, Seliger HH, eds). Amsterdam:Elsevier, 1979;495-502.
59. Yasumoto T, Oshima Y, Sugawara W, Fukuyo Y, Oguri H, Igarishi T, Fujita N. Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. *Bull Jpn Soc Sci Fish* 46:1405-1411(1980).
60. Quilliam MA, Gilgan M, Pleasance S, DeFrietas ASW, Douglas O, Fritz L, Hu T, Marr JC, Smyth C, Wright JLC. Confirmation of an incident of diarrhetic shellfish poisoning in eastern Canada In: Toxic Phytoplankton Blooms in the Sea (Smayda TJ, Shimizu Y, eds) Amsterdam:Elsevier, 1993;547-552
61. Lawrence JE, Bauder AG, Quilliam MA, Cembella AO *Prorocentrum lima*: a putative link to diarrhetic shellfish poisoning in Nova Scotia, Canada In: Harmful Algae (Reguera B, Blanc J, Fernandez ML, Wyatt T, eds) Santiago del Compostella. Xunta de Galicia and Intergovernmental Oceanographic Commission, 1998;78-79.
62. Morton SL, Leighfield TA, Haynes BL, Petipain DL. Busman MA, Moeller PD, Bean L. McGowan J, Hurst JL. Van Dolah FM Evidence for diarrhetic shellfish poisoning along the coast of Maine. *J Shellfish Res* 18:679-684(1999).
63. Gauss C-M, Sheppock JE, Naim AC, Chamberlin RA A molecular modeling analysis of the binding interactions between okadaic acid class of natural product inhibitors and the ser-thr phosphatases, PP1 and PP2A *Bioorg Med*

- Chem 5:1751-1773(1997).
64. Bagu JR, Sykes BD, Craig MM, Holmes CFB. A molecular basis for different interactions of marine toxins with protein phosphatase B1. Molecular models for bound moruporin, microcystins, okadaic acid and calyculin A. *J Biol Chem* 272:5078-5097(1997).
  65. Zhang Z, Zhao S, Long F, Zhang L, Bai G, Shima H, Nagao M, Lee EYC. A mutant of protein phosphatase-1 that exhibits altered toxin sensitivity. *J Biol Chem* 269:16997-17000(1994).
  66. Wera S, Hemmings B. Ser/thr protein phosphatases *Biochem J* 311:7-29(1995).
  67. Cohen P, Holmes CFB, Tsukitani Y. Okadaic acid a new probe for the study of cellular regulation. *Trends Biochem Sci* 15:98-102 (1990).
  68. Yasumoto T, Murata M, Oshima Y, Matsu-moto G, Clardy J. Diarrhetic shellfish poisoning. In: *Seafood Toxins* (Regalis EP, ed). Washington, DC:American Chemical Society, 1984:207-214.
  69. Saganuma M, Fujiki H, Suguri H, Yoshizawa S, Hirota M, Nakayasu M, Ojika M, Wakamatsu K, Yamada K, Sugimura T. Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc Natl Acad Sci* 85:1768-1771 (1988).
  70. Fujiki H, Saganuma M, Suguri H, Yoshizawa S, Tagai K, Uda N, Wakamatsu K, Yamada K, Murata M, Yasumoto T, et al. Diarrhetic shellfish toxin, dinophysistoxin-1, is a potent tumor promoter on mouse skin. *Jpn J Cancer Res* 79:1089--1093 (1988).
  71. Wright JLC, Boyd RK, Defrietas ASW, Falk M, Foxall RA, Jamieson WD, Laycock MV, McCulloch AW, McInnes AG, Odense P, et al Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern P.E.I. *Can J Chem* 67:481-490 (1989).
  72. Perl TM, Bedard L, Kosatsky T, Hockin JC, Todd ECD, Remic AS. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *N Engl J Med* 322:775-1780 (1990)
  73. Subba-Rao DV, Quilliam MA, Pocklington R. Domoic acid B a neurotoxic amino acid produced from the marine diatom *Nitzschia pungens* in culture. *Can J Fish Aquat Sci* 45:2076-2079 (1988).
  74. Bates SS, Bird CJ, Defrietas ASW, Foxall R, Gilgan M, Hanic LA, Johnson GR, McCulloch AW, Odense P, Pocklington R, et al Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. *J Fish Aquat Sci* 46(7):1203-1215 (1989).
  75. Murakami S, Takemoto T, Shimizu Y. Studies on the effective principles of *Dia-ganea simplex* Aq. I. Separation of the effective fraction by liquid chromatography *J Pharmacol Soc Jpn* 73:026-1028 (1953)
  76. Wright JLC, Falk M, McInnes AG, Walter JA. Identification of isodomoic acid D and two new geometrical isomers of domoic acid in toxic mussels. *Can J Chem* 68:22-25 (1990).
  77. Walter JA, Falk M, Wright JLC. Chemistry of the shellfish toxin domoic acid. characterization of related compounds. *Can J Chem* 72:430-436 (1994).
  78. Hampson OR, Huang X, Wells JW, Walter JA, Wright JLC. Interaction of domoic acid and several derivatives of kainic acid and AMPA binding sites in rat brain. *Eur J Pharmacol* 218:1-8(1992).
  79. Hampson DR, Manalo JL. The activation of glutamate receptors by kainic and domoic acid. *Natural Toxins* 6.:153-158 (1998).
  80. Xi D, Ramsdell JS. Glutamate receptors and calcium entry mechanisms for domoic acid in hippocampal neurons. *Neuroreport* 7:1115-1120 (1996).
  81. Peng YG, Taylor TB, Finch RE, Switzer RC, Ramsdell JS. Neuroexcitatory and neurotoxic actions of the amnesic shellfish poison, domoic acid. *Neuroreport* 5:981-985 (1994).
  82. Clayton EC, Peng Y.G, Means LW, Ramsdell JS. Working memory deficits induced by single but not repeated exposures to domoic acid *Toxicol* 37:1025-1039(1999).
  83. Iverson F, Truelove J, Nera E, Tryphonas L, Campbell J, Lok E. Domoic acid poisoning and mussel-associated intoxication- preliminary investigations into the response of mice and rats to toxic mussel extract *Food Chem Toxicol* 12:377-384 (1989).
  84. Preston E, Haynie I. Transfer constants for blood brain barrier permeation of the neuroexcitatory shellfish toxin, domoic acid. *Can J Neurolog Sci* 18:39-44 (1991).
  85. Fritz L, Quilliam MA, Wright JLC, Beale AM, Work TM. An out- break of domoic

## GENERAL INTRODUCTION

- acid and poisoning attributed to the pennate diatom *Pseudonitzschia australis*. J Phycol 28:438-442 (1992).
86. Work TM, Barr BB, Beale AM, Fritz L, Ouilliam MA, Wright JLC. Epidemiology of domoic acid poisoning in brown pelicans (*Pelicanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) in California. J Zool Wildlife Med 24:54-82(1993).
  87. Gulland F. 1999 Unusual Mortality Event-Domoic Acid Toxicity in California Sea Lions (*Zalophus californianus*) Stranded along the Central California Coast, May-October 1998. NOAA Technical Memorandum NMFS-OPR-8. Washington, DC:U.S. National Marine Fisheries Service, 1999;30 pp.
  88. Scholin CA, Gulland F, Doucette GJ, Benson S, Busman M, Chavez FP, Cordaro J, DeLong R, DeVogelaere A, Harvey J, et al. Mortality of sea lions along the California coast linked to a toxic diatom bloom. Nature 403:80-83 (2000).
  89. Bates SS. Bloom dynamics and physiology of domoic acid producing *Pseudo-nitzschia* species. In: Physiological Ecology of Harmful Algal Blooms (Anderson DM, Cembella AD, Hallegraeff GM, eds). New York:Springer-Verlag, 1998;267-292.
  90. Rhodes L, Scholin C, Garthwaite I. *Pseudo-nitzschia* in New Zealand and the role of DNA probes and immunoassays in refining marine biotoxin monitoring programmes. Natural Toxins 6:105-111(1998).
  91. Smith S, Noga E, Bullis RA. Mortality in *Talapia* due to a toxic dinoflagellate bloom [Abstract]. Proceedings of the Third International Colloquium on Pathology in Marine Aquaculture, Gloucester Point, Virginia. 1998;167-168
  92. Noga EJ. Fungal diseases of marine and estuarine fishes. In: Pathobiology of Marine and Estuarine Organisms (Couch JA, Fournie JW, eds). Boca Raton, FL: CRC Press, 1993;85-100.
  93. Burkholder JM, Noga EJ, Hobbs CW, Glasgow HB, Smith SA. New phantom dinoflagellate is the causative agent of major estuarine fish kills. Nature 358:407-410 (1992).
  94. Burkholder JM, Glasgow HB Jr. *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates behavior, impacts, and environmental controls. Limnol Oceanogr 42:1052-1075.
  95. Burkholder JM, Glasgow HB Jr. Trophic controls on stage transformations of a toxic amnush-predator dinoflagellate. J Euk Microbiol 44:200-205.
  96. Glasgow HB, Burkholder JM, Schmechel DE, Tester PA, Rublee PA. Insidious effects of a toxic dinoflagellate on fish survival and human health. J Toxicol Environ Health 46:101-122 (1995)
  97. Grattan LM, Oldach D, Perl TM, Lowitt MH, Matuszak OI, Dickson C, Parrot C, Shoemaker RC, Kauffman CL, Wasserman MP, et al. Learning and memory difficulties after environmental exposure to waterways containing toxin-producing *Pfiesteria* and *Pfiesteria*-like dinoflagellates. Lancet 352:532-539 (1998).
  98. Centers for Disease Control. Results of the public health response to the *Pfiesteria* workshop-Atlanta, Georgia, September 29-30, 1997. Morb Mort Weekly Rep 4:951-952(1997).
  99. Levin ED, Schmechel DE, Burkholder JM, Glasgow HB, Deamer-Melia NJ, Moser VC, Harry GJ. Persisting learning deficits in rats after exposure to *Pfiesteria piscicida*. Environ Health Perspect 101:1320-1325(1997).
  100. Pearl HW. Coastal eutrophication and harmful algal blooms: importance of atmospheric deposition and groundwater as "new" nitrogen and other nutrient sources. Limnol Oceanogr 42:1154-1165(1997).
  101. Epstein P, Sherman B, Spanger-Siegrfried E, Langston A, Prasad S, McKay B. Marine ecosystems emerging diseases as indicators of change. Year of the Oceans Special Report .Washington, DC:NOAA Office of Global Programs, 1998;85 pp.
  102. Harvell CO, Kim K, Burkholder JM, Colwell RR, Epstein PR, Grimes DJ, Hofmann EE, Lipp EK, Osterhaus AD, Overstreet RM, et al. Marine ecology-emerging marine diseases-climate links and anthropogenic factors (Review). Science 285:1505-1510 (1999).
  103. Carlton JT, Geller JB. Ecological roulette; the global transport of nonindigenous marine organisms. Science 261:78-82(1993).
  104. Rigby GR, Taylor AH, Hallegraeff GM, Mills P. Progress in research and management of ships' ballast water to minimize the transfer of toxic dinoflagellates. In: Harmful Marine Algal Blooms (Lassus P, Arzul G, Erard E, Gentien P, Marcaillou C, eds) Lavoisier:Paris, 1995;821-824.
  105. Ruiz GM, Carteton JT, Hines AH. Global invasions of marine and estuarine habitats by

- non-indigenous species: mechanisms, extent, and consequences. *Am Zool* 37:621-632 (1997).
106. Colwell R Global climate and infectious disease the cholera paradigm. *Science* 274:2025-2031 (1996).
  107. Zhang FZ, Dickman M. Mid-ocean exchange of container vessel ballast water. 1. Seasonal factors affecting the transport of harmful diatoms and dinoflagellates. *Mar Ecol -Prog Ser* 176:243-251 (1999).
  108. Dickman M, Zhang FZ Mid-ocean exchange of container vessel ballast water. 2. Effects of vessel type in the transport of diatoms and dinoflagellates from Manzanillo, Mexico to Hong Kong, China. *Mar Ecol- Prog Ser* 176:253-262(1999).
  109. Scarratt AM, Scarratt DJ, Scarratt MG. Survival of live *Alexandrium tamarense* in mussel and scallop spat under simulated conditions. *J Shellfish Res* 12:383-388 (1993).
  110. Nixon SW. Coastal marine eutrophication: a definition, social causes and future concerns. *Ophelia* 41:199-220 (1995).
  111. Reigman R. Species composition of harmful algal blooms in relation to macronutrient dynamics. In: *Physiological Ecology of Harmful Algal Blooms* (Anderson OM; Cembella AD. Hallegraeff GM, eds) New York: Springer-Verlag, 1998;475-488.
  112. Lam CWY, Ho KC. Red tides in Tolo Harbor, Hong Kong. In: *Red Tides Biology. Environmental Science and Toxicology* (Okaichi T, Anderson DM, Nemoto T, eds). New York: Elsevier, 1989;49-52.
  113. Matsuoka K. Eutrophication process recorded in dinoflagellate cyst assemblages -a case of Yokohama Port, Tokyo Bay, Japan *Sci Total Environ* 231:17-35(1999).
  114. Humbourg C, Tittkott V, Cociasu A, Bodungen B. Effect of Danube River dam on Black Sea ecosystem structure. *Nature* 386:385-388 (1997).
  115. Turner RE, Rabelais NN. Coastal eutrophication near the Mississippi Delta. *Nature* 368:619-621 (1994).
  116. Epstein PR. Perspectives. *Medicine -climate and health. Science* 285:347-348(1999).
  117. National Research Council. *Climate and weather, coastal hazards, and public health. In: From Monsoons to Microbes: Understanding the Ocean's Role in Human Health.* Washington, DC: National Academy Press, 1999;17-42.
  118. Mann ME, Bradley RS, Hughes MK. Global scale temperature patterns and climate forcing over the past six centuries. *Nature* 392:779-787 (1998).
  119. Steindinger KA. Unpublished data.

π



## Impacts of Harmful Algae on Seafarming in the Asia-Pacific Areas

Rhodora A. Corrales<sup>1</sup> & Jay L. Maclean<sup>2</sup>

<sup>1</sup> Marine Science Institute, University of the Philippines, Diliman, Quezon City, Philippines

<sup>2</sup> International Center for Living Aquatic Resources Management (ICLARM), Makati, Metro Manila, Philippines

*Key words*; harmful algae, seafarming, red tides, Asia-Pacific, marine phytoplankton, aquaculture, toxic algae, marine biotechnology

### ABSTRACT

Seafarming to produce human food has recently intensified, particularly in the Asia-Pacific region. Disastrous impacts of harmful phytoplankton blooms, however, have been experienced during the past 20 years. In extreme cases, these render shellfish and finfish toxic or cause massive fish and shrimp kills. Problems from marine algae in the region include paralytic shellfish poisoning, diarrhetic shellfish poisoning, ciguatera, tetrodotoxin poisoning, fish kills and tainting of fish and shellfish. An analysis of 72 incidents since 1934 showed that 57% were fish and shrimp kills; almost all the remainder were PSP events. By mid-1994 there had been 3164 recorded cases of human poisoning and 148 reported deaths from these events in Asia-Pacific. Economic losses may exceed one million US dollars per event, while monitoring costs may be up to \$50000 annually for each affected area. Research needs, management strategies and international cooperation are discussed. National action plan considerations include shellfish sanitation programs, public awareness and education, coastal engineering and classification of waters to protect public health.

### Introduction

Captain Cook's observation of a *Trichodesmium* bloom in the Coral Sea in 1770 and the suffering of his crew with ciguatera poisoning there in 1774 (Hallegraeff, 1990) were the first reports on algal blooms and harmful algae in the Asia-Pacific region. Since the 1970's, there has been a global increase in reports of harmful algal bloom events (Smayda, 1989; Anderson, 1989). The Asia-Pacific region is no exception, with increasing reports from e.g. Papua New Guinea and Southeast Asia (Maclean, 1989a), Korea (Park *et al.*, 1989) and Hong Kong (Lam & Ho, 1989). Globally, an average of almost 2000 cases of human poisoning from harmful algae has been recorded yearly (Hallegraeff, 1993).

Phytoplankton blooms have had

negative impacts on many coastal waters, particularly those being utilized for seafarming. From among the 5000 species of marine phytoplankton, about 200 species are known to multiply to such high numbers that discoloration of the water, or red tides occur. About 40 species, however, can produce potent toxins, which are passed through the fish and shellfish and reach ultimately the consumers, including humans (Hallegraeff, 1990).

This paper summarizes recorded harmful algal events in the Asia-Pacific region and the concomitant public health

Address correspondence to: Rhodora V. Azanza Marine Science Institute, University of the Philippines, Diliman, Quezon City, Philippines.

(This article was originally published in the Journal of Applied Phycology 7: 151-162, 1995 and reprinted here with the permission of Ms. R. Azanza)

## GENERAL INTRODUCTION

and economic impacts. Major scientific and technological advances, management measures, and needs to help cope with this expanding problem are also discussed.

### **Aquaculture/mariculture in Asia-Pacific**

Aquaculture production is increasing worldwide and will be expanding in the future. It constituted approximately 15.3% of the world's fishery production in 1990 as compared to 14% in 1989 (New, 1991, cited by Barg, 1992). Asia is the most populous region in the world; maintaining adequate food supply is a major concern. Consequently, Asia has become the major producer and consumer of aquaculture products. The region has been considered the world's leader in the field of aquaculture with Taiwan and Japan as models for aquaculture production. Asia's share in global aquaculture production rose from 80% in 1975 to 85% by 1990. The Asia-Pacific region produced  $12.9 \times 10^6$  t out of the  $15.3 \times 10^6$  world total production (Table 1). The region has shown steady and balanced growth over the past decade. Average annual growth was 7% from 1975 to 1990 with developing countries showing more rapid growth (8% ) than the developed ones (3%) (FAG, 1992; Csavas, 1993). The average per capita consumption of aquatic products in Asia is also high. As of 1986, Japan is the highest consumer in the world at 83 kg/person/year (Liao, 1992). Taiwan consumes fish products at an average of 44 kg/person/year and Southeast Asia at 27 kg/person/year (Table2).

The Philippines is a major aquaculture site for seaweeds and shellfish in the Southeast Asian region and one of the

most heavily damaged by harmful algae. It is the number one producer of seaweeds, particularly the carrageenophytes *Eucheuma Kappaphycus*, production of which comes mainly from seafarming. Shellfish which are being farmed are green mussels (*Perna viridis*) and oysters (*Crassostrea iredalei* and *C. malabonensis*).

### **Harmful algae**

The number of harmful phytoplankton species (excluding those causing depletion or clogging of fish gills) includes 27 dinoflagellates, 5 chloromonads, 2 chrysophytes, 5 prymnesioids, and 4 diatoms (Taylor, 1990). Another listing including freshwater and marine species of red tide causing organisms, other toxic species and those associated with other dominant species in red tides, in Japanese and Southeast Asian waters (Fukuyo *et al.*, 1990) contains 300 species. Worldwide there are 44 toxic eukaryotic algal species which affect shellfish (Shumway, 1990); Carmichael *et al.* (1990) list 12 genera of Cyanophyta implicated in producing acute lethal toxins.

### **Toxic microalgal products**

The toxins produced by microalgae (and by a variety of other marine organisms) have in common the property of modifying the functions of ion channels across cell membranes and could be classified as activators" stabilizers and occluders (Strichartz & Castle, 1990, cited in Maclean, 1993). Those affecting or potentially affecting seafarming are (a) activators, like the toxins causing Diarrhetic Shellfish Poisoning (DSP) and ciguatera poisoning; and (b) occluders, those causing

Table 1. Global aquaculture: continent/region production share (1990)

Source of data: Csavas (1993)

Continent/region	Production	Share (%)
Africa + Middle East (AME)	115.8	0.8
South America + Caribbean	216.6	1.4
North America (NA)	407.1	2.7
Asia + Pacific (AP)	1628.2	10.5
World Total	15322.5	100.0

Table 2. Per capita consumption of fishery products in Asia-Pacific, 1986. Source of data: Liao (1992)

Country	Per capita consumption (kg/person-1 yr <sup>-1</sup> )
Brunei	35.5
China (Mainland)	4.5
Hongkong	37.0
Indonesia	13.1
Japan	83.0
Malaysia	43.0
Philippines	41.0
Singapore	31.8
Taiwan	44.0
Thailand	18.8

Paralytic Shellfish Poisoning (PSP) (saxitoxins) and tetrodotoxin (Strichartz & Castle, 1990).

**Paralytic shellfish poisoning ( PSP )**

Poisons known collectively as saxitoxins cause PSP and include saxitoxin, neosaxitoxin, and several gonyautoxins and decarbamoyl saxitoxins (Yasumoto *et al.*, 1984; Hall *et al.*, 1990). The following marine dinoflagellates cause PSP in the Asia-Pacific region: *Alexandrium* spp., which have exhibited

toxic blooms almost all around the world (Taylor & Seliger, 1979; Anderson *et al.*, 1985; Okaichi *et al.*, 1989); *Gymnodinium catenatum* in Australia and Japan (Hall *et al.*, 1990); and *Pyrodinium bahamense* in Southeast Asia and the South Pacific.

The toxins have been found in different proportion in different algae and their consumers. Filter feeding bivalves and also gastropods (which prey on bivalves) pass the toxin through the food chain and ultimately to humans. Other

## GENERAL INTRODUCTION

pathways include zooplankton to fish to mammals and then also to birds. Some human poisonings from consumption of planktivorous fish have also been reported with neurological, gastrointestinal and respiratory symptoms (Maclean, 1993).

### **Diarrhetic shellfish poisoning (DSP)**

Ingestion of shellfish which have accumulated toxins from the dinoflagellates *Dinophysis* spp. can cause nonfatal diarrhetic (gastrointestinal) shellfish poisoning. Eleven polyether DSP toxins occur in three groups: okadaic acid and derivatives; pectenotoxin and yessotoxin (Yasumoto & Murata, 1990). DSP is a significant problem in the Asia-Pacific areas especially in Japan with incidences in Australia, India and New Zealand. *Dinophysis* spp. are ubiquitous (Fukuyo et al., 1990). Five species of *Dinophysis* have been detected recently in the Philippines but no DSP has been reported (Marasigan et al., 1993).

### **Ciguatera poisoning**

*Gambierdicus toxicus*, the alga responsible for ciguatera poisoning, causes illness to humans through tropical reef fishes which feed on such microalgae. The benthic dinoflagellate forms blooms on its macroalgal substratum. Toxic blooms in Tahiti were associated with the grazing surgeonfish *Ctenochaetus striatus* (Bagnis et al., 1990). Ciguatera has been widely reported in the South Pacific. It is less known in Asia although this may be from lack of research. It was only discovered in the Philippines, for example, during epidemiological surveys of red tide poisoning (W. Pastor, pers. comm.).

Ciguatera poisoning from farmed fish has not been reported. When sea ranching becomes practical, however, this poisoning could become a problem.

### **Amnesic shellfish poisoning (ASP)**

Short-term memory loss, gastrointestinal disorders and in some cases death, characterize the intoxication of humans from this poisoning. The neurological damage is permanent (Smith et al., 1990; Todd, 1990). Domoic acid, a neurotoxic amino acid causes ASP. It is produced by *Pseudonitzschia* (= *Nitzschia*) *pungens* but has not been detected in Asia-Pacific. Takano (1990) reported that this ubiquitous coastal species may pose a problem in aquaculture sites.

### **Tetrodotoxin (TTX)**

Tetrodotoxin has been found in *fugu* puffer fish, frogs, octopus, gastropods, starfish, crabs, flatworms, zoo-plankton, algae and bacteria (*Vibrio* spp.) (Jeon et al., 1986; Narita et al., 1987; Tamplin, 1990). It is possible that bacteria are the primary producers of this toxin which is also accumulated up the food chain. Toxicity of pufferfish in the Asia-Pacific area has been detected in those collected from the wild but not from farmed crops (Saito et al., 1984). Human poisonings are through ingestion of *fugu* or when bitten by octopus (Maclean, 1993). Poisoning from tetrodotoxin from goby fish has been reported in the Philippines (C. Gonzales, pers. comm.)

### **Other harmful effects**

When algal blooms collapse, oxygen depletion occurs causing mortalities of other organisms, a phenomenon quite common, for example, in Southern

Kattegat between Denmark and southern Sweden (Dahl *et al.* 1989). Fish kills due to clogging of gill or asphyxiation have been caused by larger diatoms with spinous skeletons such as *Chaetoceros convolutus*, *C. concavicornum* and *C. danicus*. (Mclean, 1993). Likewise, *Chatonella* poisoning in the Seto Inland Sea, Japan, of yellowtail (*Seriola quiqueradiata*) has been reported to be caused by gill damage and produce highly unsaturated fatty acids which decrease pH of the blood making gas exchange difficult in the fish (White, 1988).

*Gyrodinium aureolum* was found to kill sea-farmed rainbow trout by causing degeneration of gill tissue. Diatom blooms of *Nitzschia*, *Eucampia* and *Chaetoceros* affect nori (*Porphyra tenera*) farming in Japan. They strip the seawater of nutrients resulting in fading of color (Uno & Sasaki, 1989) and cause "diatom blight" on the seaweeds' thalli, thus lowering their commercial value. Changes in color or discoloration in sea crops have been reported as resulting from blooms of microalgae. A bloom of *Mesodinium rubrum* causing red discoloration of oysters (*Ostrea edulis*) in the Netherlands was reported by Kat (1984), who also noted previous discolorations in oysters elsewhere by either diatoms or coccolithophorids.

Tainting of fish from algal blooms is a problem for aquaculture in some areas. Stirling and Dey (1990) noted earthy tainting of pond-grown penaeid shrimp from Ecuador. In southeastern Australia in 1987, about \$1 million worth of some 500 tonnes of mussels were discarded due to a bloom of the diatom *Rhizosolenia chunii* which caused a strong bitter flavor in cultured and wild bivalves (Maclean, 1993).

### Impacts of harmful algae in the Asia-Pacific Region

Table 3 shows the percentage distribution of recorded negative impacts (fish/shrimp kills and human poisonings) of marine phytoplankton in the Asia-Pacific Region from 1934 to 1994.

#### Health impacts

By mid-1994, there had been approximately 3164 recorded cases of human poisoning and 148 reported deaths from these events in Asia-Pacific. PSP has particularly been a problem in the Philippines, Brunei and Malaysia, with *Pyrodinium bahamense* var. *compressum* as the major species apparently causing the problem. Except for 1934 and 1992 events in Australia, ciguatera poisoning has not been reported but there is a possibility that cases have been misreported or unreported in the region where the vectors, reef, fishes and seaweeds, are quite common. Table 4 shows the percentage distribution of public health impacts by phytoplankton species in Asia-Pacific region (1934-1994).

#### Economic Impacts

There are few estimates on the economic impacts of harmful algae on aquaculture/fisheries. Shumway (1990) has shown that for the shellfish industries, the losses per incident range from  $\$0.1 \times 10^6$  -  $430 \times 10^6$ . Maclean (1989b, 1993) divided economic impacts into 3 facets: occasional acute episodes, chronic situations and permanent closures. Descriptions follow.

#### Acute bloom situation

The public is taken by surprise and sometimes panics during occasional acute

## GENERAL INTRODUCTION

outbreaks of PSP. Hence, economic impacts are disproportionately large because uninformed consumers tend to avoid even uncontaminated seafood. The following account (Maclean, 1989a) of the Manila Bay, 1988 episodes clearly shows the 'halo effect' (Shumway, 1990) of impact in developing country aquaculture.

In August and September 1988, the first outbreak in Manila Bay occurred.

Thanks to the media, the whole seafood industry nearly ground to a halt while mussel growers even tried to implicate freshwater products in an effort to offset the swing of consumers to tilapias and other freshwater organism! All fish markets in Manila were depressed for over three months, similar to the case in San Francisco in 1980. Manila's seafood market handles 35% of the nation's landings. Thus the losses were large, up

Table 3. Percentage distribution of recorded negative impacts of marine phytoplankton in the Asia-Pacific Region from 1934 to 1994.

Negative impacts	% distribution
Fish kills	52.8
Paralytic shellfish poisoning (PSP)	41.7
Shrimp kills	4.2
Ciguatera fish poisoning (CFP)	1.5
Total incidents = 72	

Paralytic shellfish poisoning (PSP) Ciguatera fish poisoning (CFP)

Table 4. Marine phytoplankton and their recorded public health impacts in Asia-Pacific (1934-1994).

Phytoplankton species	Health impacts	No. of cases/ deaths (%)
<i>Pyrodinium bahamense</i>	PSP	2323 (97.16)
<i>Alexandrium tamarense</i>	PSP	63 (2.63)
<i>Gymnodinium catenatum</i>	PSP	5 (0.21)
<i>Gambierdiscus toxicus</i>	CFP	812 (25.09)
Total no. of cases and deaths		3192

to \$300 000 d<sup>-1</sup> at the height of the scare. Japan and Singapore banned shrimp imports from the Philippines for an unknown period (although they were clean) which would have meant losses of \$500 000 d<sup>-1</sup> of the produce was not subsequently sold. Losses by mussel growers for a three month-period were more modest, about

\$950 000 in all'.

Other losses from this situation in Asia include a huge *Trichodesmium* bloom in the Gulf of Thailand when \$1.16 x 10<sup>6</sup> worth of fish was affected (Suvapepun, 1989). Jaafar et al. (1989) reported a loss of \$0.1 x 10<sup>6</sup> from a mussel farm in Brunei due to high PSP levels in the shellfish.

### Chronic bloom situations

Although the public recognizes that only certain products are affected in chronic bloom situations, thus reducing the 'halo effect', large losses may still occur. The case of regular red tides in the Seto Inland Sea of Japan (Shirota, 1989; Okaichi, 1989) where large numbers of cultured fish are killed each year, is a classic example.

Costs of regular monitoring in the Asia-Pacific region are estimated at \$10000-50000 yr<sup>-1</sup> per affected area. Annual costs of monitoring in the archipelagic countries of Southeast Asia would be extremely high if all likely bloom sites were to be covered. Shumway (1990) cited a national Canadian surveillance and information program costing over \$1 x 10<sup>6</sup> yr<sup>-1</sup>.

### Permanent closure/ban

When economic sacrifices appear to be necessary to avert loss of life, permanent closures could be the only option. Some seafarmed areas in the ASEAN region have already had to be permanently closed as in the west coast of Sabah, Malaysia (Ting & Wong, 1989) and Bamnan Bay, Masinloc and Benoni Lagoon in the Philippines (C. Gonzales, pers. comm). Since people are dependent on the shellfish they collect in these areas, the permanent closure means loss of staple food to many. Toxicity of the fish *Gobius criniger*, presumably due to its feeding on microalgae, occurs sporadically without sufficient warning. The Bureau of Fisheries and Aquatic Resources of the Philippines thus has recommended to the national Inter-Agency Committee on Environmental Health a permanent ban on the catching, transporting and marketing of this species

throughout the country (C. Gonzales, pers. comm).

### Coping with harmful algal events

Expansion of harmful algal events in time and space has been explained through several hypotheses by many scientists working on this topic over many years and summarized in various regional and international workshops and symposia (Anderson, 1989; Smayda, 1989; Taylor, 1990; Hallegraeff, 1993). These include: (a) increased scientific awareness of toxic species, (b) increased utilization of coastal waters for aquaculture, (c) increase of algal blooms by cultural eutrophication, (d) stimulation of algal blooms by unusual climatological conditions and (e) transport of dinoflagellate cysts in ships' ballast water or associated with the transfer of shellfish stocks (Hallegraeff, 1990).

At the Sixth International Symposium on Toxic Phytoplankton held in France in October 1993, the following were added in support of these hypotheses: anthropogenic factors as loss of wetlands, altered predation pressure (from overfishing and fish diseases), and superimposed global change (Epstein, 1993).

Mechanisms to cope with these expanding harmful algal events so far employed by developed and developing countries can be categorized into (a) research and monitoring, (b) management measures, and (c) public information and education.

### Research and monitoring

Resource managers coping with harmful algal situations require information related to monitoring the toxicity of bodies of water or the vector

## GENERAL INTRODUCTION

organisms, predicting the onset and end of harmful algal events, and management of health and economic impacts of these events. Comprehensive scientific investigations have been done on several temperate microalgal species, but studies of this type have only started for the tropical species. There have been many occasions of missing information in the media, based on erroneous extrapolation of temperate situations/species to the tropics, for example 'red tide scares' in the Philippine media, which affected the whole seafood sector (Corrales & Gomez, 1990).

The biology of *Pyrodinium bahamense* var. *compressum*, the major PSP causing organism in the Indo-West Pacific, is not well studied. Recent developments in research on this species include the initiation of laboratory culture (Azanza-Corrales & Hall, 1990; Usup *et al.*, 1993), analysis of its toxin profile from culture of wild samples (Oshima, 1989; Usup *et al.*, 1993), and observation of its cyst initiation and germination *in vitro* (Corrales *et al.*, 1993).

Another important aspect is the study of their toxicology and toxicology. Research developments on these topics are presented in Hall *et al.* (1990) and Ravn (1993). A bacterial source of PSP toxin associated with the host (e.g., in *A. tamarense*) has been proposed several times (Kodama *et al.*, 1988; Kodama, 1990; Silva 1990). The recent finding that PSP toxin found in *A. catenella* and *A. tamarense* is genetically determined, poses more questions for research on bacteria-algae interaction (Sako *et al.*, 1993). Aside from bacterial production of saxitoxin, other marine bacteria-algal interactions were presented at the 1993 International Symposium in France,

including marine bacterial enhancement of domoic acid poisoning and algae as vectors of vibrios and other diatoms.

Monitoring affected and susceptible bodies of water should be done regularly as in many developed countries, including Australia, Japan, Taiwan and New Zealand. The Philippines and Indonesia, being endowed with large coastal areas and many enclosed bays, face the problem of very large financial and man-power costs. In Japan, monitoring and research activities concerning the problem have been coordinated. Predictive models have been constructed from data sets collected over several years of monitoring and research on affected areas. Predicting more accurately the occurrence of a red tide, however, remains difficult, though success has been achieved in predicting the possible months of occurrence of certain red tide organisms e.g. *Chatonella* (Imai *et al.*, 1989).

### Monitoring of toxicity

Bans on harvest or closure of harvest areas are based on efficient and reliable monitoring of the presence of the organism's vegetative cells and the toxicity of the affected marine organisms. The AOAC mouse bioassay is the only official standard bioassay test for PSP being used routinely for shellfish and now also for dinoflagellates. The detection limit is dependent on the mouse strain and varies from 32 to 58 µg STX-eq/100 g tissue. The health limit of PSP toxin in most countries is 80 µg STX-eq/100 g tissue (Ravn, 1993). The mouse bioassay remains the most popular monitoring technique for PSP, particularly for lack of any other standard bioassay techniques. However, since the

mice are not readily available and quite expensive in many countries, the housefly (*Musca domestica*) bioassay has also been tried (Ravn, 1993). In the Philippines, use of the blowfly bioassay was introduced for initial estimates of toxicity (to be confirmed by mouse bioassay) in order to start and maintain a nationwide monitoring effort which has been estimated to be very costly (Mendigo & Azanza-Corrales, 1993).

In view of the pressure to discontinue the use of animals for bioassay, monitoring of seafood toxins will increasingly be performed more by chemical and immunological assays or by instrumental methods of analysis (Quilliam *et al.*, 1990). There are now four HPLC-methods developed for quantification of PSP toxins: Oshima's (1988), Sullivan's (1988), Lucas' (1990) and Lawrence's (1991). The Institut Armand- Frappier has also developed a simple dipstick shellfish ELISA test kit for preliminary tests for PSP contamination and according to Prof. Yasumoto of Tohoku University, Japan, it only works well with saxitoxin (Ravn, 1993). Cheap but fast reliable methods of toxin monitoring are still needed for use across the vast aqua- culture sites in the Asia-Pacific areas. Further, availability of toxin standards for routine monitoring is also needed.

Monitoring of toxicity of water samples has been done routinely by estimates of cell density or counting of microscopic materials from plankton sampling. Considerable time and effort are required for skilled and more so for unskilled taxonomists in order to monitor the presence of dangerous species in plank- ton samples. An alternative to the microscopic count- ing is the use of

molecular probes that can bind to either internal or external sites of the target species or by visualization using a fluorescence or enzyme- linked colorimetric technique. Polyclonal and monoclonal antibodies to cell surface proteins are now being successfully used to label species such as *Aureococcus anophageferens*, *Alexandrium tamarense* and *Pseudonitzschia pungens* or even to divide between strains or varieties of species (Anderson, 1993). Sako *et al.* (1993) have lately been able to prepare monoclonal antibodies and DNA probes for *Alexandrium catenella* and *A. tamarense*. Tomas and Baden (1993) also have reported the detection of *Gymnodinium breve* in natural waters by ELISA. The development of a sensitive and specific immunoassay for detection of ciguatera fish has remained a challenge (Pauillac *et al.* , 1993).

### Management strategies

Management of the algal bloom phenomenon itself or of its harmful effects could be done at either the industry, the national, regional or international levels. At any level, the strategies or interventions are directed to (1) the human impacts and (2) the aquaculture or harvest sites.

### Human impacts

It is not the intention of this paper to deal more closely on this topic. There is a need, however, to emphasize that each country has to develop programs to meet health problems associated with harmful algae. Doctors, nurses or epidemiologists in general should be trained in the detection of and health intervention for the various types of phycotoxins. Underreporting and misreporting of cases

## GENERAL INTRODUCTION

usually result from misdiagnosis of poisonings. Likewise, health aids to victims such as respiratory support to PSP victims should be taught to coastal inhabitants/ aquaculturists. As earlier suggested (Corrales, 1994) a more permanent and regular system of educating the people through formal and non-formal means should be implemented as an important component of management strategies against harmful algal events in developing countries. A well informed public will:

1. follow government control measures, such as the timely banning of harvest and consumption of shellfish in affected areas, hence prevent sickness;
2. understand a red tide situation and will not panic, hence will continue buying and consuming unaffected fishery items;
3. cooperate with efforts toward the understanding and eventually the implementation of countermeasures against red tides/harmful algal events.

### Aquaculture/Harvest sites

Aquaculture seems to contribute to conditions favoring harmful algal blooms (Smayda, 1989; Hallegraeff, 1990). D. Anderson (pers. comm.) noted that paralytic shellfish poisoning in Balete Bay, Davao Oriental, Philippines, was reported one year after *Perna viridis* culture was introduced there. Likewise, Corrales & Gomez (1990) observed that *Pyrodinium bahamense* var. *compressum* red tides in the Philippines are mostly sites of or near mussel farms. Lately, *P. bahamense* var. *compressum* blooms were observed in Benoni lagoon, Camiguin Island, Philippines, after several years of mussel and finfish culture (C. Gonzales, pers.comm). Ronquillo (1987) felt that growing

mussels on poles too close together in the Philippines resulted in an accumulation of waste nutrients which promoted red tides. However, some areas have been affected by the species where no shellfish farming was done.

In the Indo-Pacific, most coastal aquaculture areas affected by *P. bahamense* var. *compressum* blooms are mangrove areas. *P. bahamense* var. *compressum* cysts could have been present earlier than the seafarming efforts. Studies on the presence of cysts in these aqua-culture sites should be done to elucidate this matter.

Coastal enrichment, attributed to human activities could be enhancing or sustaining algal blooms as in Seto Inland Sea, Japan (Okaichi, 1989) and in Hongkong (Lam & Ho, 1989). Aside from coastal enrichment, some organisms like *P. bahamense* var. *compressum* seem to bloom as a result of another climatological environmental factor, the El Niño event (Maclean, 1989a).

### Preventive management

To offset the impact of toxic blooms, aquaculturists can use a variety of physical and chemical techniques. Shirota (1989) has reviewed countermeasures being employed at experimental and commercial levels in Japan. Physical means include adjusting the depths of bivalves in cages. In Singapore, where affected fish cages and toxic plankton are in the upper two meters, steps to minimize contact between algae and fish include: a PVC 'skirt' at least 2 m deep to surround individual cages or the whole farm; transfer of fish to deeper nets, 4 m deep; an airlift or water pump to draw up deeper water to disperse the algae; thinning out of the stocking density of

sensitive fish; and towing of cage assemblages to safe areas (Lim, 1989, edited by Maclean, 1993). Adjusting depths or transfer of cages would not be reliable unless a hydrographic survey has been done and the biology and ecology of the causative organism studied. The uptake and depuration rates of the shellfish should be known to guide not only site selection but also physical transfers as a management option. Vertical movement of cages in considerable depth is effective only if the concentration of the noxious red tide plankton is on the surface, but it is not suitable for species having a large vertical migration or in a homogenous water mass mixed from surface to bottom. The large-scale red tide due to *Chatonella antiqua* which occurred in the southern part of Harimanada in 1987, killed more than 80% of fish in cages at a depth of 25 m, which proves only that even a net as deep as 25 m cannot keep the fish safe (Shirota, 1989).

Shirota (1989) enumerated the following prophylactic techniques: (1) improving polluted bottom mud using sand to control the elution of nutrient salts, and to restore the quality of the bottom mud, where sedimentation rate is slow; (2) cultivation of polluted bottom mud using a cultivation machine to promote the dissolution of organic substances in the polluted mud; and (3) aeration of polluted bottom mud to promote dissolution of organic substance where by jetting surface seawater saturated with oxygen into the polluted bottom mud. Conducting the above measures however, requires halting of fishery activities due to their effect on marine organisms and secondary pollution of water.

Other techniques with direct effects on the noxious organism are the following: (1) using ultrasonic waves by which the cells of red tide concentrated plankton are destroyed; (2) skimming of surface water to collect noxious red tide plankton with the use of a ship installed with pressure flotation separation equipment; (3) using a flocculant which aims to concentrate, precipitate and collect the red tide; and (4) absorbing plankton using a mixture of flocculant and iron powder with an induced magnetic field (Shirota, 1989). Chemicals used in the field to treat toxic algal blooms include copper sulfate (Hallegraeff, 1987); ammonium sulfate (White, 1988); and sodium percarbonate (Okaichi *et al.*, 1989). However, no recommended methodologies or chemicals for widespread use have emerged. Improvement of polluted bottom mud can be done using lime which has the function of reducing phosphate in seawater, eluting ammonia or sulfide from contaminated sediment and promoting the elution and dissolution of organic substances in sediment (Shirota, 1989).

With the possibility that dinoflagellate/algal cysts are transferred in ships' ballast water, Bolch & Hallegraeff (1993) experimented on chemical and physical treatments of the cysts of *G. catenatum* which causes PSP in Canada. Prevention of the germination of the cyst could be achieved with high concentration of chlorine (500 ppm) or hydrogen peroxide (5000 ppm). With the high costs and environmental safety involved, the authors consider these options still impractical. They added that heating water (30-90 minutes at 40-45 °C) is also effective and less costly and

## GENERAL INTRODUCTION

more environmentally friendly.

Rosenthal (1981) reviewed several studies which showed the effectiveness of ozone against various toxins. In the Asia-Pacific region, Gacutan *et al.* (1984) demonstrated that ozone and PVP iodide were effective in lowering toxin levels from *Perna viridis* contaminated by *Pyrodinium bahamense* var. *compressum* in the Philippines. One problem is that the ozone is dangerous to the shellfish and Schneider & Rodrick (in press) have found new methods using indirect ozone in pre-ozonated seawater which is effective.

Clean seawater provides excellent depuration of toxins from shellfish (White, 1982) but takes some time, e.g. five days in Korea (Chang *et al.*, 1988); while *Pyrodinium* toxins in the tropical oyster *Crassostrea echinata* required three weeks for removal by this means (Maclean, 1975). Shumway (1990) reported that the average time for toxicity to drop below the quarantine or detection levels *in viva* would be 4-8 weeks depending on mollusc and algal species. Because of environmental and biological constraints, the design and operation of depuration systems must meet specific requirements for each species of shellfish, as well as for public health, sanitation, and economic aspects (Furfari, 1976).

### National planning

Action plans have been implemented in both developing and developed countries in Asia-Pacific as mentioned previously. These programs involve monitoring and public awareness. In the Philippines and Hongkong 'mobile squads' or 'town criers' are used to give advice to minimize loss. The Philippines,

which is the most heavily affected in Southeast Asia, has consequently made progress in terms of monitoring and some research but it still lags behind in other areas, like public education/information, and the development of a National Shellfish Sanitation Program. However, steps are underway for legislation or possibly creation of a National Harmful Algal Bloom Center/Committee and development of a Seafood Sanitation Program.

Remedial management practices in Japan's Seto Inland Sea have not been copied in other countries in Asia-Pacific. The 1973 Seto Inland Sea Environment Conservation Law effected the reduction of problems which lessened red tides, yet fish kill events did not decrease (Okaichi, 1989).

Coastal engineering options could be considered. Dredging affected bays would change the physical habitat which previously favored the persistence of a toxic species, but changes might risk the seeding of cysts into the water column. However, where sea-farming operations are valuable enough, such measures can be compared to agriculture where such engineering options (e.g., dam construction) are common (Maclean, 1993).

Classification of waters (including marine biotoxicity as basis) to protect public health should be developed/implemented strictly, in all countries engaged in seafarming.

Shellfish sanitation programs should be legislated and implemented with government, nongovernment and fishers involvement, including financial support through schemes of self-inspection with government control or management.

### **International cooperation**

Regional cooperation and networking in all aspects of research, monitoring and management should be encouraged/initiated. Research collaborations between several countries have been made e.g. Philippine- Japan, Philippine-Canada, Japan- Thailand, Hongkong and China. An ongoing project under the ASEAN- Canada Marine Science Programme is the ASEAN-Canada Red Tide Network which aims to link ASEAN red tide specialists to exchange information on occurrence and movement of red tides, and dissemination of research information and methodologies. This regional network involves the organization of national networks and also implies networking with other regional systems to form a global or international network. An Asia-Pacific network for example, could be soon developed.

A comprehensive International Harmful Algal Bloom Program has been implemented by Intergovernmental Oceanographic Commission (IOC) since 1991 involving educational, scientific and operational elements in relation to these events and with emphasis on training and capacity building for developing countries (Enevoldsen, 1993). Also, an International Directory of Experts was made in 1989 and is currently being updated with IOC-WHOI support.

Cooperative research to model population dynamics of harmful algae which could then be useful in developing predictive systems is being done at the international level with the joint effort of IOES and IOC. The large marine ecosystems (LME) research and monitoring approach is currently being employed in some areas, such as the

Yellow Sea ecosystem (Tang, 1989), the Great Barrier Reef ecosystem (Bradbury & Mundy, 1989), and the Northwest Australian continental shelf ecosystem, in order to relate results and findings to fisheries issues, problems of pollution which include harmful algal events and coastal zone management (Sainsbury, 1988). In future, these works may help explain initiation, movement and end of certain algal bloom events.

Workshops and meetings for worldwide regulations concerning marine phycotoxins have been started to investigate inconsistencies in the monitoring and management of harmful algal bloom situations and to see if they could be standardized for public health protection and for greater harmony in international trade. One important issue is the different toxicity levels established for PSP and DSP toxins.

### **Marine biotechnology**

What will be the role of biotechnology in relation to seafarming and the problem of harmful algae: control of the blooms/algae? development of crops (shellfish/fish) that could detoxify themselves from the algae? development of fast, reliable (cheaper) chemical tests to replace the mouse bioassay toxicity test? production of toxin standards?

Biomedication technologies which are used to clean up polluted areas or treat wastewater have been developed. Environmental effects of releasing genetically engineered organisms in the field should be tested carefully and regulation of such activities should be a major concern.

Development of biosensors for harmful algae could interest marine biotechnologists as discussed previously.

## GENERAL INTRODUCTION

Most of the work is now on PSP and DSP detection. The high costs of polyclonal/monoclonal or DNA probes could be a major problem for use in extensive monitoring, especially in developing countries.

Marine microbial ecology is one of the major research areas which have grown tremendously in developed countries and will be the most helpful in relation to marine biotechnology and public health. This area needs to be fostered in developing countries.

### Acknowledgments

The senior author acknowledges the following agencies for support of her research on harmful algae: Department of

Science and Technology-Philippine Council for Marine and Aquatic Research Development (DOST-PCAMRD), World Health Organization (WHO), and ASEAN-Canada Cooperative Programme on Marine Science. The technical Assistance of Marilou Martin and Meraida Reyes is also gratefully acknowledged. Discussions with Max Taylor, Don Anderson, Yasuwo Fukuyo, Gustaaf Hallegraef, Sherwood Hall, Allan Cembella, Kazumi Matsuoka, Masaaki Kodama, Henrik Enevoldsen, Helle Ravn, Meryl Williams, Cielito Gonzales, Niño Ismael Pastor, Ma. Andrea Mendigo, Gil Jacinto, have been helpful in the preparation of this paper.

## REFERENCES

- Anderson M, Maranda L, Shimizu Y (1985) Comparison of toxicity between population of *Gonyaulax tamarensis* of Eastern North America waters. *Estuar. coast. Shelf Sci.* 21: 401-410.
- Anderson DM (1989) Toxic algal blooms and red tides: a global perspective. In Okaichi T, Anderson DM, Nemoto T (eds), *Red Tides. Biology, Environmental Science and Toxicology*. Elsevier, New York: 11-16.
- Anderson DM (1993) Identification of harmful algal species using molecular probes. Paper presented at the Sixth International Conference on Toxic Marine Phytoplankton, Nantes, France, Oct. 18- 22, 1993.
- Azanza-Corrales R, Hall S (1993) Isolation and culture of *Pyrodinium bahamense* var. *compressum* from the Philippines. In Smayda Tl Shimizu Y (eds), *Toxic Marine Phytoplankton Blooms in the Sea*. Elsevier Science Publishers, Amsterdam: 725-730.
- Bagnis R, Legrand AM, Inoue A (1990) Follow-up of a bloom of the toxic dinoflagellate *Gambierdiscus toxicus* on a fringing reef of Tahiti. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), *Toxic Marine Phytoplankton*. Elsevier, New York: 98-103.
- Barg U (1992) Guidelines for the promotion of environmental management of coastal aquaculture development. FAO Fisheries Technical Paper 328. FAO, Rome, 122 pp.
- Bolch CJ, Hallegraef GM (1993) Chemical and physical treatment options to kill dinoflagellate cysts in ships' ballast water. *J. mar. envir. Engng.* I: 23-29.
- Bradbury RH, Mundy CN (1989) Large-scale shifts in biomass of the Great Barrier Reef ecosystem. In Sherman K, Alexander LM (eds), *Biomass yields and geography of large marine ecosystems*, AAAS Selected Symposium I11. Westview Press, Boulder: 143- 167.
- Carmichael WW, Mahmood NA, Hyde EG (1990) Natural toxins from cyanobacteria (blue-green algae). In Hall S, Strichartz S (eds), *Marine Toxins. Origins, Structure, and Molecular Pharmacology*. American Chemical Society, Washington, DC: 87-106.
- Chang DS, Shin IS, Goo HY, Oh EG, Pyun IH, Park YH (1988) Studies on the distribution, characterization and detoxification of shellfish in Korea 3. Detoxification of paralytic shellfish poison in sea mussel, *Mytilus edulis*. *Bull. Korean Fish. Soc.* 21:

- 297-302.
- Corrales RA (1994) Coping with Red tides: Role of Education. Paper presented during the Annual Biology Teachers Association (BIOTA) Meeting, Central Mindanao University, 7-9 April 1994.
- Corrales RA, ED Gomez (1990) Red tide outbreak and their management in the Philippines. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier, New York: 453-458.
- Corrales RA, Reyes M, Martin M (1993) Notes on the encystment and excystment of *Pyrodinium bahamense* var. *compressum* *in vitro*. Paper presented at the Sixth International Conference on Toxic Marine Phytoplankton, Nantes, France, Oct. 18-22, 1993.
- Csavas I (1993) Aquaculture development and environmental issues in the developing countries of Asia. In Pullin RSV, Rosenthal H, Maclean JL (eds), Environment and aquaculture in developing countries. ICLARM Conf. Proc. 31: 74-101.
- Dahl E, Lindahl O, Paasche E, Throdsen I (1989) The *Chrysochromulina polyepis* bloom in Scandinavian waters during Spring 1988. In Cosper EM; Bricelj VM, Carpenter EI (eds), Novel phytoplankton blooms. Causes and impacts of recurrent brown tides and other unusual blooms. Springer-Verlag. Berlin: 383-405.
- Epstein PR (1993) Highlights of Sixth International Conference on Toxic Marine Phytoplankton. Nantes, France, 18-22 October 1993. In Harmful Algae News No.7. 1993.
- Enevoldsen H (1993) Harmful Algal Blooms: International Programme Development. Paper presented at the Sixth International Conference on Toxic Marine Phytoplankton, Nantes, France, Oct. 18-22, 1993.
- FAO (1992) Aquaculture production (1984-1990). FAO Fish Circ. 815. Rev. 4, 206 pp.
- Fukuyo Y, Takano H, Chihara M, Matsuoka K (eds) (1990) Red tide organism in Japan. An illustrated taxonomic guide. Uchida Rokakuho, Tokyo, 407 pp.
- Furfari SA (1976) Shellfish purification: A review of current technology, FAO Technical Conference on Aquaculture, FIR AQ/Conf/76/R 11, 16pp.
- Gacutan RQ, Tabbu MY, de Castro T, Gallego AB, Bulalacao M, Arafles L, Icatlo Jr F (1984) Detoxification of *Pyrodinium* generated paralytic shellfish poisoning toxin in *Perna viridis* from Western Samar, Philippines. In White AW, Anraku M, Hooi KK (eds), Toxic red tides and shellfish toxicity in Southeast Asia. Southeast Asian Fisheries Development Centre. Singapore: 80-85.
- Hall S, Strichartz G, Moczydlowski E, Ravindran A, Reichardt PB (1990) The saxitoxins. Sources, chemistry, and pharmacology. In Hall S, Strichartz G (eds), Marine Toxins. Origins, Structure, and Pharmacology, American Chemical Society, Washington, D.C.: 29-65.
- Hallegraeff GM (1987) Red tides in the Australian region. CSIRO Mar. Lab. Rep. 187, 14 pp.
- Hallegraeff GM (1990) Status of harmful algal blooms in the Indo-West Pacific region. Paper presented to OSLR/OIC meeting of the Group of Experts on Harmful Algal Blooms, 31 January- 2 February 1990, Paris, 10 pp.
- Hallegraeff GM (1992) Harmful algal blooms in the Australian region. Mar. Pollut. Bull. 25: 186-190.
- Hallegraeff GM 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32: 79-99.
- Imai I, Itoh K, Anraku M (1989) Dormancy and maturation of *Chatonella* spp. {Raphidophyceae} red tide flagellates in the Inland sea of Japan. In Okaichi T, Anderson DM, Nemoto T (eds), Red tides. Biology, Environmental Science, and Toxicology. Elsevier, New York: 289-292.
- Jaafar MH, De Silva MWRN, Sharifuddin PHY (1989) *Pyrodinium* red tide occurrences in Brunei Darussalam. In Hallegraeff GM, Maclean JL (eds), Biology, epidemiology and management of *Pyrodinium* red tides. ICLARM Conf. Proc. 21: 9-17.
- Jeon JK, Miyazawa K, Noguchi T, Narita H, Matsubara S, Nara M, Nara M, Ito K, Hashimoto K (1986) Occurrence of paralytic toxicity in marine flatworms. Bull. Japan. Soc. Sci. Fish. 52: 1065-1069.
- Kat M (1984) 'Red' oysters (*Ostrea edulis* L.) caused by *Mesodinium rubrum* in Lake Grevelingen. Aquaculture 38: 375-377.
- Kodama M, Ogata T, Sato S (1988) Agric. Biol. Chem. 52: 1075-1077.

## GENERAL INTRODUCTION

- Kodama M (1990) Possible links between bacteria and toxin production in algal blooms. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier, New York: 52-61.
- Lam CWY, Ho KC (1989) Red tides in Tolo Harbour, Hongkong. In Okaichi T, Anderson DM, Nemoto T (eds), Red tides. Biology, Environmental Science, and Toxicology. Elsevier, New York: 49-52.
- Liao C (1992) Aquaculture in Asia: Status, Constraints, Strategies, and Prospects. In Liao C, Chung-Shyu, Nai-Hsien Chao (eds), Proceedings of the 1990 APO Symposium in Aquaculture: 13- 27.
- Maclean JL (1973) Red tide and paralytic shellfish poisoning in Papua New Guinea Agric. J. 24: 131-138.
- Maclean JL (1975) Paralytic shellfish poison in various bivalves, Port Moresby 1973. Pac. Sci. 29: 349-352.
- Maclean JL (1989a) Indo-Pacific red tides, 1985-1988. Mar. Pollut. Bull. 230: 304-310.
- Maclean JL (1989b) Economic aspects of *Pyrodinium* red tides in the western Pacific. In Hallegraeff OM, Maclean JL (eds), Biology, epidemiology and management of *Pyrodinium* red tides. ICLARM Conf. Proc. 21: 179-185.
- Maclean JL (1993) Developing-country aquaculture and harmful algal blooms. In Pullin RSV, Rosenthal H, Maclean JL (eds), Environment and aquaculture in developing countries. ICLARM Conf. Proc. 31: 252-284.
- Marasigan AN, Fukuyo Y, Kodama M (1993) Distribution of *Dinophysis* in Sapien Bay, Capiz, Philippines. Abstracts. Paper presented at the NRCT-JSPS Joint Seminar on Marine Science, Songkhla, Thailand, 2-3 December 1993, 52 pp.
- Mendigo MA, Azanza-Corrales R (eds), Field and Laboratory Manual on Philippine Red Tide Monitoring and Data Management, 178 pp.
- Narita H, Matsubara S, Miwa N, Akahane S, Murakami M, Ooto T, Nara M, Nogochi T, Saito T, Ishida Y, Hashimoto K (1987) *Vibrio alginolyticus*, a TTX-producing bacterium isolated from the starfish *Astropecten polyacanthus*. Nippon Suisan Oukkaishi 53: 617-621.
- Okaichi T (1989) Red tide problems in the Seto Inland Sea, Japan. In Okaichi T, Anderson DM, Nemoto T (eds), Red tides. Biology, Environmental Science, and Toxicology. Elsevier, New York: 137-142.
- Okaichi T (1991) The studies on the cause of the fish kill due to red tide organisms to establish the countermeasure to avoid the damage. In Park JS, Kim O (eds), Recent approaches on Red tides. Proc. of 1990 Korean-French Seminar on Red tides. National Fisheries Research and Dev. Agency, 159 pp.
- Oshima Y (1989) Toxins in *Pyrodinium bahamense* var. *compressum* and infested marine organism. In Hallegraeff GM, Maclean JL (eds), Biology, epidemiology and management of *Pyrodinium* red tides. ICLARM Conf. Proc. 21: 73-79.
- Park JS, Kim HO, Lee SO (1989) Studies on red tide phenomena in Korean coastal waters. In Okaichi T, Anderson DM, Nemoto T (eds), Red Tides. Biology, Environmental Science, and Toxicology. Elsevier, New York: 37-40.
- Pauillac S, Blehaut J, Legrand AM (1993) Recent advances in detection of Ciguatoxins in French Polynesia. Abstracts. Paper presented at the Sixth International Conference on Toxic Marine Phytoplankton, Nantes, France, Oct. 18-22, 1993.
- Quilliam MA, Ayer SW, Pleasance S, Sim PO, Thibault P, Marr JC (1990) Recent developments in instrumental analytical methods for marine toxins. NRCC No.31652. Chapter for proceedings of Seafood 2000 Conference.
- Ravn H (1993) Toxicological and Toxin Chemistry Aspects of Phytoplankton. In IOC-DANIDA Training Course Manual on Taxonomy of Marine Phytoplankton, Univ. of Copenhagen, 16- 28 August 1993, 32 pp.
- Ronquillo IA (1987) Fish kills in Philippine lakes and coastal waters. Paper presented at the Seminar-workshop on Disseminating Information on Environmental Hazards, Mines and Geo- Science Bureau, 23-25 September 1987, Quezon City, Philippines, 14 pp.
- Rosenthal H (1981) Ozonation and sterilization. In Tiews K (ed.), Aquaculture in heated effluents and recirculation systems. Vol. I. Heenemann Verlagsgesellschaft mbH, Berlin: 219-274.
- Sainsbury KJ (1988) The ecological basis of multispecies fisheries and management of a demersal fishery in tropical Australia. In Gulland JA (ed), Fish population dynamics,

- 2nd edn., John Wiley & Sons, New York, N. Y.: 349-382.
- Saito TJ, Maruyama J, Kanoh S, Jeon JK, Noguchi T, Harada T, Murata O, Hashimoto K (1984) Toxicity of the cultured pufferfish *Fugu rubripes rubripes* along with resistibility against tetrodotoxin. Bull. Japan. Soc. Sci. Fish. 50: 1573-1575.
- Sako Y, Adachi M, Ishida Y (1993) Monoclonal antibodies and DNA probes specific for the toxic dinoflagellate *Alexandrium*. Paper presented at the Sixth International Conference on Toxic Marine Phytoplankton, Nantes, France, Oct. 18-22, 1993.
- Schneider KR, Rodrick GE (in press) The use of ozone to degrade *Gymnodinium breve* toxins. Aquat. Living Resour.
- Shirota A (1989) Red Tide Problem and Countermeasures I. Int. J. Aquat. Fish. Technol. I: 25-38.
- Shumway SE (1990) A review of the effects of algal blooms on shellfish and aquaculture. J. World Aquacult. Soc. 21: 65-104.
- Silva ES (1990) Intracellular bacteria: the origin of dinoflagellate toxicity. J. Envir. Pathol. Toxicol. Oncol. 10: 124-128.
- Smayda TJ (1989) Primary production and the global epidemic of phytoplankton blooms in the sea: a linkage. In Cospser EM, Bricelj VM, Carpenter EJ (eds), Novel phytoplankton blooms, causes and impacts of recurrent brown tides and unusual blooms. Springer-Verlag: Berlin: 449-483.
- Smith JC, Cormier R, Worms J, Birds CJ, Quilliam MA, Pocklington R, Angus R, Hanic L. (1990) Toxic blooms of domoic acid containing diatom *Nitzschia pungens* in the Cardigan River, Prince Edward Island, in 1988. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), Toxic marine phytoplankton. Elsevier, New York: 227-232.
- Stirling HP, Dey T (1990) Impact of intensive cage fish farming on the plankton and periphyton of a Scottish freshwater loch. Hydrobiologia 190: 193-214.
- Strichartz G, Castle N (1990) Pharmacology of marine toxins. Effects on membrane channels. In Hall S, Strichartz G (eds), Marine toxins. Origins, structure, and molecular pharmacology. American Chemical Society, Washington, D.C.: 2-20.
- Suvapepun S (1989) Status of red tide in Thailand. Paper presented at the Management and Training Workshop on *Pyrodinium* Red tides, 23-30 May, 1989, Brunei Darussalam, 3 pp.
- Tamplin ML (1990) A bacterial source of tetrodotoxins and saxitoxins. In Hall S, Strichartz G (eds), Marine toxins. Origins, structure, and molecular pharmacology. American Chemical Society, Washington D.C.: 78-86.
- Tang Q (1989) Changes in biomass of the Yellow Sea ecosystems. In Sherman K, Alexander LM (eds), Biomass yields and geography of large marine ecosystems. AAAS selected Symposium 111. Westview Press, Inc., Boulder, Co.: 7-35.
- Takano H (1990) Bacillariophyceae. In Fukuyo Y, Takano H, Chi-hara M, Matsuoka K (eds), Red tide organism in Japan. An illustrated taxonomic guide. Uchida Rokakuho, Tokyo: 163-331.
- Taylor DL, Seliger HH (eds) (1979) Toxic dinoflagellate blooms. Elsevier-North Holland, New York, USA.
- Taylor FJR (1990) Red tides, brown tides and other harmful algal blooms: the view into the 1990's. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier, New York: 527-533.
- Ting TM, Wong JTS (1989) Summary of red tides and paralytic shellfish poisoning in Sabah, Malaysia. In Hallegraef GM, Maclean JL (eds), Biology, epidemiology and management of *Pyrodinium* red tides. ICLARM Conf. Proc. 21: 19-26.
- Todd ECD (1990) Amnesic shellfish poisoning - a new seafood toxin syndrome. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds). Toxic Marine Phytoplankton. Elsevier. New York: 504- 508.
- Tomas CR & Baden DG (1993) Detection of *Gymnodinium breve* in natural waters by using an ELISA detection technique. Paper presented at the Sixth International Conference on Toxic Marine Phytoplankton. Nantes. France. Oct. 18-22. 1993.
- Uno S, Sasaki K (1989) Volumetric considerations of some species of diatoms composing red tides in the nori culture area in Ariake bay. In Okaichi T, Anderson DM, Nemoto T (eds). Red tides. Biology. Environmental Science. and Toxicology. Eisevier. New York: 157-160.

## GENERAL INTRODUCTION

- Usup G, Kulis D, Anderson DM (1993) Toxin production by the dinoflagellate *Pyrodinium bahamense* var. *compressum* clone PBGUOI in laboratory cultures. Paper presented at the Sixth International Conference on Toxic Marine Phytoplankton, Nantes, France. Oct. 18-22, 1993.
- White AW (1982) Intensification of *Gonyaulax* blooms and shellfish toxicity in the Bay of Fundy. Can. Tech. Fish. Aquat. Soc. 1064, 12pp.
- White AW (1988) Blooms of toxic algae worldwide: their effects on fish farming and shellfish resources. In Jensen A (moderator) The impact of toxic algae on mariculture. Proceedings of Aquanor 87. Norske Fiskeoppdretteres Forening, Trondheim, Norway: 9-14.
- Yasumoto T, Raj U, Bagnis R (1984) Seafood poisonings in tropical regions. Faculty of Agriculture, Tohoku, University, Japan.
- Yasumoto T, Murata M (1990) Polyether toxins involved in seafood poisoning. In Hall S, Strichart G (eds), Marine Toxins. Origins, Structure, and Molecular Pharmacology. American Chemical Society, Washington D.C.: 120-132.

π

---

## The ASEAN-CANADA Red Tide Network: 1993 -1998.

Rhodora V. Azanza

Marine Science Institute, University of the Philippines

1101 Diliman, Quezon City, Philippines

### ABSTRACT

An ASEAN-Canada Red Tide Network was formally started in 1993 under the ASEAN-Canada Cooperative Programme on Marine Science -Phase II (CPMS-II). The Network has been considered to be the world's first and only regional network on harmful algae linking seven (7) ASEAN countries (Brunei Darussalam, Indonesia, Malaysia, Philippines, Singapore, Thailand, and its newest member, Vietnam) and Canada.

With the initial objectives of (1) providing a linkage/awareness among ASEAN countries (represented by their respective country anchors) about occurrence and movement of a Red Tide/harmful algal blooms for public health purposes, and (2) dissemination of research information and methodologies, the Network's objectives have expanded to include (3) creation of a regional historical database and (4) using this database as a basis for geographic information systems (GIS).

Data available from publications and alert reports show that *pyrodinium bahamense* var. *compressum* has been the main toxic red tide causative organism in the Philippines, Malaysia and Brunei. Species of *Trichodesmium*, *Gymnodinium*, *Noctiluca*, *Ceratium*, *Alexandrium*, *Chatonella*, *Cochlodinium*, *Gonyaulax*, *Dinophysis*, *Mesodinium*, *Peridinium* and *Gambierdiscus* have also been reported in some Southeast Asian waters.

---

### Introduction

Cognizant of the need to exchange information concerning local harmful algal blooms/Red Tide events, all ASEAN countries/representatives formally signed on July 1993 an agreement for regional networking. It was initiated as one of the project components of the ASEAN-Canada Cooperative Programme on Marine Science -Phase II (CPMS-II). The Network links seven ASEAN countries (Brunei, Malaysia, Philippines Singapore, Thailand, Indonesia and its newest member, Vietnam) and Canada. The Network facilitates the exchange of information concerning regional harmful algal blooms (HABs)/Red Tide events in the region. latest research breakthroughs, and updates on possible trainings and fellowships.

The Network is divided into two sub-networks: an "Information Network" and an "Alert Network". The Alert Network serves to inform about the occurrence of Red Tides in any member country. The Information Network aims to encourage exchange of information on research findings and to announce availability of pertinent reference materials. It also disseminates other news relevant to HABs and toxic Red Tides.

---

Address correspondence to: Rhodora V. Azanza Marine Science Institute, University of the Philippines, Diliman, Quezon City, Philippines.

(This article was originally published in Watson, I., Vigers, G., Ong, K.S., McPherson, C., Millson, N., Tang, A., Gass, D. (eds.) Towards Sustainable Development and Integrated Management of the Marine Environment in the ASEAN. Fourth ASEAN Canada Conference on Marine Science Proc., Malaysia, Oct. 26-30, 1998.

## GENERAL INTRODUCTION

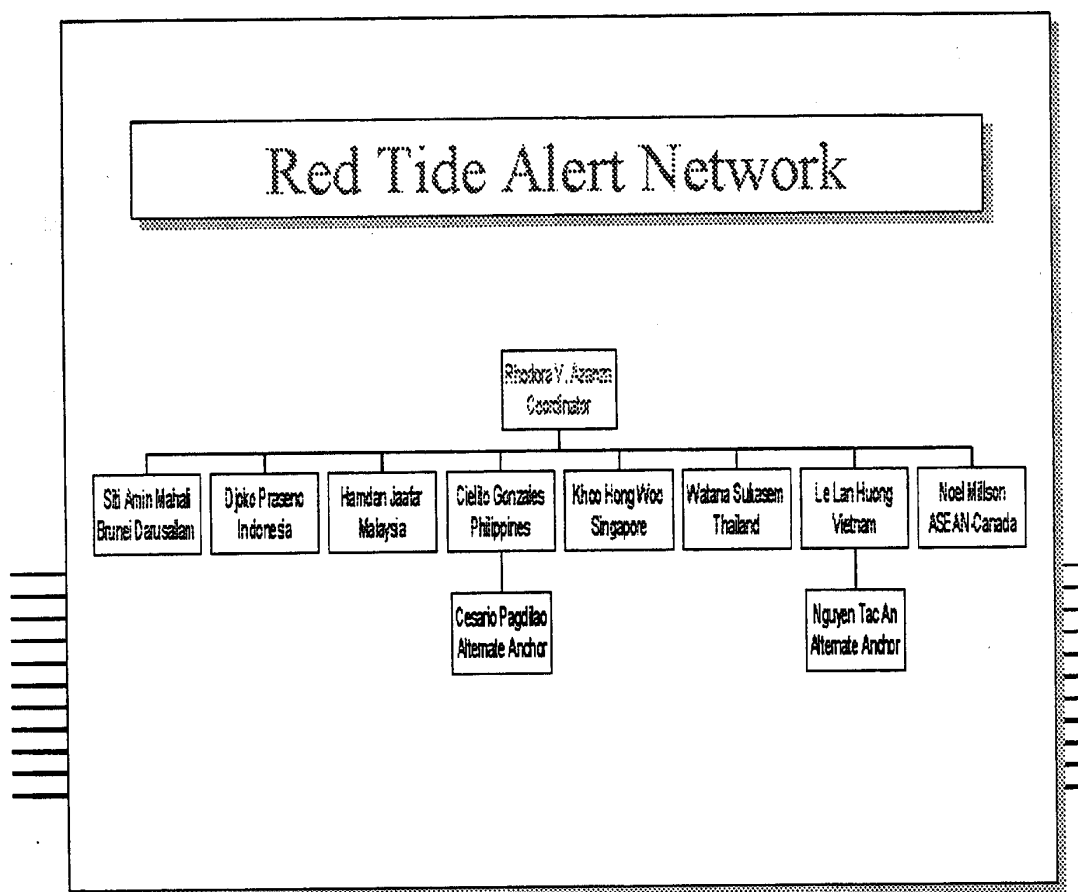
The Network is composed of the "Network Coordinator", "Anchors" and "Alternate Anchors" for every member country (Figure 1). The "Network Coordinator" is the focal point of the Network and the "Anchors" report regularly in the "Alert Network" even during the non-occurrence of HABs. The author has acted as Coordinator since 1993.

### The Red Tide Alert Network

Confidential alert reports are trans-

mitted to the country anchors through the Network Coordinator based in the Philippines when harmful algal bloom events occur within the region. Figures 2 to 8 show the frequency of HAB occurrences in Southeast Asia and Table 1 presents the harmful algal species identified on every HAB occurrences based on the literature (1976-1997) and reports from the country anchors (1993-1998). *Pyrodinium bahamense* var. *compressum* has been identified as the the main toxic organism, which blooms in the bays of the Philippines, Malaysia, Brunei and Indonesia.

The medium of communication is by facsimile. Alert reports are faxed to the Network Coordinator in standardized



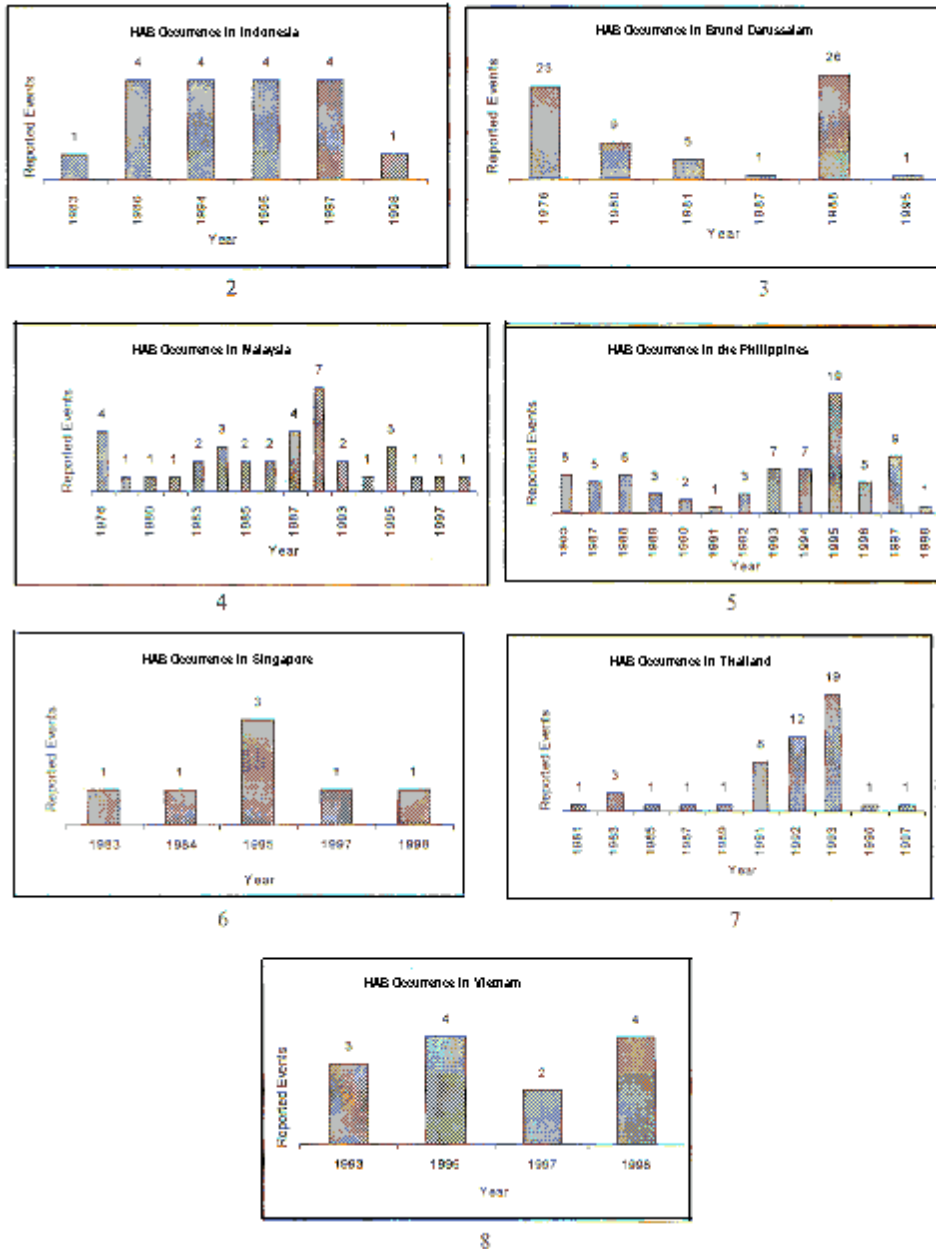
mitted to the country anchors through the Network Coordinator based in the Philippines when harmful algal bloom events occur within the region. Figures 2 to 8 show the frequency of HAB occurrences in Southeast Asia and Table 1 presents the harmful algal species identified on

formats. The Network Coordinator then broadcasts the report by fax to all member countries of the Network. With the advent of electronic communication, the electronic mail (e-mail) is being used as a back-up means of communication between the Network Coordinator and the

country anchors of the ASEAN member countries. Aside from being the focal point of the Network, the Coordinator is also responsible for entry of all data and the creation of a historical database for HABs in the ASEAN region. This database, which can be accessed in the

ASEAN-Canada Red Tide Network web site (<http://msi01.cs.upd.edu.ph/asean-canada/>), is meant to be reviewed and analyzed by experts to enhance present management strategies for toxic Red Tide/HAB problems.

Figures 2-8 Harmful algal bloom occurrences in Southeast Asia as reported in the Alert Network and publications.



## GENERAL INTRODUCTION

### The Red Tide Information Network

Regular publication and distribution of the Southeast Asian Harmful Algal Bulletin (SEAHAB) has occurred since 1993. A growing number of people and agencies involved in HAB management and research have become interested and signed up to receive the newsletter.

Red Tide/HAB management has been enhanced and expanded through exchange of information between the Network Coordinator and the country anchors. Usual activities include regular monitoring of Red Tide/HAB affected areas to detect the toxicity of shellfish and other bivalves, timely banning and lifting of bans on the harvest of shellfish imposed by the

authorities and public awareness on the Red Tide phenomenon through information drive and formal/non-formal education of the public.

In the Philippines, a “Shellfish Sanitation Program” has been designed for legislation by government through consultation with non-government organizations and the fishermen. Likewise, HABs have been considered as part of Environmental Impact Assessments and Coastal Zone Management projects implemented by government agencies. Further, financial report through provision of alternative sources of food and income has been started.

Table 1. Harmful Algal Species Reported in Southeast Asia based on the Alert Network and Publications

Country	Year	Organism
Singapore	1983	<i>Chatonella</i> sp.
	1984	Unknown sp.
	1995	Unknown sp.
	1997	<i>Trichodesmium thibautii</i>
		<i>Gambierdiscus yamamotoi</i>
	1998	<i>Gambierdiscus yamamotoi</i> , <i>Trichodesmium thibautii</i>
Brunei	1976	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1980	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1981	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1987	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1988	<i>pyrodinium bahamense</i> var. <i>compressum</i>
Vietnam	1993	<i>Trichodesmium</i>
	1995	<i>Ceratium</i> , <i>Noctiluca</i> , <i>Dinophysis</i>
	1998	<i>Oscillatoria</i> , Unknown sp., <i>Peridinium</i>
Indonesia	1983	Unknown sp.
	1986	<i>Ceratium</i> , <i>Dinophysis</i> , <i>Noctiluca</i> , <i>pyrodinium bahamense</i> var. <i>compressum</i>
	1994	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1995	<i>Trichodesmium</i> , <i>pyrodinium bahamense</i> var. <i>compressum</i> , <i>Noctiluca</i> , Unknown sp.
	1997	<i>Trichodesmium</i> , <i>Gymnodinium</i> , <i>Alexandrium</i> , <i>Gonyaulax spinifera</i> , <i>Gambierdiscus toxicus</i> , <i>pyrodinium bahamense</i> var. <i>compressum</i> , <i>Gymnodinium catenatum</i>

Country	Year	Organism
	1998	<i>Gymnodinium catenatum</i>
<b>Thailand</b>	1981	<i>Noctiluca, Ceratium</i>
	1983	<i>Trichodesmium</i> , Unknown sp., <i>Protogonyaulax</i>
	1985	<i>Alexandrium</i>
	1987	<i>Noctiluca, Trichodesmium</i>
	1989	<i>Trichodesmium</i>
	1991	<i>Mesodinium, Noctiluca, Trichodesmium</i>
	1992	<i>Noctiluca</i>
	1993	<i>Cochlodinium, Noctiluca, Trichodesmium</i>
	1996	<i>Chatonella</i>
	1997	<i>Noctiluca</i>
<b>Malaysia</b>	1976	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1979	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1980	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1981	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1983	<i>pyrodinium bahamense</i> var. <i>compressum</i> , <i>Chatone//a</i>
	1984	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1985	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1986	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1987	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1988	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1993	<i>pyrodinium bahamense</i> var. <i>compressum</i> , Unknown sp.
	1994	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1995	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1996	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1997	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1998	<i>pyrodinium bahamense</i> var. <i>compressum</i> , <i>Alexandrium tama-yavanichi</i> , <i>Dinophysis caudata</i>
<b>Philippines</b>	1983	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1987	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1988	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1989	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1990	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1991	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1992	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1993	<i>pyrodinium bahamense</i> var. <i>compressum</i> , <i>Gymnodinium</i>
	1994	<i>pyrodinium bahamense</i> var. <i>compressum</i>
	1995	<i>pyrodinium bahamense</i> var. <i>compressum</i> , Unknown sp.
	1996	<i>pyrodinium bahamense</i> var. <i>compressum</i> , <i>Gymnodinium catenatum</i> , <i>Dinophysis</i>
	1997	<i>Gymnodinium</i> , Unknown sp.
	1998	<i>pyrodinium bahamense</i> var. <i>compressum</i> , <i>Gymnodinium catenatum</i>

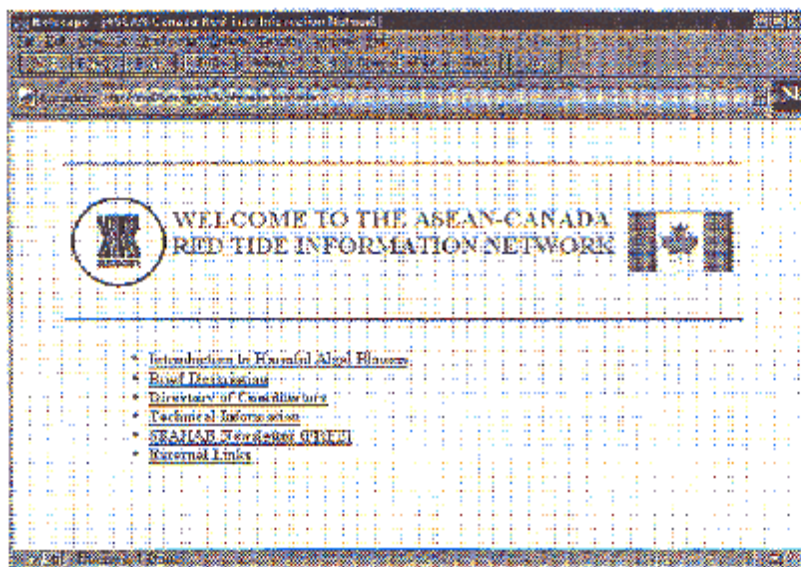


Figure 9 The ASEAN-Canada Red Tide Information Web Page

### The ASEAN-Canada Red Tide Information Web Page

To ensure faster communication and greater access to available information, The ASEAN-Canada Red Tide Information Web Page was established. This includes icons on introduction to harmful algal blooms, brief description of the Network, technical information, directory of coordinators, SEAHAB Newsletter and external links to other homepages in relation to HABs. External linkages established are with the homepages of Asia-Pacific Economic Conference (APEC) Secretariat, The Department of Fisheries and Oceans (DFO) Canada, The Harmful Algae Page, S.T.A.R.T. Solutions to End Red Tide, United Nation Educational, Scientific and Cultural Organization (UNESCO) and the Intergovernmental Oceanographic Commission (IOC) -Science and Communication Centre on Harmful Algae. Future plans include collaboration with publishers for the establishment of HAB/Red Tide ref-

erence database and inclusion of an on-line description of HAB/Red Tide organism identification, chemical and biological assay procedures in the homepage (Figure 9).

### Future Directions and Needs

Red Tide/harmful algal bloom activities in the region should be institutionalized with appropriate legislation and government support provided on a more permanent basis.

Research, monitoring and management should be expanded considering all potential problems from harmful algal blooms. To economize or minimize on costs, time and energy, these should be done in collaboration or consonance with existing or planned activities as in the country's Environmental Impact Analysis (EIA) systems, Coastal Zone Management and others. Networking in the region should be enhanced and linkages with other regional and international programs developed.

A coordinating center as a "SEAHAB Center" should be developed -a proposal recently put forward by the University of the Philippines' Marine Science Institute for the rest of Southeast Asia. The Center is envisioned to continue not only the present activities of the ASEAN-Canada Red Tide Network but also help in the coordination and implementation of regional (and international) Red Tide HAB effects.

Research to enhance and help in the

understanding of Red Tide/HAB phenomena has been carried out. These include studies on basic understanding of the causes and behavior of HABs. Roles of anthropogenic nutrients/climate variability, seeding/cysts and critical stages of bloom formation/propagation, however, should be addressed as well. The roles/impacts of parasites/predators and physico-chemical processes as they relate to HAB management should also be dealt with.

$\pi$





---

## Receptors and Receptor Assay Principles

Lourdes J. Cruz

Marine Science Institute, College of Science  
University of the Philippines, Diliman, Q. C. 1101

---

### Introduction

Receptors are biological macromolecules that are involved directly in chemical signalling between and within cells. They play a critical role in the regulation of cellular activity by hormones, neurotransmitters and other modulators. The interaction of specific molecules (ligands) with a receptor may influence cellular activity through a change in membrane permeability, a change in enzyme activity or an alteration in gene transcription. Most lipophilic hormones interact with intracellular receptor proteins whereas water-soluble ligands such as peptide hormones and neurotransmitters target cell-surface receptors.

In the transmission and integration of signals in the nervous system two types of ion channels play important roles. These are the ligand-gated ion channels such as acetylcholine receptors, glutamate receptors, glycine receptors etc., which open in response to specific molecules, the neurotransmitters. In addition there are ion channels that are voltage-gated. As electrical signals are transmitted down the nerve and muscle, voltage-sensitive ion channels open in response to changes in the distribution of charges across the cellular membrane. Since ion channels are mostly located on cell surfaces, they are readily accessible for interaction with extracellular molecules including drugs and toxins.

J. N. Langley of UK and P. Erlich of Germany are both credited for introducing the receptor concept. To explain the effect of nicotine and curare on nerve stimulation but their lack of direct effect on muscle contraction, Langley (1898) conceptualized an accessory substance, the “**receptive substance**”, which receives stimuli and transfers it to the contractile material. At about the same time, Paul Erlich screened 600 organometallic compounds and discovered salvarsan, an anti-syphilis drug with relatively minor side effects on humans. To explain the selectivity of salvarsan, he stated that toxins react with corresponding chemical organs of the protoplasm, which he referred to as “**receptors**”. “When the poisons and the organs sensitive to it do not come into contact, or when sensitiveness of the organs does not exist, there can be no action. ... Toxic actions can only occur when receptors fitted to anchor the toxins are present.” (Jenkinson DH, 1996).

“Receptor” is now a widely used term encompassing sensory receptors such as mechanoreceptors, baroreceptors and telereceptors; T-cell receptors; etc. (Black JW, 1996)

## GENERAL INTRODUCTION

### Toxins acting on ion channels

In the struggle for survival, many organisms have developed toxins as armaments for defense and offense. The toxins are used by animals for catching prey and warding off prey, and by plants as a defense against herbivory. Interference with key elements of the neuromuscular junction is an excellent strategy for incapacitating other organisms (see Table

1 for a summary of selected toxins and their targets). Apparently, voltage-gated sodium channels are the favorite target of toxin producers. These channels are essential in the generation and propagation of action potentials and therefore critical for effective movement in animals.

Table 1. Marine toxins and their ion channel targets (Myers et al, 1993; Shoon et al., 1994; Craig et al., 1998). The interaction of toxins with specific channel subtypes are believed to be primarily responsible for the characteristic symptoms observed in victims of poisoning and envenomation (Olivera BM et al., 1999).

Ion channel	Toxin	Chemical Type	Mode of Action
Voltage-sensitive calcium channels	$\omega$ -Conotoxins	Peptide (24-27 AA)	Block neuronal subtypes of Ca channels; $\omega$ -GVIA very specific for N-subtype
Acetylcholine receptors	$\alpha$ -Toxins of sea snakes	Peptide (61-74 AA)	Competitive antagonist
	$\alpha$ -Conotoxins	Peptide (13-15 AA)	Competitive antagonist
	Lophotoxin of gorgonian corals	Diterpene lactone	Noncompetitive antagonist
	Neosurugatoxin (Jap. Ivory snail)	Glycoside	Noncompetitive antagonist
Voltage-sensitive sodium channels	Tetrodotoxin (TTX)	Heterocyclic guanidinium compound	Block of ion channel (Site 1)
	Saxitoxins (STX)	Heterocyclic guanidinium cpds.	Block of ion channel (Site 1)
	$\mu$ -Conotoxins	Peptide (22-23 AA)	Block of ion channel (Site 1)
	Sea anemone toxins	Peptide (47-49 AA)	Inhibit inactivation (Site 3)
	Brevetoxins	Polyether ladder	Channel activator (Site 5)
	Ciguatoxins	Polyether ladder	Channel activator (Site 5)
	$\delta$ -Conotoxins	Peptide (27-29 AA)	Delay inactivation (Site 6)
Glutamate receptors	Domoic acid	Tricarboxylic imino acid	Agonist of kainate subtype of glutamate receptors
	Conantokin	Peptide (17-21 AA)	Antagonist of NMDA subtype of glutamate receptors

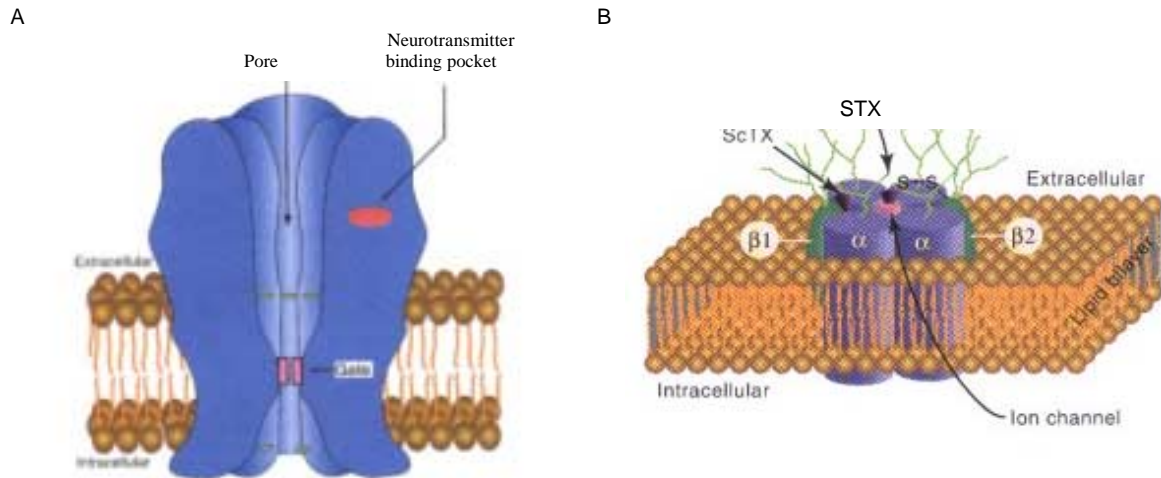
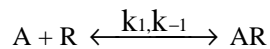


Figure 1. Models of receptors. A. Acetylcholine receptor as an example of ligand-gated ion channel. B. Na channel as an example of voltage-gated ion channels (from Zigmond et al., *Fundamental Neuroscience*, 1999).

### Receptor Binding Assays (Jenkinson, DH; Hylett DG, 1996)

The simplest model for the combination of a ligand, A with a receptor, R is:



where  $k_1$  and  $k_{-1}$  are the rate constants for the forward and backward reactions. The rate of the reaction is proportional to the product of reactant concentration and at equilibrium,

$$k_1 [A][R] = k_{-1} [AR]$$

Based on this relationship and its variations, several equations (Hill-Langmuir equation, Hill plot, Scatchard plot, Schild equation) to quantify binding of ligands to macromolecules have been derived. With the use of radio-labeled ligands, it is possible to increase the sen-

sitivity of measurement to femtomole ( $10^{-15}$  M) levels.

When studying a new receptor or ligand, radioligand binding studies are useful for measuring dissociation equilibrium constants, association and dissociation rate constants and receptor density. In the presence of a toxin or an inhibitor, binding competition assays can be done to measure its affinity for the receptor and to quantify the amount of toxin present in a given sample.

Binding of ligands to a particular receptor is referred to as specific binding, whereas the binding to nonreceptor tissue components is termed nonspecific binding. As show in Figure 2, specific binding is saturable whereas nonspecific binding is non-saturable at least within the range of concentrations used for the ligand of interest.

## GENERAL INTRODUCTION

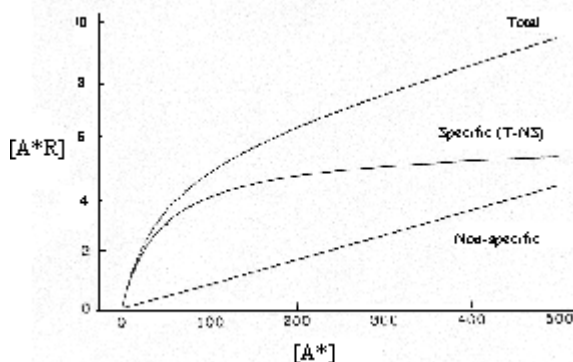


Figure 2. Specific and nonspecific binding of a radioligand

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

Reduction of the binding of the radioligand to the receptor (formation of the A\*R complex) depends on the con-

centration of the unlabeled ligand as shown in Figure 3. This is the basis for receptor binding assays used to quantify the amount of toxin in test samples as compared to reference standards.

Competition binding assays may also be used in characterizing new toxins for which no radiolabeled derivative is yet available. Using the Schild equation, the dissociation constant of the receptor-toxin complex can be estimated from a series of competition binding assays.

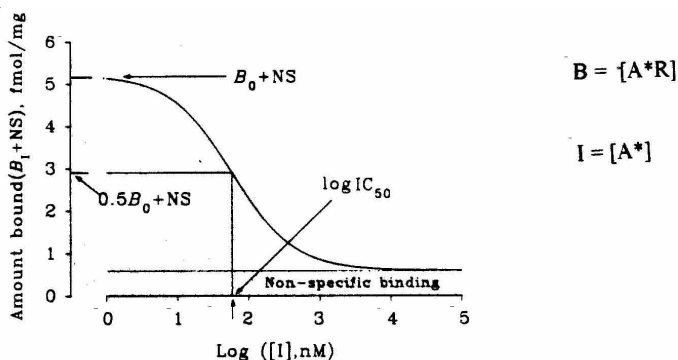
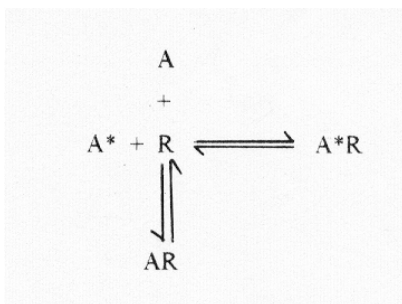


Figure 3. Illustration of a competition experiment

**References**

- Black JW (1996) in Foreman JC & Johansen T (eds.) Receptor Pharmacology, CRC Press, Inc. NY, pp.277-285.
- Craig AG, Zafaralla G, Cruz LJ, Santos AD, Hillyard DR, Dykert J, Rivier JE, Gray WR, Imperial JS, Dela Cruz RG, Sporning A, Terlau H. West PJ, Yoshikami D & Olivera BM (1998) Biochemistry 37: 16019-16025.
- Haylett D (1996) in Foreman JC & Johnsen T (eds.). Receptor Pharmacology, CRC Press, Inc., NY, pp. 121-149.
- Jenkenson DH (12996) in Foreman JC & Johansen T (eds.). Receptor Pharmacology, CRC Press, Inc., NY, pp. 3-62
- Myers RA, Cruz LJ & Olivera BM (1993) Chem. Rev. 93: 1923-1936.
- Olivera BM, Cruz LJ & Yoshikami D (1999) Current Opinion in Neurobiology, in press.
- Shon K-J, Hasson AH, Spira ME, Cruz LJ, Gray WR & Olivera BM (1994) Biochemistry 33: 11420-11425.

 $\pi$



# LABORATORY PROCEDURES



---

## Receptor Binding Assay for Saxitoxin

---

### ◆ *STX Receptor Binding Assay: Microplate or Traditional Scintillation Counting*

#### *A. Apparatus and Supplies*

- Traditional or microplate scintillation counter
- micropipettes (1-1000  $\mu$ l variable volumes)
- multichannel pipettes (5-200  $\mu$ l variable volume) and pipette tips
- 96-well microtiter filter plate with 0.65 mm Durapore membrane and type C glass fiber filter (Millipore, Bedford, MA, USA; cat. no. MAFC NOB 50)
- Multiscreen vacuum manifold (Millipore; cat. no. MAVM 096 01)
- vacuum pump or house vacuum
- 15 and 30 ml plastic centrifuge tubes
- dilution tubes
- reagent reservoir
- ice bucket
- vortex
- sealing tape

For traditional counter only:

- Multiscreen disposable punch tips (Millipore; cat. no. MADP 196 10)
- Multiscreen punch kit B for 4 ml vials (Millipore cat. no. MAPK 896 OB)
- Scintillation vials, 4 ml

#### *B. Reagents*

- [ $^3$ H] STX diacetate (Amersham, Arlington, Heights, ILL, USA; cat. no. TRK922)
- STX diHCl Calibration Solution (NRC/Inst. for Marine Biosciences, Halifax, N.S., CA)
- Buffer, pH7.5: 75 mM HEPES (Sigma, St. Louis, MO; cat. No. H9136) /140 mM NaCl
- rat brain synaptosome preparation (page 75)

For traditional counter:

- Scintiverse liquid scintillation cocktail (or equivalent)

For microplate counter:

- Optiphase liquid scintillation cocktail (Wallac, Inc.)

#### *C. Preparation of Stock Solutions and Standards*

- Radioligand solution:  
[ $^3$ H]STX diacetate working solution.  
Note: Amersham stock is provided at 14.9 Ci/mmol, 50  $\mu$ Ci, 200  $\mu$ L volume, which is (50  $\mu$ Ci/14.9  $\mu$ Ci/nmol ) or 3.35 nmol.

## LABORATORY PROCEDURES

STX conc. = 3.35 nmol/0.2 ml.  
= 16.8  $\mu$ M.

The working stock concentration of [ $^3$ H]STX is 13.25 nM.

To make enough working stock solution for one 96-well plate, add 3  $\mu$ L [ $^3$ H]STX to 3.8 mL HEPES/NaCl buffer.

Keep on ice.

The final assay concentration of [ $^3$ H]STX will be 2.2 nM.

- Unlabelled STX working solution:

**Note:** *National Research Council Canada/Institute for Marine Biosciences (NRC/IMB) STX Calibration Solution is provided at a concentration of 25  $\mu$ g/ml (= 67.2  $\mu$ M). Standard curve is made from this working stock using the follow dilution series (this is sufficient for one standard curve run in triplicate):*

- 
- Standard curve:

$\mu$ l of Standard	$\mu$ l of Buffer	Concentration *	Concentration in assay
11 $\mu$ l 67.2 $\mu$ M STX	+ 114 $\mu$ l	6.0 x 10 <sup>-6</sup> M	1.0 x 10 <sup>-6</sup> M
14 $\mu$ l 6.0x10 <sup>-6</sup>	+ 126 $\mu$ l	6.0 x 10 <sup>-7</sup> M	1.0 x 10 <sup>-7</sup> M
14 $\mu$ l 6.0x10 <sup>-7</sup>	+ 126 $\mu$ l	6.0 x 10 <sup>-8</sup> M	1.0 x 10 <sup>-8</sup> M
7 $\mu$ l 6.0x10 <sup>-7</sup>	+ 133 $\mu$ l	3.0 x 10 <sup>-8</sup> M	5.0 x 10 <sup>-9</sup> M
28 $\mu$ l 3.0x10 <sup>-8</sup>	+ 112 $\mu$ l	6.0 x 10 <sup>-9</sup> M	1.0 x 10 <sup>-9</sup> M
14 $\mu$ l 6.0x10 <sup>-9</sup>	+ 126 $\mu$ l	6.0 x 10 <sup>-10</sup> M	1.0 x 10 <sup>-10</sup> M
Reference	+ 140 $\mu$ l	Buffer Only	0

\*All standard solutions are diluted 1/6 in the assay.

---

- Interassay calibration standard (QC check)

Make up STX standard at 3.0 x 10<sup>-8</sup> M in 0.003 M HCl. Store refrigerated (as advised by supplier). Include in the assay as a sample (final concentration in assay 5.0 x 10<sup>-9</sup> M) to serve as a QC check to confirm day-to-day performance of the assay.

- Rat brain synaptosome preparation

Dilute rat brain synaptosome preparation (page 75) with ice cold 75 mM HEPES/140 mM NaCl, pH 7.5, to yield a final protein concentration of approximately 0.5 mg/ml (this is usually 1/8).

### D. Performing the Assay

- Plate Setup and Incubation

## RECEPTOR ASSAY PROCEDURE

When possible, use a multichannel pipette to minimize pipetting effort. Standard curve, QC check, and samples are run in duplicate. Multiple dilutions of samples may need to be analyzed before a dilution will fall on the linear part of the competition curve,

which is required for quantification.

For ease of analysis, it is convenient to use a standard plate layout which maximizes the number of samples and standards which can be analyzed on one plate:

Sample plate layout:

1	2	3	4	5	6	7	8	9	10	11	12
$10^{-6}$	$10^{-6}$	$10^{-6}$	U1 1/2	U1 1/2	U1 1/2	U3 1/10	U3 1/10	U3 1/10	U6 1/5	U6 1/5	U6 1/5
$10^{-7}$	$10^{-7}$	$10^{-7}$	U1 1/5	U1 1/5	U1 1/5	U4 1/2	U4 1/2	U4 1/2	U6 1/10	U6 1/10	U6 1/10
$10^{-8}$	$10^{-8}$	$10^{-8}$	U1 1/10	U1 1/10	U1 1/10	U4 1/5	U4 1/5	U4 1/5	U7 1/2	U7 1/2	U7 1/2
3x $10^{-9}$	3x $10^{-9}$	3x $10^{-9}$	U2 1/2	U2 1/2	U2 1/2	U4 1/10	U4 1/10	U4 1/10	U7 1/5	U7 1/5	U7 1/5
$10^{-9}$	$10^{-10}$	$10^{-10}$	U2 1/5	U2 1/5	U2 1/5	U5 1/2	U5 1/2	U5 1/2	U7 1/10	U7 1/10	U7 1/10
$10^{-10}$	$10^{-11}$	$10^{-11}$	U2 1/10	U2 1/10	U2 1/10	U5 1/5	U5 1/5	U5 1/5	U8 1/2	U8 1/2	U8 1/2
Ref	Ref	Ref	U3 1/2	U3 1/2	U3 1/2	U5 1/10	U5 1/10	U5 1/10	U8 1/5	U8 1/5	U8 1/5
QC	QC	QC	U3 1/5	U3 1/5	U3 1/5	U6 1/2	U6 1/2	U6 1/2	U8 1/10	U8 1/10	U8 1/10

U = unknown sample

1. Add in the following order to each of the 96 wells:  
35  $\mu$ L [ $^3$ H]STX  
35  $\mu$ L STX standard, QC check, or sample  
140  $\mu$ L synaptosome preparation
2. Cover and incubate plate at 40°C for 1 hour.
  - Assay Filtration
    1. Place 96-well plate on the Multiscreen vacuum manifold. Turn on vacuum. Optimum vacuum will pull the wells to dryness in

## LABORATORY PROCEDURES

- 2-5 sec. Pull contents of well through until liquid is removed.
  2. Rinse each well once with 200  $\mu$ L ice cold HEPES/NaCl using multichannel pipette.
  3. Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.
- Counting
    - a. Counting in traditional scintillation counter:
      1. Set plate in the Multiscreeen punch system. Place disposable punch tips on top of plate. Punch wells into vials pre-filled with 4 mL scintillation cocktail (Scintiverse or equivalent). Put caps on vials, vortex.
      2. Allow vials to sit overnight.
      3. Count on tritium window in traditional scintillation counter.
    - b. Counting in microplate scintillation counter:
      1. Place microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape.
      2. Add 25  $\mu$ L Optiphase scintillation cocktail. Seal top of plate with sealing tape.
      3. Allow to sit 30 min before counting in microplate counter.
      4. Count in microplate scintillation counter.



## Data Analysis

Curve fitting for binding competition data is best fitted using a 4 parameter logistic fit (4PL), which can be carried out using commercially available curve fitting software packages. General graphing packages capable of

4PL curve fitting include Graph Pad Prism (Lotus) and Sigma Plot (Jandel). Specialized programs for receptor assay applications include Ligand (Biosoft, Milltown, NJ) and Multicalc (Wallac, Gaithersburg, MD).

Sample Calculation:

Sample concentration is calculated in  $\mu\text{g}$  STX equivalents/ 100 g shellfish, using the following formula:

$$(\text{nM equiv STX}) \times (\text{sample dilution}) \times \frac{210 \text{ ul total volume}}{35 \text{ ul sample}} = \text{nM equiv STX in extract}$$

$$(\text{nM equiv STX in extract}) \times \frac{1\text{L}}{1000 \text{ mL}} \times \frac{372 \text{ ng}}{\text{nmol}} \times \frac{1 \text{ ug}}{1000 \text{ ng}} = \mu\text{g equiv. STX per mL}$$

$$\mu\text{g STX equivalent} \times \frac{\text{ml extract}}{\text{g shellfish extracted}} \times 100 = \mu\text{g STX equiv./ 100g shellfish}$$



## Assay Quality Control

---

- **Precision**

Precision of an assay is defined as: “A measure of the variation in estimating a sample, e.g. the intraassay (within assay) coefficient of variation” (see below for definition). Alternatively, this may be explained as the range of possible values obtained by an assay for the true value of a sample. If the assay variation is large, then the range of values obtained for the true value will be large. That is, if the true value of a sample is 10, and the assay varies as much as 20%, the assay values obtained may range from 8 to 12 for that sample. Intraassay coefficient of variation is usually measured by analyzing a number of samples (they can be actual samples, or “quality control” samples) multiple times, and calculating the coefficient of variation for each of the samples. Acceptable intraassay variation is less than 10%.

### Coefficient of variation

Coefficient of variation is defined as: “The standard deviation of multiple estimates of a sample, divided by the mean of the multiple estimates, times 100”.

MEAN of 20 replicates: 6.9  
Standard Deviation : 0.61

$$\%CV = \frac{0.61}{6.9} \times 100 = 8.8$$

- **Accuracy**

Accuracy of an assay is defined as: “The ability of an assay system to assess the true value of a sample”. That is, the ability to estimate the concentration of a toxin in a sample that is identical to the actual concentration of toxin in the sample, according to the best methods available serving as reference standards. Since few methods are absolute in their determinations, an acceptable method of testing for agreement between values of a sample obtained by receptor binding assay and the “true value” of the sample is to prepare a large pool sample and assess the value of the sample by a variety of methods and compare them. If there is reasonable agreement between different methods, then the value obtained must be acceptably close to the “true value.”

- **Interassay Variation**

The “interassay” or between assay variability is extremely important since its control is the only way to ensure that STX values from

A suitable interassay calibration standard (QC check) is an STX standard at  $3.0 \times 10^{-8}$  M in 0.003 M HCl. This is included in the assay as a sample (final concentration in assay  $5.0 \times 10^{-9}$  M) to serve as a QC check to confirm day-to-day performance of the assay.

- **Critical Control Points**

1. For a ligand which interacts specifically at one receptor site, the slope of the resulting competition curve should theoretically be 1.0. If the slope of the curve for a given assay is outside of the acceptability range of 0.8-1.2, linearity of the assay will be compromised and quantitation of the unknowns will be incorrect. Therefore, the assay should be re-run.

a series of samples reflects its toxic potency and are not the variation due to drift between assay.

2. The QC check standard should fall within  $\pm 20\%$  of the stated value (5nM). If the QC check standard does not fall within acceptable limits, the assay should be re-run.
  3. Sample dilutions which fall on the linear part of the curve ( $B/B_0 = 0.2-0.8$ ) can be accurately quantified. In the event that no sample dilutions fall within the linear range (i.e. concentration is too high,  $B/B_0 < 0.2$ ), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e.,  $B/B_0 > 0.8$ ), the sample must be reported as below limit of detection.
- 





---

## Shellfish Extraction Protocol For Saxitoxin

---

### A. Apparatus and Supplies

- Vortex mixer, Blender or Polytron
- Centrifuge, 3000g
- Hot Plate
- Pipette, 10 ml, plastic
- Vials
- 50 ml. Polypropylene conical tube
- pH paper

### B. Reagents

- 0.1 N sodium hydroxide solution
- 0.1 N and 0.3 N hydrochloric acid

### C. Procedure

1. Weigh out 100 grams of shucked, drained shellfish meats. Homogenize thoroughly using a blender or polytron.
2. Subsample the homogenized sample by weighing 10g homogenate directly into a 50mL polypropylene conical tube. (Try to obtain exactly 10.0g of each sample. If a 10g-sample is not obtained, record exact weight taken.)
3. To each tube, add 10 ml of 0.1N HCl (or a volume equal to the sample weight i.e., 9.5 mL for 9.5g sample) using a disposable

10mL- plastic pipette to avoid “wetting” error.

4. Cap the tubes tightly and mix.
5. Check the pH of each sample with narrow range pH paper. The pH should be between 2.0 and 4.0, preferably around 3.0. To lower the pH, add 3N HCl dropwise and mix. To raise the pH, add 0.1N NaOH.
6. **Insure that the tubes are tightly capped.** Transfer the tubes (5 at a time) to a beaker or dish of boiling water. Once the temperature of the water reaches 100°C after the tubes have been added, start a 5 minute countdown.
7. Remove the tubes to a container of cold water and allow to cool to room temperature. Mix the contents of the tubes. Check and adjust the pH again, if necessary, as in step #5.
8. Centrifuge the tubes until a clear supernatant is obtained (15-20min at 3000g).
9. Pipet off 1-2mL of the supernatant and place in a clean marked tube or vial. Keep tightly capped at 4°C.

π



## Rat Brain Membrane Preparation

### A. Apparatus and Supplies

- Teflon/glass homogenizer
- Motorized homogenizer
- Ultracentrifuge
- Swinging bucket rotor
- Ultracentrifuge tubes, polyallomer 12 ml
- 15 ml plastic tubes with lids
- 500 ml beaker

### B. Reagents

- 20 Rat brains, male Holzman, six week old (Harlan Bioproducts, Indianapolis, IN)
- 20 mM Tris/140 mM NaCl, pH 7.1 (for 1 L 2.422 g Tris, 8.182 g NaCl, 1 mL 1.0 M PMSF\* pH 7.1 with HCl )

[\*Dissolve 0.1749 PMSF (phenyl methylsulfonyl fluoride; Sigma Cat. No. P7626) in 1 mL isopropanol; larger volumes may be made and stored in 1- mL aliquots at 20°C.]

### C. Procedure

1. Remove medulla from each brain and discard.
2. Place cerebral cortex in Tris/NaCl on ice until ready to proceed.
3. Homogenize two brains in 25 ml Tris/NaCl buffer in glass/teflon homogenizer at 70% full speed (385 rpm). Keep tube in ice at all times. Pour homogenized tissue into 250 ml beaker on ice and re-

peat procedure with remaining cortices.

4. Pool homogenized tissue. Transfer to polyallomer ultracentrifuge tubes.
5. Centrifuge at 54000 x g for 15 min at 4°C.
6. Aspirate supernatant. Resuspend pellets in Tris/NaCl buffer.
7. Pool resuspended membrane preparation in 200 ml total. (Keep on ice.)
8. Polytron at 70% full speed for 20 sec. (Keep on ice.)
9. Aliquot 2 ml/tube in polypropylene tubes.
10. Freeze and store at -80°C. This preparation is stable for at least 6 months.

### D. Determination of Tissue Linearity

1. Make dilutions of membrane preparation (1/2, 1/4, 1/6, 1/8, 1/10, 1/12, 1/16) in HEPES/NaCl buffer. Add 140 µl of each dilution to triplicate wells of a 96 well plate containing:
  - 35 µl HEPES/NaCl
  - 35 µl [<sup>3</sup>H]STX (To make enough working stock solution for one 96-well plate, add 3 µl [<sup>3</sup>H]STX to 3.8 mL

## LABORATORY PROCEDURES

HEPES/NaCl buffer. The final assay concentration of [<sup>3</sup>H]STX will be 2.2 nM)

2. Incubate for 1 h at 4°C.
3. Filter and count as per assay protocol.
4. Plot CPMs vs dilutions to determine the maximum dilution that

gives adequate binding (> 1000 CPM desired), such that a minimum of membrane preparation is used in each assay. This dilution must be on the linear part of the curve.

### Reference

Hartshome, R.P., and Catterall, W.A. (1984) *J. Biol. Chem.* 259: 1667-1675.

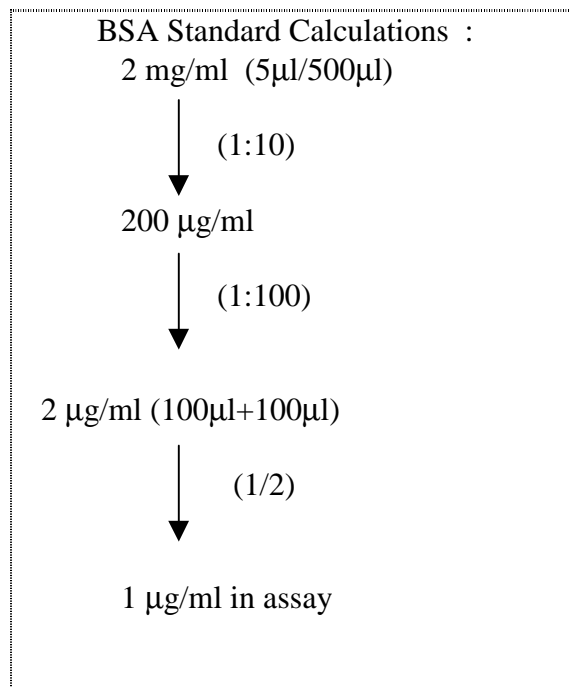
## Microplate Protein Assay

### A. Apparatus and Supplies

- Titertek Multiskan Microplate Reader
- Micropipettors (20 - 1000 uL)
- Micro BSA Protein Assay Reagent Kit, Pierce, Rockford, Ill
- Flat- bottom 96-well microtiter plate (Costar, cat# 3595)
- Reagent ABC
- Oven/incubator

### B. Procedures

1. Prepare Standard Curve  
Make 1:10 dilution of supplied BSA



1/10 Standard ( $\mu$ l)	distilled H <sub>2</sub> O (ml)	Concentration ( $\mu$ g/ml)	Reagent ABC (ml)
5 (10)	0.495 (0.490)	1 ( 2)	0.1
10 (20)	0.490 (0.480)	2 ( 4)	0.1
15 (30)	0.485 (0.470)	3 ( 6)	0.1
20 (40)	0.480 (0.460)	4 ( 8)	0.1
25 (50)	0.475 (0.450)	5 (10)	0.1
30 (60)	0.470 (0.440)	6 (12)	0.1
35 (70)	0.465 (0.430)	7 (14)	0.1
40 (80)	0.460 (0.420)	8 (16)	0.1
45 (90)	0.455 (0.410)	9 (18)	0.1

2. Add 0.1 ml of each standard to a well of flat bottom 96 well plate (Costar, cat#3595).
3. Prepare unknown dilutions (total volume required = 0.1 ml/well).

## LABORATORY PROCEDURES

4. Add 0.1ml of each prepared unknown dilution to a well of the 96-well plate.
5. Prepare reagent ABC (2 parts C : 48 parts B : 50 parts A) in that order.
6. Add 0.1 ml reagent ABC to each well containing a sample or standard.
7. Incubate in oven at 60 °C for 1 hour or in incubator at 37 °C for 4 hours.
8. Measure absorbance in spectrophotometer (Titertek Multiskan) with 570 nm filter in place.

$\pi$

## APPENDICES



## Radioisotopes - Characteristics, Handling, Measurement, Calculations

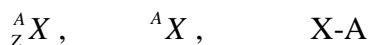
Estelita G. Cabalfin  
 Philippine Nuclear Research Institute  
 Quezon City, Philippines

### BASIC ATOMIC STRUCTURE

An atom is composed of:

1. the nucleus, which in turn is composed of
  - a. protons: charge = +1, mass = 1
  - b. neutrons: charge = 0, mass = 1
2. orbiting electrons: charge = -1, mass = 0

NUCLIDE - atomic species characterized by number of protons and mass number



where X, symbol of element  
 Z, atomic number = number of protons  
 Each element has a unique atomic no.  
 N, no. of neutron  
 A, mass number = Z + N

Example:  ${}^{12}_6 C$ ,  ${}^{12} C$ , C-12

ISOTOPES - Atoms with the same Z, but different N and therefore different A

ex.  ${}^1_1 H$ ,  
 ${}^2_1 H$  (deuterium)  
 ${}^3_1 H$  (tritium)

**All isotopes of a particular element have identical chemical properties.**

### RADIOACTIVE DECAY

The nuclei of some nuclides are not stable. They disintegrate or undergo nuclear transformation spontaneously and are called "radioactive".

Radioactive nuclides are called **radionuclides**.

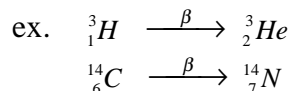
Radioactive isotopes are called **radioisotopes**.

Upon disintegration, radioactive nuclides may emit:

- a. alpha ( $\alpha$ ) particles - fast moving helium nuclei
- b. beta ( $\beta$ ) particles - fast moving electrons
- c. gamma ( $\gamma$ ) rays - electromagnetic radiation of very short wavelength

Beta decay is equivalent to the nuclear transformation of a neutron into a proton and an electron. The proton remains in the nucleus, while the electron is emitted. The ejected electron is called a beta ( $\beta$ ) particle.

Beta particles are emitted with a distribution of energies up to a maximum which is characteristic of a particular radionuclide



### ACTIVITY

$$\text{ACTIVITY (dps)} = \frac{dN}{dt} = -\lambda N$$

## APPENDICES

$$\lambda = \frac{0.693}{T_{1/2}}$$

N, no. of radioactive atoms  
 $\lambda$ , decay constant  
 dps, disintegration per second

### INTERACTION OF $\beta$ PARTICLES WITH MATTER

#### Radioactive Decay Law

$$A = A_0 e^{-\lambda t}$$

$A_0$ , activity at time,  $t = 0$   
 A, activity at any time,  $t$

SI unit for activity: becquerel (Bq)  
 (1 Bq = 1 dps)

Non-SI unit for activity: curie (Ci)

$$1 \text{ Ci} = 3.7 \times 10^{10} \text{ dps}$$

$$1 \text{ Ci} = 3.7 \times 10^{10} \text{ Bq}$$

Half-life - time to reduce the amount of a radionuclide to half its original value

#### 1. IONIZATION

When the energy a  $\beta$  particle transfers to an orbital electron is enough to eject the electron from the atom, an ion pair is formed: the atom with a positive charge and the ejected electron with negative charge

#### 2. EXCITATION

When energy transferred to an orbital electron is just enough to raise the electron to an excited state

### PROPERTIES OF SOME COMMONLY USED $\beta$ - EMITTERS

	$^3\text{H}$	$^{14}\text{C}$	$^{35}\text{S}$
Half-life	12.3 y	5730 y	88 d
Max $\beta$ energy, keV	18	154	167
Ave $\beta$ energy, keV	6	50	49
Range in air, cm	0.61	30	30
Range in water, cm	0.0005	0.029	0.032
Dose rate, mSv/h, due to 1 kBq at 1 cm	-	0.51	0.48
Fraction transmitted through dead layer of skin (0.0007 cm)	-	0.11	0.16
Dose rate to basal cells, mSv/h, due to 1 kBq/cm <sup>2</sup>	-	0.35	0.48

## LIQUID SCINTILLATION COUNTING

Best method for counting  $\beta$  particles coming from  $^3\text{H}$  and  $^{14}\text{C}$

The radioactive sample is combined with a liquid scintillation cocktail or solid scintillator. Part of the kinetic energy of the  $\beta$  particle is transferred to the scintillator, which converts the energy into light, which is then detected by the LS system.

The scintillations produced are detected by a photomultiplier tube (PMT), which converts them into electrical pulses.

The intensity of the scintillation produced is proportional to the absorbed energy from the beta particle.

Efficiency, E, is the fraction of disintegration, which is detected by the counter

Eff. (E) = cpm/dpm

cpm = counts/minute

dpm = disintegration/minute

Efficiency can be affected by:

1. presence of any color in the sample
2. if mixture of sample and scintillator is not homogeneous
3. if radioactive material is trapped in solids

## SAFETY MEASURES

### A. EXTERNAL RADIATION HAZARD

$\beta$  particles with very low energies do not present a serious external hazard

### B. INTERNAL RADIATION HAZARD

Unsealed sources of radioactive material can be internal hazards.

Possible routes of entry of radioactive material into the body:

1. inhalation of airborne contamination
2. ingestion through the mouth
3. absorption through the skin
4. entry through open wound

Techniques for controlling internal hazards:

1. minimization of activity handled
2. administrative control
  - a. Classification of work areas
  - b. Rules and conditions of work
    - i. no eating, drinking, smoking or applying cosmetics
    - ii. good housekeeping
    - iii. use of protective clothing
    - iv. area and personnel contamination monitoring
  - c. source inventories
3. physical control
  - a. use of trays, barrier, glove boxes, fume hoods
  - b. containment of radioactive waste

## SOME CALCULATIONS

### 1. DECAY

Activity on label: 7 MBq  $^{35}\text{S}$  on 2 April 1999, what is the activity on 30 November 1999

$t = 242$  days  $\lambda = \frac{0.693}{88}$

$T_{1/2} (^{35}\text{S}) = 88$  days

$$\begin{aligned} A &= A_0 e^{-\lambda t} = 7 \times e^{-0.693 \times 242 / 88} \\ &= 7 \times 0.149 \\ &= 1.04 \text{ MBq} \end{aligned}$$

## APPENDICES

### 2. SPECIFIC ACTIVITY

Specific activity for radioligand is usually given as curies/mmole

$$\begin{aligned}1 \text{ fmole} &= 10^{-15} \text{ mole;} \\1 \text{ mmole} &= 10^{-3} \text{ mole;} \\1 \text{ Ci} &= 2.22 \times 10^{12} \text{ dpm}\end{aligned}$$

To convert to Z Ci/mmole to Y cpm/fmole

$$Y \frac{\text{cpm}}{\text{fmole}} = Z \frac{\text{Ci}}{\text{mmole}} \times 2.22 \times 10^{12} \frac{\text{dpm}}{\text{Ci}} \times 10^{-12} \frac{\text{mmole}}{\text{fmole}} \times E \frac{\text{cpm}}{\text{dpm}}$$

$$Y \frac{\text{cpm}}{\text{fmole}} = Z \frac{\text{Ci}}{\text{mmole}} \times 2.22 \times E \frac{\text{cpm}}{\text{dpm}}$$

### 3. CONCENTRATION OF LIGAND

Measure cpm of a small volume of radioligand stock solution,

C cpm in V ml  
Y cpm/fmole as calculated above

$$\text{Conc. in pM} = \frac{C \text{ cpm}}{V \text{ ml}} \times \frac{\text{fmole}}{Y \text{ cpm}} \times \frac{\text{pmole}}{1000 \text{ fmole}} \times \frac{1000 \text{ ml}}{\text{liter}}$$

$$\text{Conc. in pM} = \frac{C \text{ cpm}}{V \text{ ml} \times Y \text{ cpm/fmole}}$$

$\pi$

---

**Items Required for Receptor Assay**


---

ITEM	REMARK
<ol style="list-style-type: none"> <li>1. vacuum flask and tubing</li> <li>2. multichannel pipettor (2)</li> <li>3. pipettors (10<math>\mu</math>l, 200<math>\mu</math>l, 1000<math>\mu</math>l)</li> <li>4. rechargeable pipettor &amp; charger</li> <li>5. pipet tips (P200, P1000, 5mL)</li> <li>6. 15mL &amp; 50mL centrifuge tubes</li> <li>7. cryomarker, pens, pencil, scissors</li> <li>8. latex gloves</li> <li>9. 500 mL bottles for waste (2)</li> <li>10. paper towels</li> <li>11. mini-dilution tubes</li> <li>12. ice bucket</li> <li>13. detergent</li> <li>14. marking tape, radiation tape</li> <li>15. 96 well microtiter plates</li> <li>16. reagent reservoirs (large &amp; small)</li> <li>17. microplate carriers for MicroBeta</li> <li>18. assay barcode, stop barcode, plate #2 barcode</li> <li>19. filtermat A &amp; plain filter paper</li> <li>20. meltilex scintillant or liquid scintillant</li> <li>21. sealing bags</li> </ol>	

**APPENDICES**

ITEM	REMARK
22. timer	
23. calculator	
24. filter manifold	
25. lab coat & radiation badge	
26. bench paper & bench diaper	
27. MultiCalc program disks, MicroBeta operating disks	
28. assay protocols & blank assay sheet	
29. copy of rad license	
30. normalization plate = to ensure no shipping damage	
31. sealing tape for STX assay	
32. rad wipe towelettes	
33. for shipboard use rope, bungees, velcro, duct tape	
34. plug strip, extension cord	
35. vortex	
36. heat sealer	
37. slide warmer	
38. hot plate	
39. vacuum pump	
40. microfuge?	
41. <sup>3</sup> H label ( <sup>3</sup> HPbTX, <sup>3</sup> HKA, <sup>3</sup> HSTX)	
42. rat prep or insect prep	
43. standards and QC checks (PbTX3, DACS, STX)	
44. assay buffer	

---

**Items Required for Receptor Assay**  
 (alphabetical list)
 

---

ITEM	REMARK ( )
<ul style="list-style-type: none"> <li>• <math>^3\text{H}</math> label (<math>^3\text{HPbTX}</math>, <math>^3\text{HKA}</math>, <math>^3\text{HSTX}</math>)</li> <li>• assay barcode, stop barcode, plate #2 barcode</li> <li>• assay buffer</li> <li>• assay protocols &amp; blank assay sheet</li> <li>• bench paper &amp; bench diaper</li> <li>• bottles for waste (2) 500 mL</li> <li>• calculator</li> <li>• centrifuge tubes, 15mL &amp; 50mL</li> <li>• copy of rad license</li> <li>• cryomarker, pens, pencil, scissors</li> <li>• detergent</li> <li>• filter manifold</li> <li>• filtermat A &amp; plain filter paper</li> <li>• for shipboard use rope, bungees, velcro, duct tape</li> <li>• heat sealer</li> <li>• hot plate</li> <li>• ice bucket</li> <li>• lab coat &amp; radiation badge</li> <li>• latex gloves</li> <li>• marking tape, radiation tape</li> </ul>	

**APPENDICES**

<b>ITEM</b>	<b>REMARK ( )</b>
<ul style="list-style-type: none"> <li>• meltilex scintillant or liquid scintillant</li> <li>• microfuge?</li> <li>• microplate carriers for MicroBeta</li> <li>• microtiter plates, 96 well</li> <li>• mini-dilution tubes</li> <li>• MultiCalc program disks, MicroBeta operating disks</li> <li>• multichannel pipettor (2)</li> <li>• normalization plate = to ensure no shipping damage</li> <li>• paper towels</li> <li>• pipet tips (P200, P1000, 5mL)</li> <li>• pipettors (10<math>\mu</math>l, 200<math>\mu</math>l, 1000<math>\mu</math>l)</li> <li>• plug strip, extension cord</li> <li>• rad wipe towelettes</li> <li>• rat prep or insect prep</li> <li>• reagent reservoirs (large &amp; small)</li> <li>• rechargeable pipettor &amp; charger</li> <li>• sealing bags</li> <li>• sealing tape for STX assay</li> <li>• slide warmer</li> <li>• standards and QC checks (PbTX3, DACS, STX)</li> <li>• timer</li> <li>• vacuum flask and tubing</li> <li>• vacuum pump</li> <li>• vortex</li> </ul>	

### Saxitoxin Receptor Binding Assay Worksheet

DATE \_\_\_\_\_  
 ANALYST \_\_\_\_\_

BOOK \_\_\_\_\_ PAGE \_\_\_\_\_

SAMPLES

PLATE LAYOUT

Plate \_\_\_\_\_ of \_\_\_\_\_

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

**REAGENTS:**

<sup>3</sup>HSTX

STX \_\_\_\_\_ DATE \_\_\_\_\_ CPM \_\_\_\_\_ ( \_\_\_\_\_  $\mu$ l <sup>3</sup>HSTX + \_\_\_\_\_ mL Hepes buffer)

Rat Prep

DATE \_\_\_\_\_ PROTEIN \_\_\_\_\_  $\mu$ g/ml DIL \_\_\_\_\_ ( \_\_\_\_\_ mL prep + \_\_\_\_\_ mL Hepes buffer)

NRC STX 100 Mm (standard curve) DATE \_\_\_\_\_ LOT # \_\_\_\_\_

QC 5.0 nM in assay

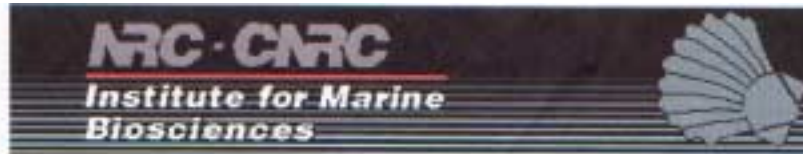
**COMMENTS**

**RESULTS**

SLOPE \_\_\_\_\_ ED-50 \_\_\_\_\_ REF \_\_\_\_\_ QC \_\_\_\_\_



## Saxitoxin Dihydrochloride Standard

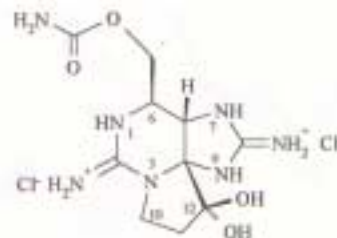


### STXdiHCl-C

#### *Saxitoxin Dihydrochloride Calibration Solution for Bioassay*

##### Test Substance

Saxitoxin dihydrochloride  
 CA Registry Number: 35554-08-6  
 Molecular Formula:  $C_{10}H_{16}N_7O_4Cl_2$   
 Molecular Weight: 372.2



##### Concentration

67  $\mu$ M saxitoxin in 0.003 M hydrochloric acid,  
 20.6  $\mu$ g/mL free base or 25  $\mu$ g/mL as the dihydrochloride.  
 Each ampoule contains 0.2 mL of solution.

##### Recommended Use

As a calibration solution for bioassay methods such as the mouse bioassay. For mouse bioassay, this solution may be diluted in 0.001 M hydrochloric acid to make working solutions in the appropriate concentration range. For other assays, dilute in the appropriate solvent, but keep the pH below 7 if possible.

##### Recommended Storage Conditions

Refrigerator at approximately +4 °C.

##### Cost

CAD\$60 per set of four ampoules.

##### For further information, please contact

NRC Certified Reference Materials Program  
 Institute for Marine Biosciences  
 1411 Oxford Street  
 Halifax, NS, B3H 3Z1, Canada  
 Tel : (902) 426-8281  
 Fax : (902) 426-5426  
 Email: [crm.imb@nrc.ca](mailto:crm.imb@nrc.ca)




STXdiHCl- C

••• NRC Certified Reference Materials Program



## Commercially Available Tritium-labeled Saxitoxin Diacetate

### SAMPLE PRODUCT DATA SHEET

<p><b>CAUTION - RADIOACTIVE MATERIAL</b></p> <h1>Radiochemical batch analysis</h1> <p>Before using this product, please read the instructions overleaf for safe handling, storage and disposal. This product has been manufactured to the quality assurance standard ISO9002</p>	<p>Amersham Pharmacia Biotech UK Limited Amersham Place Little Chalfont Buckinghamshire HP7 9NA England</p>   <p>Certificate No. 925289</p>								
<p><b>[11-<sup>3</sup>H]Saxitoxin diacetate</b> <b>Code TRK922</b> <b>Batch 1</b> <b>Pack Size 50µCi</b></p>									
<p><b>BATCH TECHNICAL DATA</b></p> <table> <tr> <td>Specific activity</td> <td>551 GBq/mmol, 14.9 Ci/mmol 1.31 GBq/mg, 35.5 mCi/mg</td> </tr> <tr> <td>Molecular weight</td> <td>419 (unlabelled)</td> </tr> <tr> <td>Radioactive concentration</td> <td>9.25 MBq/ml, 250 µCi/ml</td> </tr> <tr> <td>Radiochemical purity</td> <td>by ion pair high performance liquid chromatography (HPLC) on a Pierce RP-300 10µm Aquapore Octyl column with gradient elution from 0.001M trifluoroacetic acid containing 0.001M heptane sulphonic acid to 0.001M trifluoroacetic: acetonitrile (25:75) containing 0.001M heptane sulphonic acid. : 98.1 %</td> </tr> </table> <p>Analysed 9th June 1999</p>		Specific activity	551 GBq/mmol, 14.9 Ci/mmol 1.31 GBq/mg, 35.5 mCi/mg	Molecular weight	419 (unlabelled)	Radioactive concentration	9.25 MBq/ml, 250 µCi/ml	Radiochemical purity	by ion pair high performance liquid chromatography (HPLC) on a Pierce RP-300 10µm Aquapore Octyl column with gradient elution from 0.001M trifluoroacetic acid containing 0.001M heptane sulphonic acid to 0.001M trifluoroacetic: acetonitrile (25:75) containing 0.001M heptane sulphonic acid. : 98.1 %
Specific activity	551 GBq/mmol, 14.9 Ci/mmol 1.31 GBq/mg, 35.5 mCi/mg								
Molecular weight	419 (unlabelled)								
Radioactive concentration	9.25 MBq/ml, 250 µCi/ml								
Radiochemical purity	by ion pair high performance liquid chromatography (HPLC) on a Pierce RP-300 10µm Aquapore Octyl column with gradient elution from 0.001M trifluoroacetic acid containing 0.001M heptane sulphonic acid to 0.001M trifluoroacetic: acetonitrile (25:75) containing 0.001M heptane sulphonic acid. : 98.1 %								
<p><b>STABILITY AND STORAGE RECOMMENDATIONS</b></p> <p>To minimize decomposition, stocks of [11-<sup>3</sup>H]saxitoxin diacetate solution at the above radioactive concentration should be stored at -140°C. Under these conditions decomposition is not expected to exceed 0.4% per week in the first 17 weeks after the initial analysis, but thereafter may accelerate. This increase in the rate of decomposition during storage is characteristic of many tritium labelled compounds. The rate of decomposition is likely to be higher than that indicated above in a more concentrated solution, in a different or unpurified solvent or as a solid. Storage at -20°C may also result in a higher rate of decomposition. Storage as a solution at +2°C, whilst minimizing radiolytic decomposition allows the loss of tritium in the form of tritiated water at the rate of 4-5% per week.</p>									
<p>All goods and services are sold subject to the terms and conditions of sale of the company within the Amersham Pharmacia Biotech group which supplies them. A copy of these terms and conditions is available on request. © Amersham Pharmacia Biotech UK Limited 1999 - All rights reserved.</p> <p>Amersham is a trademark of Nycomed Amersham plc Pharmacia and Drop design are trademarks of Pharmacia &amp; Upjohn Inc</p>  <p><i>amersham pharmacia biotech</i></p>									



---

## Analyzing and Graphing Radioligand Data

---

### Sample Software: GraphPad PRISM

---

#### GraphPad Prism

---

All the graphs in this booklet were created using GraphPad Prism, a general-purpose curve fitting and scientific graphics program for Windows. Although GraphPad Prism was designed to be a general purpose program, it is particularly well-suited for analyses of radioligand binding data.

- Prism provides a menu of commonly-used equations (including equations similar to all those mentioned in this booklet). To fit a curve, all you have to do is pick the right equation. Prism does all the rest automatically, from picking initial values to graphing the curve.
- Prism can automatically compare one- and two-site models. Prism automatically calculates  $K_i$  from  $IC_{50}$ .
- Prism's manual and help screens explain the principles of curve fitting with nonlinear regression and help you interpret the results. You don't have to be a statistics expert to use Prism.

Please visit our web site at <http://www.graphpad.com>. You can read about the Prism and download a free demo. Or contact Graph Pad Software to request a brochure and demo disk by phone (619-457- 3909), fax (619-457-8141) or email ([sales@graphpad.com](mailto:sales@graphpad.com)). The demo is not a slide show -it is a functional version of Prism with no limitations in data analysis. Try it out with your own data, and see for yourself why Prism is the best solution for analyzing and graphing scientific data.

From: The GraphPad Guide to Analyzing Radioligand Binding Data