Proceedings of the Fifth Canadian Workshop on Harmful Marine Algae

R. W. Penney (Ed.)

Department of Fisheries and Oceans
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Les numéros 1 à 456 de cette série ont été publiés à titre de rapports techniques de l'Office des recherches sur les pêcheries du Canada. Les numéros 457 à 714 sont parus à titre de rapports techniques de la Direction générale de la recherche et du développement, Service des pêches et de la mer, ministère de l'Environnement. Les numéros 715 à 924 ont été publiés à titre de rapports techniques du Service des pêches et de la mer, ministère des Pêches et de l'Environnement. Le nom actuel de la série a été établi lors de la parution du numéro 925.

Les rapports techniques sont produits à l'échelon régional, mais numérotés à l'échelon national. Les demandes de rapports seront satisfaites par l'établissement auteur dont le nom figure sur la couverture et la page du titre. Les rapports épuisés seront fournis contre rétribution par des agents commerciaux.

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MADHU A. PARANJAPE (1939-1996)

Madhu's career in the the Department of Fisheries and Oceans began with the brand new Marine Ecology Laboratory, at the Bedford Institute of Oceanography, in a new oceanographic study of St. Margaret's Bay, Nova Scotia beginning in January 1968 and extending over three years to April 1971. The importance of microzooplankton in the pelagic food web was becoming recognized and Madhu began to apply his considerable skill in optics and photography toward their study in Canadian waters. During the early and mid-1970s Madhu developed considerable expertise with the biology and culture of marine protozoans, especially the tintinnids. In the 1970s and 80s, Canada began to look seriously at the marine chemistry and biological oceanography of its polar oceans and Madhu produced pivotal papers on the role of microzooplankton in these environments. In 1987 he joined the staff of a new biological oceanography unit at the Northwest Atlantic Fisheries Centre at St. John's, Newfoundland. Madhu recognized the importance of long-term monitoring in consideration of the effects of climate change on biological systems and was instrumental in re-instating Continuous Plankton Recorder (CPR) collections in the Region. He also established a biological sampling program at Station 27, the region's long-term oceanographic monitoring location and put in place a seasonal sampling program in off-shore waters. In late summer 1995, Madhu found out that he had inoperable cancer. He apparently responded well to chemotherapy initially and looked forward to returning to work up to a few weeks before his death. His presence and scientific balance were key in all research projects with which he was associated. He will be sadly missed by all his colleagues, and, especially by his wife Chitra and daughters Kena and Renita.

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SPECIAL WORKING GROUP REPORTS

Abstract

Penney, R. W. (Ed.). 1996. Proceedings of the Fifth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2138. xiii + 195p

The Fifth Canadian Workshop on Harmful Marine Algae, sponsored by the Department of Fisheries and Oceans, and co-hosted by the Ocean Sciences Center of Memorial University, was held at the Delta Hotel and Convention Center in St. John's, Newfoundland, on September 11-13, 1996. The workshop was attended by 65 participants representing all the DFO Regions of Canada as well as 14 foreign countries, including a significant contingent from the ASEAN countries working on the joint CIDA-sponsored ASEAN-Canada Cooperative Programme on Marine Science - Phase II. Participation and the sessions reflected the many and diverse aspects of harmful algal bloom research and management, including analytical methodology, monitoring programs, physiology and biochemistry of toxic species, analysis of bloom events, the role of bacteria as toxin producers, toxicology, and a review of research and monitoring programs from a number of national agencies. This report contains the extended abstracts of the oral and poster sessions as well as the reports of the Special Working Groups held during the workshop.

Résumé

Penney, R. W. (Ed). 1996. Proceedings of the Fifth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2138. xiii + 195p

Le cinquième Atelier de travail canadien sur les algues nuisibles, organisé sous l'égide du ministère des Pêches et des Océans avec le soutien de l'Ocean Sciences Center de la Memorial University of Newfoundland, s'est tenu du 11 au 13 septembre 1996 au Centre des congrès de l'hôtel Delta de St. John's (Terre-Neuve). Quelque 65 délégués, comprenant des représentants de toutes les régions du MPO au Canada et des chercheurs de 14 autres pays, y compris une importante délégation des pays de l'ANASE oeuvrant pour la phase II du Programme de coopération ANASE-Canada sur la science de la mer, ont pris part à l'atelier. La composition des participants et la teneur des séances ont témoigné du nombre et de la diversité des recherches et des facteurs de gestion associés à la prolifération d'algues nuisibles, y compris la méthodologie analytique, les programmes de surveillance, la physiologie et la biochimie des espèces toxiques, l'analyse des proliférations, le rôle toxicogène des bactéries, la toxicologie et la revue des programmes de recherche et de surveillance d'un certain nombre d'agences nationales. Le présent rapport contient les résumés détaillés des exposés oraux et des communications affichées, ainsi que les rapports des groupes de travail spéciaux qui se sont réunis au cours de l'atelier.

Acknowledgements

The Fifth Canadian Workshop on Harmful Marine Algae was the latest in a series sponsored by the Department of Fisheries and Oceans, Phycotoxin Working Group. Members of the PWG provided much helpful advice and encouragement in the months leading up to the workshop itself. I especially thank Kats Haya and Jennifer Martin who graciously offered their time and assistance with organization of the session schedules and Glen Harrison for his invaluable assistance with computer graphics.

The 5th CWHMA was jointly co-hosted by the Department of Fisheries and Oceans, Newfoundland Region and the Ocean Sciences Center of Memorial University. Staff of both organizations assisted with the logistical and local events. The Local Organizing Committee included: Cynthia MacKenzie (OSC), Jim Helbig (DFO), and Conrad Powell (DFO). Staff of the OSC who contributed to the events were: Fiona Harper, Jeanette Wells, Karen Whalen, Mike Riehl, and Miranda Pryor. Contributing staff from DFO included: Charles Bromley, Stefan Tarrant, Dan Lane, Moira Hynes, and Marlene Carroll.

Special thanks to the Newfoundland Department of Fisheries and Aquaculture which donated seafood for the evening social event.

Introduction

Marine algal-derived bio-toxins have appeared in all Canadian provinces which border both the Atlantic and Pacific coasts of Canada. PSP toxins are endemic to many areas. ASP, caused by domoic acid, is widespread among molluscs on both coasts, and outbreaks of DSP have occurred in Nova Scotia and Newfoundland. As well, other algal toxin producers such as *Heterosigma carterae* and *Chaetoceros* spp., have recently been implicated in economically serious mass mortalities of farmed salmonids in British Columbia (Forbes, 1994). Several flagellated algal species (*Mallomonas, Mallomonopsis, Chrysochromulina bergeri*) are believed responsible for fish kills in the Bras D'Or lakes region of Nova Scotia (Bates and Keizer, 1996).

The coastlines of the world have some of the most heavy concentrations of human population. While Canada is not thought of as a densely populated country by global standards, many of the population centers in the Atlantic and Pacific provinces are on or near the coasts. Human-derived waste effluents, a variety of heavy industrial discharges, agriculture run-off, and some of the richest fisheries in the world all co-occur within the estuaries and nearshore regions of both coasts. In recent years, this activity has broadened to include a rapidly growing shellfish and finfish aquaculture industry. Such a diverse mix of activity and inputs have the potential for pronounced and complex interactions with the population functions and metabolic dynamics of toxin producing algal species and thus, through the food chain, with the economically important shellfish and finfish fisheries and aquaculture industries nearby.

The Department of Fisheries and Oceans maintains, at great public expense (and growing industry expense), a network for monitoring shellfish toxins to ensure the safety of consumers of both wild fisheries and aquaculture produced seafood. Yet, amidst the growing challenges of toxin events, there is increasing concern that recent downsizing and fiscal restraints within the Government of Canada are negatively impacting on the ability of affected government agencies to respond to these challenges. Resourcing for toxic algal monitoring and a variety of phycotoxin research programs have been adversely affected.

These Proceedings constitute the written record of the Fifth Canadian Workshop on Harmful Marine Algae, held at St. John's, Newfoundland, Canada, September 11-13, 1996. Hosting this Workshop Series is an integral part of the activities of the Department of Fisheries and Oceans' Phycotoxin Working Group (PWG). The 5th CWHMA was co-hosted by the Ocean Sciences Center of Memorial University. The PWG was established after the first recorded outbreak of domoic acid poisoning in Canada in 1987. Initially, its role was to coordinate scientific research responses to that event. Since then, the PWG's role has expanded and it is now the Department's national advisory and management body on matters pertaining to the science of phycotoxins, harmful algal blooms, and the species which cause them in Canada.

Previous Workshops in this series were held at Sidney, B. C. in 1994 (Forbes, 1994), Mont-Joli, Quebec in 1992 (Therriault and Levasseur, 1992), Dartmouth, N. S. in 1990 (Gordon,

1991), and Moncton, N. B. in 1989 (Bates and Worms, 1989). PWG members have also sponsored regional workshops dealing with phycotoxin issues on both the Atlantic (Bates and Keizer, 1996) and Pacific coasts (Forbes, 1991) of Canada. The Proceedings of the 5th CWHMA contain the abstracts or extended abstracts (authors' choice) of both the oral and poster presentations, as well as the reports of four Special Working Group Sessions held during the Workshop. These written submissions have been reprinted without editing of content. Minor typographic changes in style were made where necessary to ensure consistency of presentation.

The 5th CWHMA and its Proceedings are dedicated in memory of Madhu Paranjape (1939-1996), Research Scientist at DFO St. John's and Newfoundland Region's representative on the PWG for many years.

Previous Workshop Proceedings in this Series and Related PWG Activities

Bates, S. S. And P. D. Keizer. 1996. Proceedings of the Workshop on Harmful Algae Research in the DFO Maritimes Region. Gulf Fisheries Center, Moncton, N. B., June 19, 1996. Can. Tech. Rep. Fish. Aquat. Sci. 2128: v + 44p.

Forbes, J. R. (Ed.). 1994. Proceedings of the Fourth Canadian Workshop on Harmful Marine Algae. Institute of Ocean Sciences, Sidney, B. C., May 3-5, 1994. Can. Tech. Rep. Fish. Aquat. Sci. 2016: 92p.

Therriault, J.-C., and M. Levasseur (Eds.). 1992. Proceedings of the Third Canadian Workshop on Harmful Marine Algae. Maurice Lamontagne Institute, Mont-Joli, Quebec, May 12-14, 1992. Can. Tech. Rep. Fish. Aquat. Sci. 1893: 154p.

Gordon, D. C. Jr. (Ed.). 1991. Proceedings of the Second Canadian Workshop on Harmful Marine Algae, Bedford Institute of Oceanography, Dartmouth, N. S., October 2-4, 1990. Can. Tech. Rep. Fish. Aquat. Sci. 1799: 66p.

Forbes, J. R. (Ed.). 1991. Pacific coast research on toxic marine algae. Can. Tech. Rep. Hydrogr. Ocean. Sci. 135: 76p.

Bates, S. S., and J. Worms (Eds.). 1989. Proceedings of the First Canadian Workshop on Harmful Algal Blooms, Gulf Fisheries Center, Moncton, N. B., September 27-28, 1989. Can. Tech. Rep. Fish. Aquat. Sci. 1712: 58p.

ABSTRACTS

ORAL SESSION 1

ANALYTICAL METHODOLOGY

THE ELECTROCHEMICAL DETECTION OF PSP TOXINS

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Introduction

High performance liquid chromatography (HPLC) is a powerful tool for analysis of PSP toxins. These toxins have only a weak natural chromophore and must be modified prior to detection. The post column reaction system (PCRS) is the current method of choice (Sullivan and Wekell, 1984). In this system, the effluent from the HPLC column is mixed with a chemical oxidant (periodic acid, pH 9.0), passed through a reaction coil, and acidified to form a fluorescent product. This approach is highly sensitive but suffers from several complications. The oxidant is unstable and changes in pH affect the fluorescence yield of the individual toxins. Janiszewski and Boyer (1993) reported the oxidation to form fluorescent products could also be done using electrochemical techniques (ECOS), eliminating the need for post column reagent pumps and the use of unstable reagents. In the ECOS system, the toxins are still detected using fluorescence detection. The electrochemical oxidation of the toxins also results in a signal that can be used for the direct electrochemical detection of the toxins (DECD). Here we compare the selectivity and sensitivity of PCRS, ECOS, and DECD for the measurement of PSP toxins.

Materials and Methods

The toxins were separated using an 5µ Inertsil C8 (150 mm x 4.6 mm) column and the isocratic mobile phases of Oshima *et al.* (1989) [STX and NeoSTX; 2 mM sodium heptane sulfonate in 30 mM ammonium phosphate (pH 7.1) with 5.7% of ACN, Gonyautoxins; 2 mM sodium heptane sulfonate in 10 mM ammonium phosphate (pH 7.1)]. In the PCRS, a 1 ml reaction coil in a 50 °C heating block was installed after column. Periodic acid (7 mM in 50 mM pH 9.0 sodium phosphate) was added before the reaction coil and acetic acid (0.5 M) was added after the coil. For the ECOS and DECD, the reaction coil was replaced with a ESA Coulochem-II 5010A electrochemical cell. Fluorescence was detected in the PCRS and ECOS using 330 nm excitation and 390 nm emission wavelengths. Standards of STX, neoSTX and GTX 1-4 were purchased from NRC - Canada. Toxin mixture PSP-4 was obtained from cultures of *Alexandrium tamarense*. Cells were lysed by sonication in 0.03 N acetic acid and filtered through a 10,000 NMW cutoff filter prior to use. The toxin mixture WH0304 was from the cyanobacterium *Aphanizomenon flos-aquae* and processed similarly to *A. tamarense*.

Results and Discussion

All six PSP toxins tested were electrochemically active. Increasing the oxidation potential increased the size of the peak. It also increased the background signal. Hydrodynamic voltammograms (Figure 1) showed the optimal voltage for STX and NeoSTX was 0.9 v while the optimum for the gonyautoxins was 0.8 v. All the N-hydroxy compounds showed a characteristic two-step oxidation starting at potentials > 0.3 V. This may lead to the higher sensitivity observed for these compounds (Table 1). The three different methods showed different selectivity for identical injections of STX, NeoSTX (Figure 2) and the gonyautoxins (Figure 3). Compared to the PCRS, the ECOS system was more sensitive for STX and GTX-2/3 whereas DECD was more sensitive for NeoSTX and the N-hydroxy gonyautoxins. This suggests that the optimal conditions for ring cyclization to form the pyrimido-purine are not the same as the optimal conditions responsible for the initial ring opening.

Table 1. Detection limits for the three methods for the analysis of the PSP toxins in pmol on column.

Toxin	PCRS[1]	ECOS[2]	DECD
STX	0.30	0.66;	0.31
NeoSTX	2.20	2.40	0.08
GTX-1	2.80	0.39	0.08
GTX-2	0.10	0.15	0.59
GTX-3	0.10	0.09	0.72
GTX-4	2.80	nd	0.08

Applied potentials for STX and NeoSTX were 0.9 v, applied potential for GTX was 0.8v. HPLC conditions: Inertsil C8 column running the Oshima A and B solvents. [1] PCRS data is from Sullivan and Wekell. [2]ECOS data is from Janiszewski and Boyer.

While the sensitivity of the three different methods differed slightly, the analysis of natural samples by the three techniques gave similar results (Table 2). Electrochemical oxidation replaces the need for periodate and the reaction coil. However direct electrochemical detection is not without its own problems. Long equilibration times are required to achieve steady background voltages. For these reasons, electrochemical oxidation coupled with fluorescent detection (ECOS) probably represents the best compromise technique.

Table 2. A comparison of the three methods for the analysis of six PSP toxins in two algal samples (μM).

Toxin	PCRS	ECOS	DECD
A. tamarense PSP-4			
STX	11.87	11.44	11.82
NeoSTX	3.89	3.48	3.80
GTX-2	0.17	0.20	0.14
GTX-3	0.28	0.25	0.29
GTX-4	nd	nd	0.025
Aph. flos-aquae WH0304			
STX	2.20	2.16	1.80
NeoSTX	8.86	8.49	9.19
GTX-2	0.033	0.031	nd
GTX-3	0.022	0.023	nd

Acknowledgments

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Sullivan, J. J., and M. M. Wekell. 1984. Determination of paralytic shellfish poisoning toxins by high pressure liquid chromatography. p. 197-205. In: E. P. Ragelis (ed.) Seafood Toxins, American Chemical Society, Washington DC.

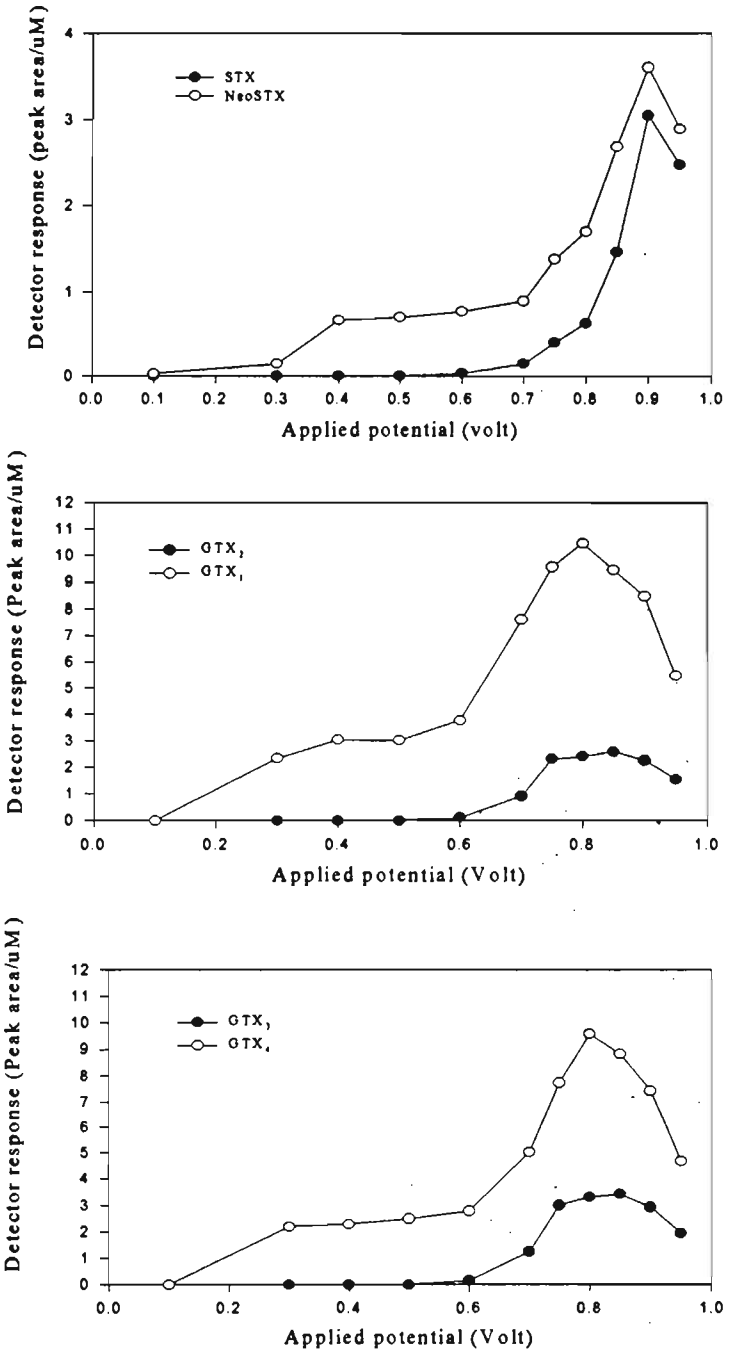

Figure 1. Hydrodynamic voltammograms for (a) STX and NeoSTX. (b) GTX-1 and GTX-2, (c) GTX-3 and GTX-4.

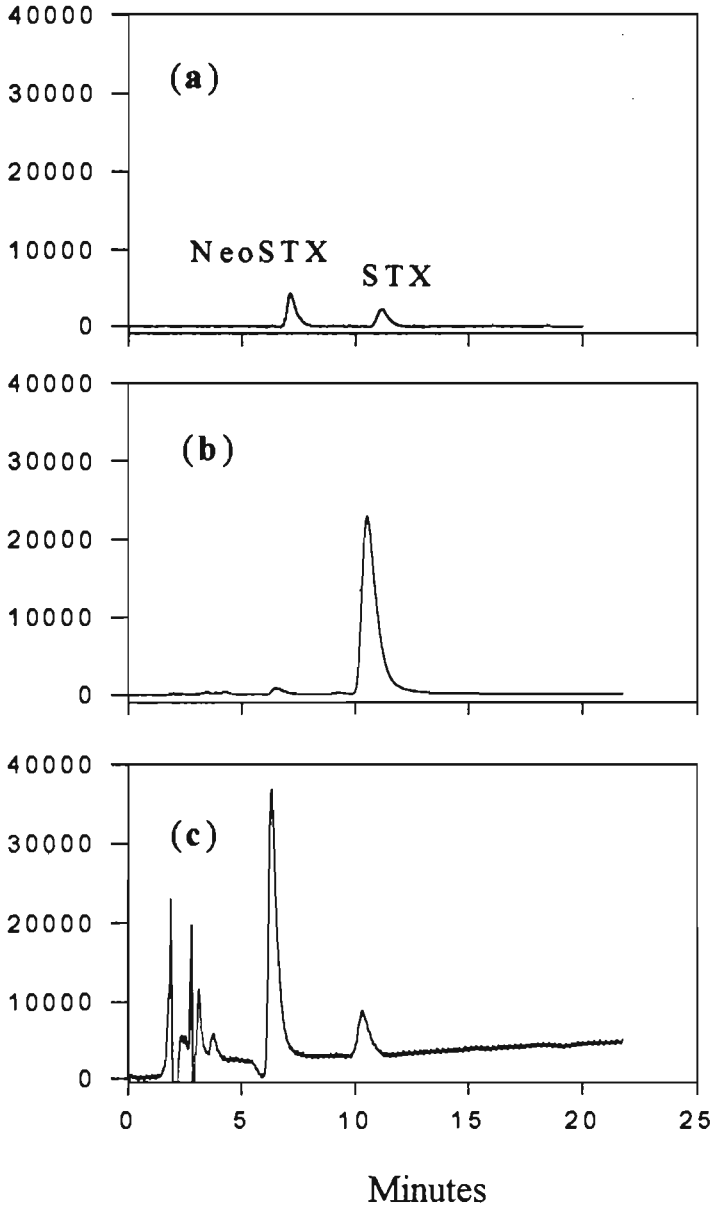

Figure 2. Chromatographs of STX (2 μM) and NeoSTX (3 μM) using (a) PCRS, (b) ECOS, and (c) DECD.

USE OF THE MOUSE NEUROBLASTOMA ASSAY TO DETECT PSP

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Paralytic shellfish toxins are potent neurotoxins which block sodium channels in mammalian nerve cells. This mechanism can be utilised in the development of quantitative assays for their detection. One such method uses mouse neuroblastoma cells which express active sodium channels. Chemicals, which cause a sodium influx, are added to a cell monolayer leading to cell death, which is blocked if paralytic shellfish toxins are present. The response can be quantified using vital dyes and an ELISA plate reader. Three variations of this technique currently exist and their differences will be discussed. In addition, preliminary data will be presented on the use of the assay for PSP monitoring.

THE MARITIME IN VITRO SHELLFISH TESTS (MIST) FOR PSP: SHIPPABLE, USER-FRIENDLY FORMATS

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The cell bioassay as a quantitative tool for the detection of Paralytic Shellfish Poisoning (PSP) toxins was first described by Kogure et al. (1988), and by Sato et al. (1988), who both counted morphologically changed cells in each well to determine the saxitoxin equivalents. The cell bioassay for PSP was automated by Jellett et al. in 1992 with a crystal violet endpoint and by Gallacher and Birkbeck (1992) with a neutral red endpoint. Both new endpoints were automated using a microplate reader. The Jellett et al. (1992) method was subsequently used in a collaborative study with the National Research Council of Canada to determine the specific toxicities of pure saxitoxin, neosaxitoxin, gonyautoxin II, gonyautoxin II/III and decarbamoylsaxitoxin relative to the US FDA pure saxitoxin standard (Jellett et al. 1995). The Gallacher and Birkbeck (1992) method was used to detect sodium-channel-blocking (SCB) bacteria in seawater and shellfish and to quantify the toxins (Gallacher and Birkbeck 1992 and others). A third endpoint, MTT, was used with this cell bioassay by Manger et al. (1995), who adapted the method to also detect ciguatoxin and brevetoxins, in addition to saxitoxins, in seafood extracts. The cell bioassay has several advantages over the mouse bioassay which is now the standard method worldwide for PSP toxins (Table 1).

Table 1. Comparison of the neuroblastoma cell bioassay for PSP with the standard whole animal mouse bioassay.

Item	mouse bioassay	cell bioassay
detection limit	40 μg/100g	2 μg/100g
animal use	yes	no
cost per sample	$30	$30
automatable	no	yes
user kits available	no	yes
extract	AOAC boiling acid	AOAC boiling acid
incubation time	15 min	8 h
specificity	all toxins	PSP, TTX[1]

[1]TTX, tetrodotoxin

The cell bioassay is similar to the mouse bioassay in that they both measure total toxicity directly and are similar in cost. The cell bioassay, however, does not require any animals, is 20 times more sensitive than the mouse, and is performed in a standard 96-well tissue culture dish. The cell bioassay can be automated and has been performed in its entirety by a robotic instrument. The cell bioassay is very specific for PSP toxins and other sodium channel toxins such as tetrodotoxin, while the mouse will detect all toxic materials in the aqueous extract. The mouse bioassay can be performed in 15 minutes while the cell bioassay requires an 8 h incubation period. The longer incubation time can be a disadvantage for stat samDles but is not unreasonable considering the general turnaround time for most mouse bioassay facilities. Approximately 100 extracts can be tested in the cell bioassay in one morning. For convenience, these can be incubated overnight and read the following morning. Current developments include a 3 hour version of the cell bioassay.

One of the disadvantages of using the cell bioassay is the requirement for tissue culture facilities and expertise. Laboratories interested in using this technologies must make a large capital outlay for lamellar flow hoods and clean-room facilities, as well as the other equipment required. Trained tissue culture personnel must be acquired or existing personnel must be retrained. Furthermore, maintenance of the neuroblastoma cells in a state such that they continuously express a consistent number of sodium channels has also proven difficult for many of those who have attempted to use the technology. Because of the facilities and equipment requirements, the technology has been restricted up to now to use in central laboratories, and was previously unavailable to end-users like aquaculturists.

To overcome these technology drawbacks, and in response to industry needs, the MIST™ (Maritime *In Vitro* Shellfish Test) has been developed into user-friendly test kit formats. Patents have been filed for the test kit formats. All the tests are based on the same basic bioassay theory. Neuroblastoma cells are the basis of the bioassays because they contain high numbers of sodium channels, the receptor sites for the PSP toxin molecules. The PSP toxins block the sodium channels of mammalian cells. The competitive reagents used in the bioassay activate sodium channels and cause the cells to swell and lyse if there is no saxitoxin present. The end-point of the MIST™ products is colorimetric. Cells that survive (or are protected by saxitoxins) stain dark purple with crystal violet (or red with neutral red) in the 96-well dishes, while wells with no PSP toxins appear pale purple (or red) to clear.

The MIST_ Kit for PSP has been developed into three versions, each capable of providing different types of information and each requiring different types of equipment (Table 2). The three versions are 1) fully quantitative known as the Quantitative MIST™ Kit for PSP, 2) semi-quantitative, known as the Semi-Quantitative MIST™ Kit for PSP and 3) qualitative, known as Mini-MIST™ .

Table 2: Equipment requirements and costs for the MIST™ Kits for PSP.

Equipment Required	Quantitative	Semi-quantitative	Mini-MIST™
Plate Reader	+	-	-
37°C Incubator	+	+	+/-
Multi-Channel Pipettor	+	+	-
Incubator Mat	-	-	+
Total Equipment Costs (Cdn $)	$8-9000	$1000-1500	$150

The largest kit is fully quantitative, requires a microplate reader, a multi-channel pipettor, a 37°C incubator. A standard curve and four samples are assayed in duplicate on each microplate. The semi-quantitative version can be read visually without the aid of the microplate reader; toxicity falls in one of six ranges read in μg/100g in the kit, 0-2, 2-10, 10-50, 50-250, 250-1250, 1250-6000 (Fig . 1)

The newly developed Mini-MIST™ Kit for PSP gives mainly qualitative information, although approximate levels can be inferred by comparison of the sample colour with the colour in the positive control well (Fiq. 2). If the colour produced bv the sample is as dark as or darker than the positive control well, then the sample contains 50 μg/100g or more of PSP toxins.

Although providing less information, this version is much simpler, and requires only a small incubation mat for equipment. The Mini-MIST™ is incubated on a tiny incubation mat, the only piece of equipment required by this version of MIST™. The shelf life varies for the kits from 7 days (Semi-quantitative MIST™ and Mini-MIST™) to 3 weeks (Quantitative MIST™). All versions are performed with an 8 h incubation time, and can be done by end users at their "kitchen sink" thanks to the antibiotics which are incorporated in the medium. The tests can be incubated for up to 24 h without any change in the results obtained after 8 h. Recent improvements include the completion of the test within 3 h, although this modification is still in the development stage.

The fully quantitative MIST™ Kit for PSP is currently being validated through an AOAC intercollaborative laboratory study, which is scheduled to be completed in late 1997. Twelve laboratories from 8 different countries will participate in this trial. In addition, parallel trials with the mouse bioassay are underway in the USA, and shipping trials to the EEC are also underway. A more extensive parallel trial with the mouse bioassay is scheduled for summer 1997. We anticipate full validation of the technology by late 1997. Jellett Biotek Ltd. is currently developing a similar battery of MIST™ Kits for DSP. This technology requires only 3 hours of incubation,

and is scheduled to enter validation trials in about 1 year.

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Figure 1: Interpretation of the MIST_ semi-quantitative test plate. Dark indicates purple colour. More toxin produces more purple colour when the samples are serially diluted across the plate. Samples which have little toxin quickly lose their colour on dilution (samples 1 and 2) while samples which have higher toxicity (samples 3 and 4) retain their colour farther across the dilution series.

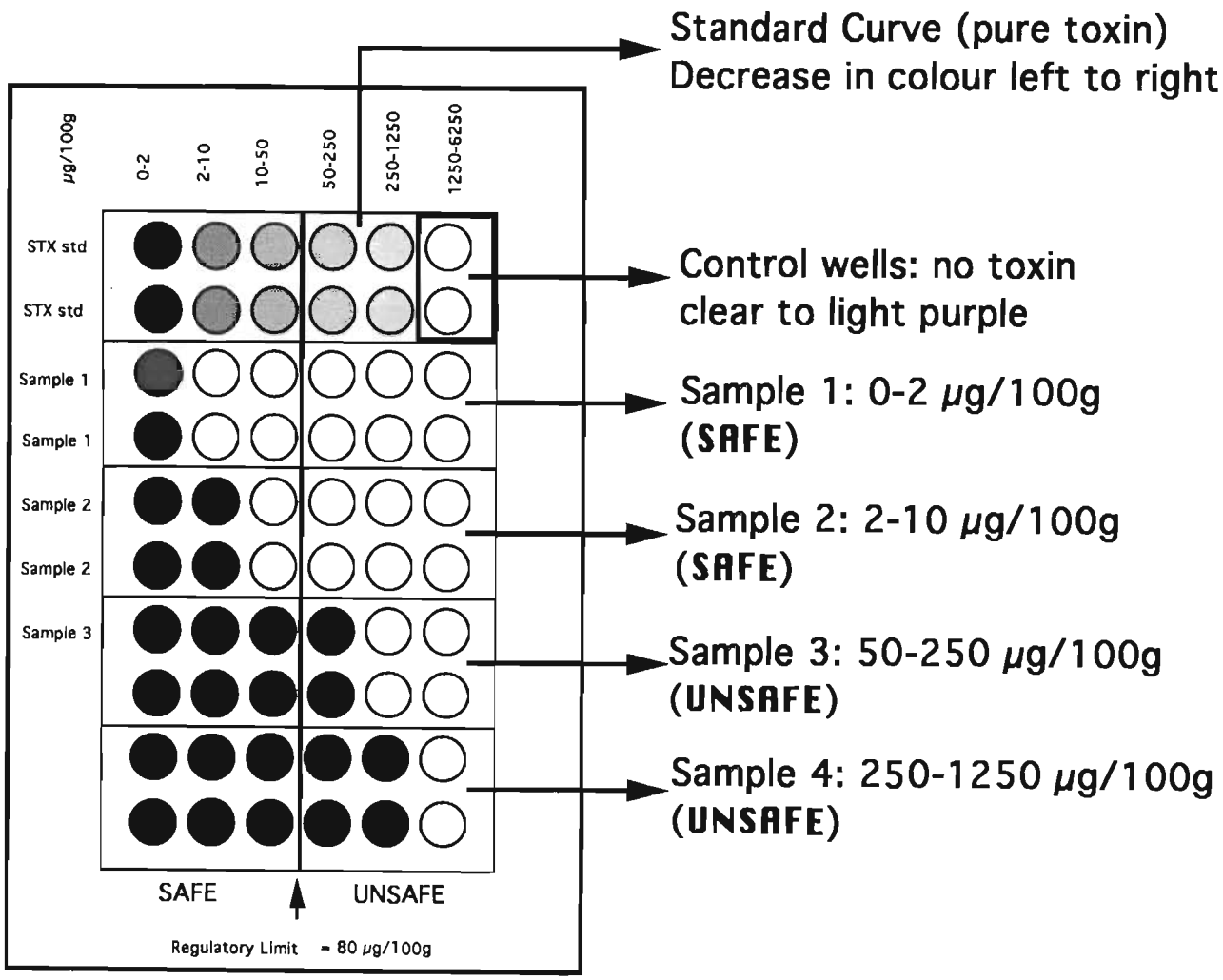
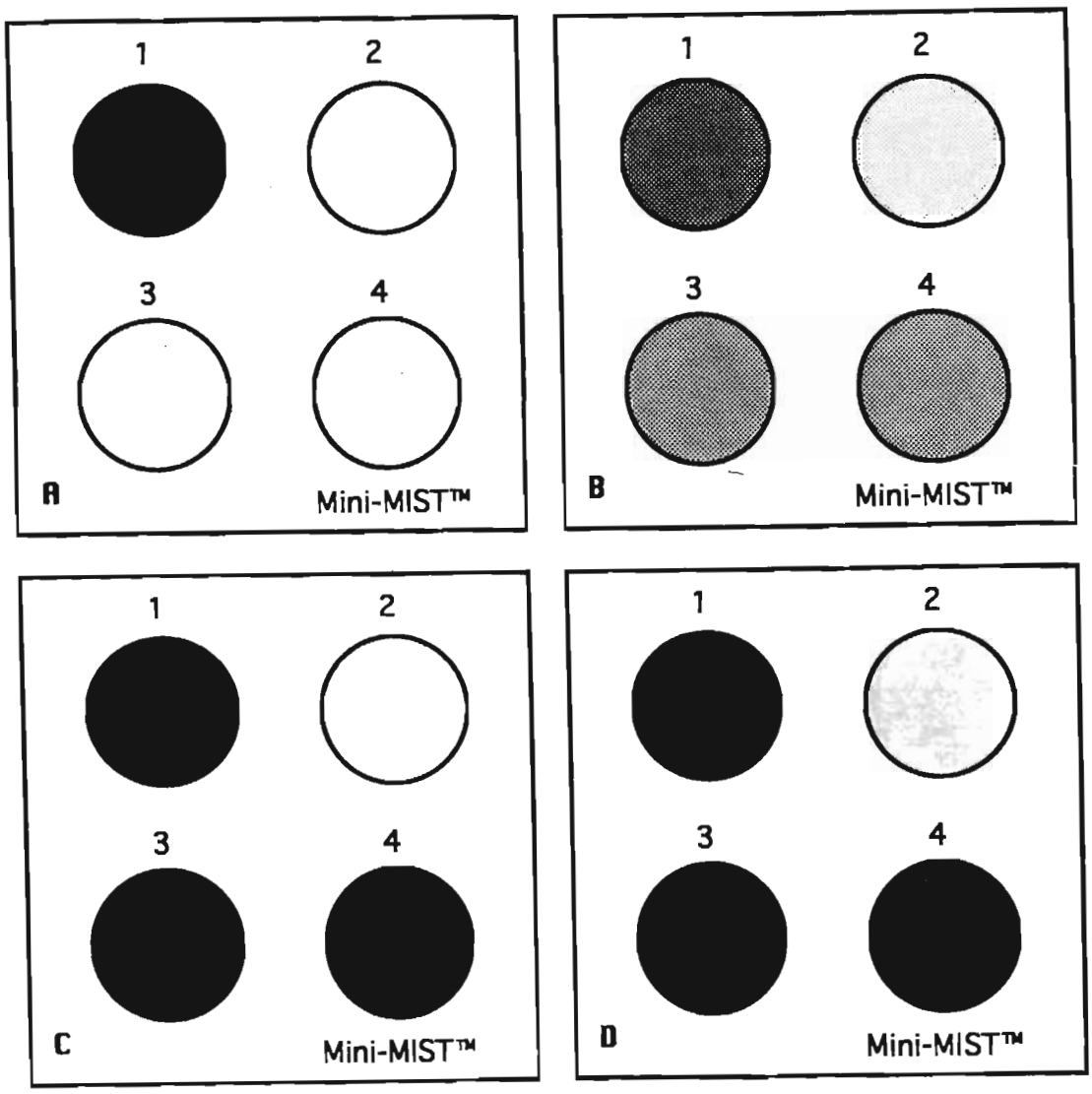


Figure 2: Interpretation of the Mini-MIST™ test plates. Dark indicates purple colour. Well 1 is the positive control and shows the amount of colour produced if the sample contains 50 μg/100g. Well 2 is the negative control and should be pale purple to clear if the test is performed correctly. Wells 3 and 4 are samples, plated in duplicate on each plate. Although the Mini-MIST™ is mainly designed to show if the sample is above or below 50 μg/100g, the amount of purple colour produced by the sample can be used to approximate the level of toxicity when compared to the colour in the positive control well. A. The sample contains less than 2 μg/100g PSP toxins. B. The sample contains >2 μg/100g but <50 μg/100g. C. The sample contains approximately 50 μg/100g and is therefore approaching the regulatory limit. D. The sample contains >50 μg/100g and is probably well over the regulatory limit.



FATTY ACID MARKERS OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM FUNDYENSE*

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Biomarkers are chemical components of organisms which are used to qualitatively and quantitatively determine *in situ* biomass. Fatty acid biomarkers are often used to signal the presence of a particular organism and, in this study, research is being conducted to identify a biomarker for the toxic dinoflagellate, *Alexandrium fundyense*.

In Notre Dame Bay, Newfoundland, a former aquaculture site has been infected with the toxic alga, *Alexandrium fundyense*. In this area, it is the encysted algae that are responsible for the toxicity of the shellfish. As a result, a specific biomarker for *Alexandrium* cysts that could be detected gas chromatographically would prove useful. However, the problem is compounded by the presence in the same area of a harmless dinoflagellate, *Scripsiella trochoidea*, that has a similar chemical composition. A marker, or ratio of markers, that applies to *Alexandrium* but not to *Scripsiella* is necessary to differentiate between the two organisms.

Various methods have been developed to directly measure toxins produced by algae. These include bioassays using mice or rats[1], as well as several liquid chromatographic techniques[2,3]. However, the gas chromatographic analysis of biomarkers of the toxic organism offers several advantages. The most obvious is the ready availability of the required gas chromatograph with flame ionization detection. Also, many aquaculture sites carry out fatty acid analyses to determine the nutritional quality of the shellfish and their food. This GC procedure would also allow determination of the biomarker. Finally, the preparation of samples for fatty acid analyses is relatively simple and rapid.

Fatty acid analyses of vegetative cells of both *Alexandrium* and *Scripsiella* were carried out in order to establish any differences in lipid composition in the two organisms. The polyunsaturated fatty acid, 18:5ω3, has been associated with toxicity in other studies[4] and it was initially proposed as a possible biomarker for *Alexandrium*. Unfortunately, this acid is also present in large proportions (>15%) in *Scripsiella*. Examination of the data did reveal that a ratio of 18:3ω3/18:2ω6 functions as a marker for vegetative cells of *Alexandrium*. This ratio is two to three times larger both in cultures of *Alexandrium* and in field samples predominantly containing *Alexandrium* than in the respective *Scripsiella* samples. The value of this marker ratio is also low for *Scripsiella* results in the current literature.[5,6]

A marker was also necessary for cysts of *Alexandrium*. Pure cultures of *Alexandrium* cysts were not available and, to obtain data on the cysts, it was necessary to examine purified sediment samples containing known amounts of cysts. Surprisingly, 18:5ω3 was not detected in any sediment

samples, suggesting that 18:5ω3 has no relationship with toxicity in this case. The ratio of 18:3ω3/18:2ω6 also did not correlate with numbers of cysts, but a new ratio, 20:4ω6/22:5ω6, was found to correlate significantly with cyst number.

In order to investigate the biochemistry of the rare fatty acid, 18:5ω3, compound - specific isotope ratio mass spectrometry was carried out on selected samples. In all samples examined, the acid most enriched in ^{13}C was 18:5ω3. This is surprising as the longer the biochemical pathway required to produce an acid, the more depleted in ^{13}C that acid becomes. It has been assumed that the production of 18:5ω3 would require a large series of steps but this data suggests otherwise. Another possibility is the occurrence of a reverse isotope fractionation during double bond formation, resulting in enriched products. If this reverse fractionation is occurring, 18:5ω3 must have 18:4ω3 as a precursor, and not 20:5ω3 as previously assumed.[7]

Acknowledgements

We thank Alan Cembella, NRC, Halifax for providing *Alexandrium* and *Scripsiella* cultures, and Jun Abrajano, MUN, St. John's for use of the isotope ratio mass spectrometer.

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PRODUCTION OF SIGNATURE AND POTENTIALLY TOXIC FATTY ACIDS BY *GYMNODINIUM CF. NAGASAKIENSE* AND *PSEUDO-NITZSCHIA MULTISERIES*

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Lipid analyses of toxic algae can reveal marker compounds useful in screening water or seafood samples. Unusual signature compounds can be used to indicate the presence of the alga or of organisms that have consumed the alga. Some algal lipids or their oxidation products have been shown to possess bioactive properties including ichthyotoxicity[1], haemolytic activity[2,3], suppression of algal growth[3,4], and mouse toxicity[5].

We measured the lipid class and fatty acid composition of two species of toxic marine algae grown in batch culture under different environmental conditions: The dinoflagellate *Gymnodinium* cf. *nagasakiense*, a strain isolated from Brest Bay, France, and the diatom *Pseudo-nitzschia multiseries*, a strain isolated from Cardigan Bay. The dinoflagellate was grown at 13 or 18°C and at 35 or 75 $\mu E/m^2/s$ while the diatom was grown in nutrient-replete culture and under conditions of silica limitation. Lipids were extracted in chloroform-methanol and lipid classes were determined using the Chromarod-Iatroscan TLC-FID system[6]. Fatty acids in the lipid extracts were determined by gas chromatography after methylation[7].

Using the Chromarod-Iatroscan TLC-FID system, several important lipid classes were identified in *G.* cf. *nagasakiense* and *P. multiseries*. These classes consisted of storage triacylglycerol and membrane associated phospholipids and glycolipids. Each class has in common a glycerol backbone to which one or more fatty acids is esterified. Gas chromatographic analyses revealed the prominence in both *G.* cf. *nagasakiense* and *P. multiseries* of the common fatty acid, 16:0 which is a saturated 16 carbon fatty acid. Fatty acids are denoted as the ratio of carbon atoms to double bonds. Fatty acids can occur in the free (unesterified) form, but most are esterified in lipid classes. Another 16 carbon fatty acid, this time with 4 double bonds, 16:4ω1, was prominent in *P. multiseries* while 18:5ω3 was prominent in *G.* cf. *nagasakiense*. Usually, in unsaturated fatty acids the position of the first double bond from the methyl end is given as n or ω. Unsaturated fatty acids have been implicated in ichthyotoxicity[1] (16:4 and 18:4), haemolysis[2,3] (18:5 and 20:5), suppression of diatom growth[8] (18:5ω3, 20:5ω3, 22:6ω3), and mouse toxicity[5] (16:1ω7, 18:4ω3 and 20:5ω3).

Under conditions of comparatively high temperature and low light, *G.* cf. *nagasakiense* had unusually high levels of 18:5ω3: up to 34% (Figure 1). 18:5ω3/18:4ω3 is a good marker: this ratio is in the range 20-113 in *G.* cf. *nagasakiense* depending on environmental conditions *cf.* 3.0±2.6 in 18 species of microalgae[8,9] including 14 dinoflagellate species. 18:5ω3 has been shown to be haemolytic[2,3] and an even more potent repressor of diatom growth[8] than 20:5ω3 or 22:6ω3. The

importance of environmental conditions on the production of 18:5ω3 may help explain the large variability observed in the toxicity of *G.* cf. *nagasakiense*.

Under conditions of silica limitation, *P. multiseries* had high levels of 16:2ω6, 16:4ω1, and 20:5ω3 (Figure 2). The levels of 16:4ω1 were unusually high: up to 11% of the fatty acids. 16:4ω1/18:2ω6 is a good marker: this ratio has a value of 18.3±3.6 (n=3) in silica limited *P. multiseries cf.* 2.9±3.8 in 18 species of diatom in which both 16:4ω1 and 18:2ω6 were detected[8,10] including 2 separate determinations of *Nitzschia closterium*. The highest proportion of the marker occurs when the diatom is most toxic[11]. Bioactive properties ascribed to the major polyunsaturated fatty acid, 20:5ω3, include haemolysis[3], mouse toxicity[5], suppression of diatom growth[3], and production of bioactive bacillariolides in *P. multiseries*[12]. It is interesting that 20:5ω3 triples under conditions of silica limitation (Figure 2) when *P. multiseries* produces the most domoic acid[11] as these bacillariolides are thought to work synergistically with domoic acid. It is also significant that the novel bacillariolides were not found in the non-toxic *P. pungens*[13].

In summary, both species had high contents of unusual polyunsaturated fatty acids: 18:5ω3 accounted for up to 34% of the fatty acids in *G.* cf. *nagasakiense* under conditions of comparatively high temperature and low light, while 16:4ω1 accounted for up to 11% of the fatty acids in *P. multiseries* under conditions of silica limitation. When taken in ratio to other polyunsaturated fatty acids, these compounds could be used as signatures of these organisms. In addition to being useful marker compounds, the same or similar fatty acids or their oxidation products have previously been shown to be ichthyotoxic, toxic to mice, haemolytic, and to adversely affect growth in several marine species. Toxic fatty acids seem to occur in glycolipids[2] or in the free form[2,3,5]. Their toxicity, however, seems to be mediated through oxidation products. Polyunsaturated free fatty acids and superoxide (O_2^-) cause destruction of gill cells, while polyunsaturated fatty acids and lipoxygenase in *P. multiseries* produce bioactive bacillariolides[12]. Some fatty acids, or their derivatives are thought to work synergistically with other known toxins such as domoic acid[12,13] or DSP[5,14].

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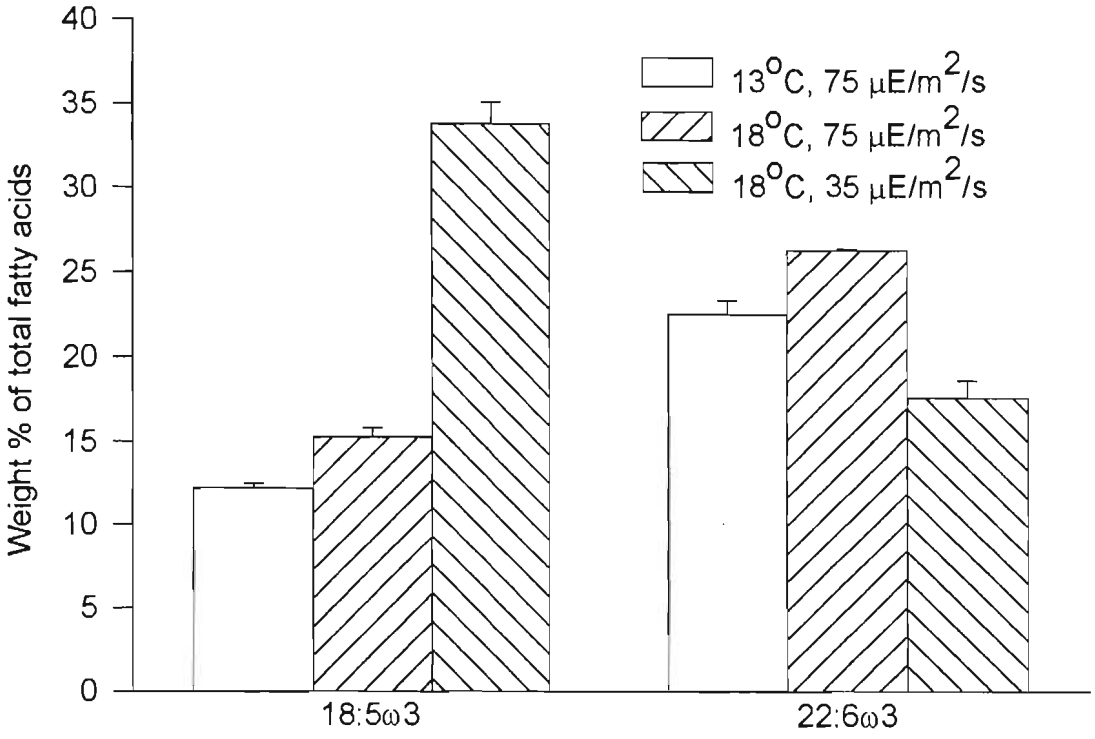

Figure 1. The major (>10%) unsaturated fatty acids in *G.* cf. *nagasakiense*.

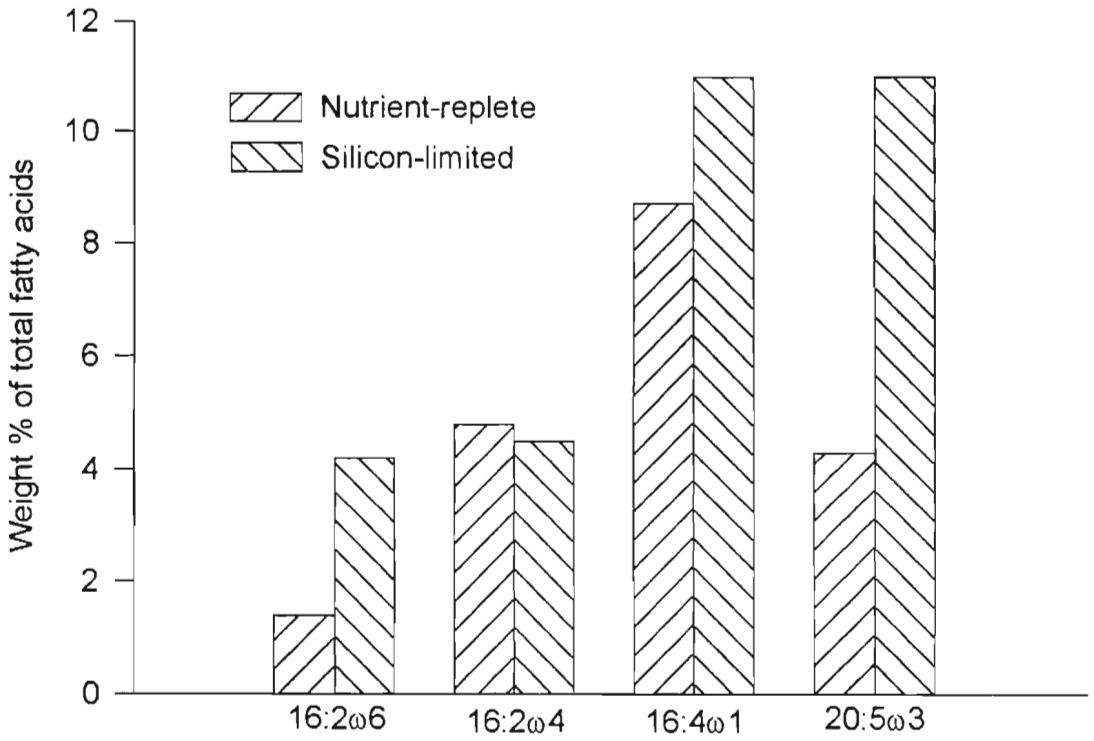

Figure 2. The major (>4%) polyunsaturated fatty acids in *P. multiseries*.

IMMUNOCHEMICAL RECOGNITION OF DSP CAUSING ORGANISMS

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Phytoplankton monitoring to identify those organisms responsible for causing diarrhetic shellfish poisoning (DSP) raises an interesting problem. While certain species of *Dinophysis* and *Prorocentrum* are known to produce the toxins associated with DSP, those organisms producing toxins cannot always be discriminated from their non-toxic relatives by morphological features alone. Potential toxin-producers often occur together in the water column, making it very difficult to determine the organism(s) responsible for DSP-toxicity.

We have found immuno-labelling to be a promising method for discriminating between toxic and non-toxic dinoflagellates. The method relies on a commercially available antibody (6/50; Rougier Bio-tech, Montreal, Canada) which recognizes the DSP-toxin okadaic acid, and many okadaic acid derivatives (Chin et al., 1995). This antibody is tagged with a fluorochrome and is visualized under epifluorescence microscopy (Figure 1). The primary antibody is applied to thin sections of resin embedded cells instead of whole cells because *Prorocentrum sp.*, one of the potential DSP-toxin producers, has a very thick and impermeable theca. We did not find it possible to permeate the theca without compromising the ultrastructure and/or mobilizing the toxin. Immuno-labelling of toxins within individual sectioned cells allows toxic species to be identified within a larger phytoplankton community, and also allows the localization of the organelles within phytoplankton cells which are associated with toxin synthesis and/or storage.

Phytoplankton samples can either be fresh or chemically fixed prior to further preparation for immuno-labelling. Each sample is filtered onto a membrane filter by vaccuum aspiration. The cells are then freeze-fixed by quickly plunging the filter into liquid propane.The sample is transferred to pre-cooled methanol, and allowed to freeze-substitute at -80°C for 72 hours. At this point, the samples are allowed to warm to 21°C over a 2 day period. The cells are then scraped from the filter into the methanol. Through alternate centrifugation and decantation, the cells are rinsed and infiltrated with LR White resin. The resin is cured in a vacuum oven. Embedded cells are thick sectioned using a diamond knife. 100 nm sections are collected and transferred to a water droplet on a glass microscope slide and dried at 68°C for one hour.

To prepare the sections for immuno-labelling of toxins, the sections are initially blocked for one hour with antibody buffer (AbB). The sections are then incubated overnight with the primary antibody, 6/50 (Rougier Bio-tech, Montreal, Canada), in AbB. This antibody recognizes okadaic acid and several other okadaic acid derivatives. Following three rinses with AbB, the sections are incubated for one hour with a biotinylated secondary antibody. This solution is rinsed

off with three AbB washes, and the sections incubated with FITC-conjugated streptavidin. The slides are viewed under epiflurescence microscopy for fluorescent labelling of cells.

We have tested this method on a number of laboratory cultures with promising results. Upon immuno-labelling separately processed strains of toxic *Prorocentrum lima* (strains Pa and KP200), and non-toxic *Prorcentrum micans*, we were able to successfully discriminate between the non-toxic organisms and the toxic organisms by fluorescent signal alone (Figure 2). In a laboratory mixture containing three species: a DSP-toxic *P. lima*, a PSP-toxic *Alexandrium sp.*, and a non-toxic *P. micans*, we were able to identify the DSP-toxic *P. lima* as the only species tagged with the fluorescent signal. Both the PSP-toxin producer and the non-toxic organism did not pick up the fluorescent signal. Within all of the toxic *Prorocentrum lima*, labelling of the toxin was localized to the chloroplasts, and especially the chloroplast region surrounded by the pyrenoid.

We are currently testing the method on field samples of *Dinophysis sp.* and *Prorocentrum sp.* Applications and limitations will be discussed.

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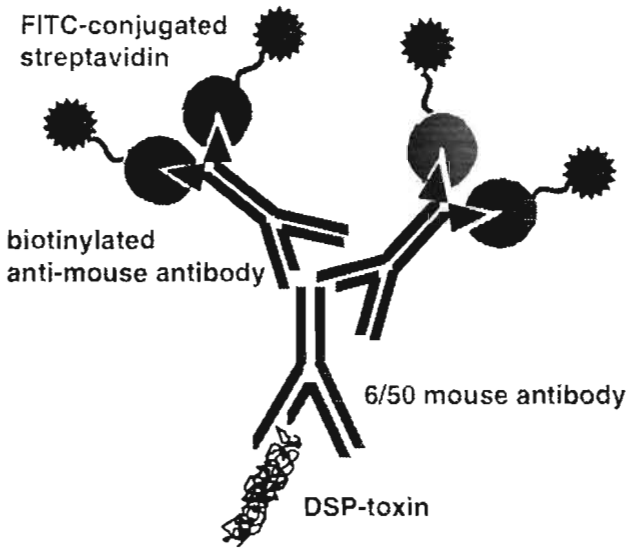

Figure 1. Antibody application for visualization of DSP-toxins.

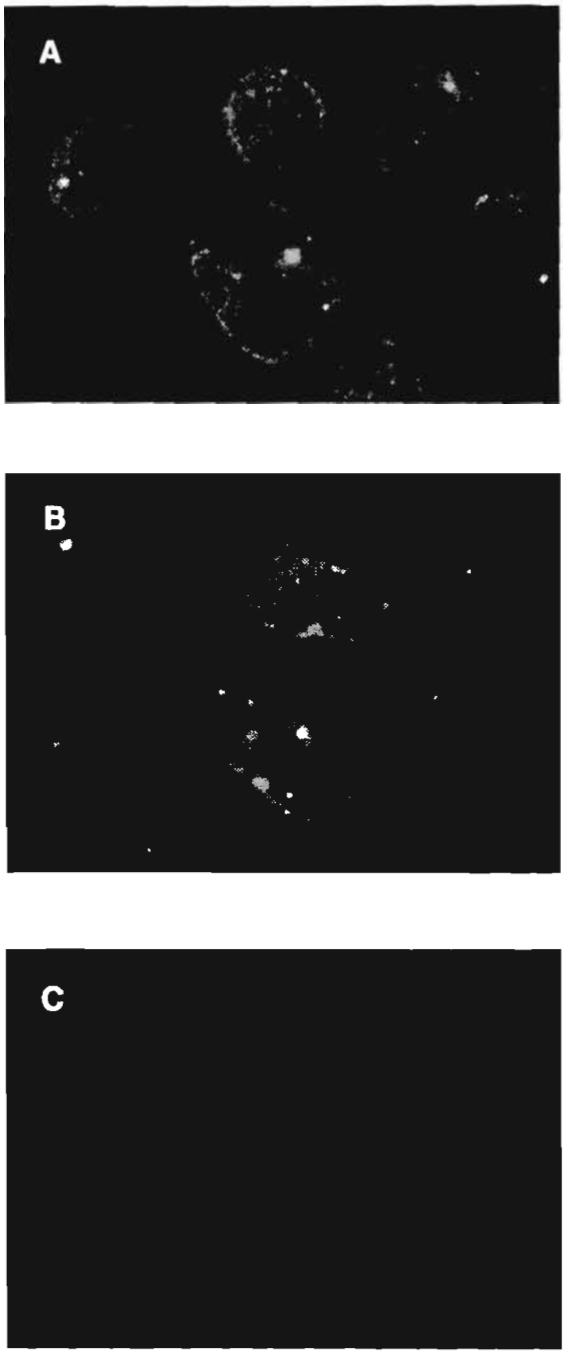

Figure 2. Cell sections of *Prorocentrum spp.* immuno-labelled with fluorescently tagged anti-DSP-toxin antibody. Sections photographed under epifluorescence microscopy.
A. Toxic *Prorocentrum lima* strain Pa. **B.** Toxic *Prorocentrum lima* strain KP 200.
C. Non-toxic *Prorocentrum micans*.

ABSTRACTS

ORAL SESSION 2

MONITORING AND MANAGEMENT PROGRAMS

HARMFUL ALGAL BLOOMS IN THE ASIA-PACIFIC REGION - SCOPE OF THE PROBLEM AND MANAGEMENT APPROACHES

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During 1994 and 1995 the Marine Resource Conservation Working Group of Asia-Pacific Economic Cooperation (APEC) reviewed the scale and nature of harmful algal blooms, as well as management capabilities, in the eighteen member economies, which skirt the Pacific rim. Production by APEC members of bivalve shellfish, the product most at risk from shellfish toxins, was 4.2 million metric tonnes in 1992. This represented 80% of world production and was valued at $6.9 billion. APEC members' exports also account for 66% of the world export market. Estimates from individual economies or events provide some measure of the scale of the regional HAB problem:

- Mortality of farmed yellowtail and other fish species during HAB outbreaks in the Seto Inland Sea of Japan between 1972 and 1991 exceeded $161 million in value.
- In the Philippines there have been over 1500 reported illnesses and 84 fatalities from PSP since 1983. Outbreaks in 1983, 1987 and 1988 resulted in direct losses to the mussel industry of $5 million each year, with equivalent indirect losses due to lack of consumer confidence in seafood products.
- In 1917 the shellfish industry in Alaska produced 5 million pounds of product. Today the State's commercial shellfish industry is virtually non-existent as a direct result of persistent contamination by PSP; the value of the sustainable shellfish resources in Alaska is estimated to be at least $50 million per year.
- In Malaysia, the economic loss to shrimp farms following mass mortality during HAB events in 1983 and 1985 was estimated to be approximately $25 million.

The review of the capacity of APEC members to undertake research and manage HAB problems identified several themes:

- There is a great disparity in the capabilities of different economies to respond to threats from marine biotoxins and harmful blooms; however, among the economies the full range of capacities exists, with some having unique expertise which should be exploited in a regional program. For example Australia has worked extensively on ciguatera problems, Canada on domoic acid and ASP, and New Zealand on NSP.
- Approximately half of the economies have some sort of information exchange system, ranging from electronic bulletin boards, and fax systems, to telephone networks. There is interest in developing a broader regional information exchange network.
- Most economies identified a need for training. Identified needs varied considerably, but common topics included bioassay techniques; HPLC and other advanced analytical techniques; taxonomy;

field sampling methodologies; monitoring program design; and management and mitigation strategies for affected fisheries resources, including workshops for fishers. In addition, several respondents requested assistance in developing legislation and regulations for shellfish toxins.

Based on this review, APEC has developed a five-year program designed to assist each APEC economy develop a scientific and regulatory infrastructure sufficient to certify that its seafood is safe from algal toxins, using procedures and standards that are acceptable to all regional economies. Areas of work include: development of complementary regulations; monitoring programs for fish and shellfish; detection of algal toxins and harmful algae; development of HAB research capability and enhancement of capabilities to manage and mitigate HAB effects.

ASEAN-CANADA COOPERATIVE PROGRAMME ON MARINE SCIENCE - PHASE II: ACTIVITIES AND ACHIEVEMENTS OF THE RED TIDE TECHNICAL STUDY

Kah-Sin Ong

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1. INTRODUCTION

The ASEAN-Canada Cooperative Programme on Marine Science - Phase II (CPMS-II) is a CIDA-funded, 5-year (1991-1996) programme with a total budget of CND 8.4 million. With the entry of Vietnam as a new member of ASEAN in 1995, and to provide for additional regional training activities, CIDA recently granted a 2-year extension (1997-1998) of the Programme with an additional allocation CND 2.5 million.

The Programme was formulated with the theme "Establishment of Environmental Criteria for Development and Management of Living Marine Resources and Human Health Protection". CPMS-II's goal is to support the regional effort of ASEAN countries to cooperatively optimize marine resource-based benefits through environmental maintenance in a manner that ensures the integrity of the resource base and promotes human health.

The overall objective is to upgrade ASEAN marine environmental management capabilities through cooperative endeavours jointly undertaken by participating ASEAN countries and Canada. This is to be achieved through training, and the execution of three major Technical Studies, viz:

1. development of tropical Marine Environmental Quality Criteria,
2. undertaking marine Pollution Monitoring and Baseline studies, and
3. investigation of toxic Red Tides which cause contamination of shellfish, marine fish kills and also human deaths.

This report gives an overview of the activities and achievements of the CPMS-II Red Tide Technical Study implemented in the ASEAN countries.

2. MAIN ACTIVITIES

The main activities of the Red Tide Technical Study include the execution of technical workplans in ASEAN countries, training in monitoring of shellfish toxicity and identification of HAB organisms, establishment of RT information network, and dissemination of information pertaining to RT management for human health protection.

The technical workplans carried out in the ASEAN countries encompass the following:

- RT Network and Information Management;
- RT Response Framework;
- Technical Support Services: Identification & Analysis;
- GIS-based Mapping of HAB locations in ASEAN;
- RT Information Management in Brunei Darussalam;
- RT Monitoring in Indonesia;
- Monitoring & Toxicology of HAB in Peninsular Malaysia;
- Baseline Study of HAB in Sarawak, Malaysia;
- Baseline Study of Ciguatera in Sabah, Malaysia;
- Nutrient Studies in Manila Bay, Philippines;
- *Pyrodinium bahamense* in Maqueda/Villareal Bays, Philippines;
- Life History of *Pyrodinium bahamense* var. *compressum*;
- Bioassay & Screening Protocols for HAB Toxins;
- Distribution of Phytoplankton in the Upper Gulf of Thailand; and
- HAB in Shrimp Farms in Thailand

The main training activities include:

- Training in Plankton Taxonomy comprising training workshops held in ASEAN countries and training of ASEAN HAB researchers by practical attachment to UBC Department of Oceanography & Botany;
- Training in Field Methods & Monitoring Strategies conducted in ASEAN;
- Training in Marine Toxin Detection and Analysis, particularly in standard mouse bioassay of PSP toxin in shellfish;
- Technical Planning Workshops and Technical Conferences held in ASEAN countries at which research findings are presented and discussed.

3. MAJOR ACHIEVEMENTS

While CPMS-II activities are on-going, considerable progress and achievements have been made in the following areas:

3.1 Quality assurance / quality control procedures

ASEAN laboratories participating in the Programme's activities have attained higher standards in their work as a result of the training given and emphasis on quality assurance / quality control procedures associated with all field and laboratory techniques, particularly in the application of the standard mouse bioassay for paralytic shellfish toxins.

3.2 Enhancement of harmful algal bloom (HAB) and shellfish toxicity monitoring programmes

Blooms of harmful algae including those causing toxic red tides are of frequent occurrence in ASEAN, particularly in the Philippines. Through training workshops on the identification of marine phytoplankton with emphasis on harmful algal species and in proper monitoring procedures, as well as training on marine toxin detection and analysis using the standard mouse bioassay, ASEAN countries have been able to enhance both their HAB and shellfish toxicity monitoring programmes to ensure human health protection. This is reflected in a number of new red tide locations observed in recent years, e.g., in Melaka, Malaysia and Ambon Bay, Indonesia.

3.3 Establishment of HAB network in ASEAN

To improve communication of information on harmful algal blooms within ASEAN, an HAB Alert & Information Network has been set up. The Network which operates through fax machines will enable ASEAN countries to inform each other promptly of HAB occurrences and take appropriate management measures. CPMS-II has also initiated a regional newsletter on harmful algal blooms called SEAHAB and four issues of this newsletter have been published.

3.4 Transfer of technology

The detailed requirements for technical training, supply of materials and equipment as well as on-site technical assistance are formulated by the Red Tide Working Group composed of ASEAN and Canadian specialists, and reviewed by the Programme's Project Steering Committee. Careful planning and proper implementation have resulted in a very effective transfer of technology from specialists and centres of excellence in Canada to participating scientists and institutions in ASEAN.

3.5 Dissemination of information

Many marine scientists and research centres in ASEAN do not have access to all the foreign literature relevant to their particular technical studies. Through the information acquisition / dissemination services provided by the Programme, participating ASEAN specialists and institutions have been able to acquire appropriate publications and keep abreast of current developments.

4. OVERALL CONTRIBUTIONS

4.1 Closer cooperation in Marine Science

The Programme has brought about closer technical cooperation between Canada and ASEAN and within ASEAN in the field of HAB studies and in marine science in general. There is growing information exchange between all the participating scientists who are now better able to keep abreast with relevant developments in other countries.

4.2 Upgrading of ASEAN Marine Science capabilities

The Programme has contributed significantly towards the upgrading of technical capabilities in ASEAN for the monitoring and management of harmful algal blooms and shellfish toxicity. This upgrading is reflected in the number of training activities successfully conducted and the number of technical workplans implemented in ASEAN countries, as well as the number and quality of the scientific papers generated by the Programme in the field of HAB studies, including those listed in this paper.

4.3 Standardization of methodologies

Through training courses, practical attachments and workshops, supported by the Programme, ASEAN specialists have made considerable progress towards a standardization of methodologies used in the region for the study on harmful algal blooms and monitoring of shellfish toxicity.

4.4 QA/QC and data comparability

The emphasis given to Quality Assurance/Quality Control procedures in all field sampling and laboratory techniques and the standardization of the shellfish toxicity bioassay methodology including the use of saxitoxin standards, have contributed much towards attaining data reliability and comparability in all participating laboratories.

4.5 RT management framework

The development and dissemination of a RT management framework provides a useful guide for ASEAN countries to adopt appropriate steps for the proper monitoring and management of HAB events in the region.

4.6 Protection of human health

The establishment of marine environmental quality criteria and standards, the Red Tide Alert Network, and the enhancement of marine pollution monitoring programmes including the monitoring of toxic algal blooms and shellfish toxicity in the ASEAN region will contribute significantly towards the protection of human health, by ensuring quality/safety of seafood and the coastal environment for recreational use.

5. CONCLUSION

The ASEAN-Canada Cooperative Programme on Marine Science has made significant contributions towards upgrading the capabilities of ASEAN countries to carry out relevant activities in marine science and marine environmental management in ASEAN, including in the monitoring of harmful algal blooms and shellfish toxicity and the management of red tide events for human health protection. Based on the foundation of progress achieved so far, and with the 2-year extension granted by CIDA, the Programme will be able to further enhance cooperative endeavours in ASEAN, particularly through the the further implementation of regional training activities and the execution of priority field activities in Vietnam as have already been carried out in the other ASEAN countries. Through the Programme, ASEAN HAB workers have been able to establish many useful contacts with specialists from Canada as well as many other parts of the world, and this networking will help them keep abreast with the advances and developments in the field of HAB research and management throughout the world.

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THE ASEAN-CANADA RED TIDE NETWORK: A REVIEW

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Red tides can involve coastlines of adjacent countries. The harmful ones can cause grave negative health and economic impacts particularly if management measures are not in place. An ASEAN-Canada Red Tide Network has been operational since early 1994 linking six ASEAN countries (Brunei Darussalam, Indonesia, Malaysia, Philippines, Singapore, and Thailand). Funded by the Canadian International Development Agency (CIDA) through the ASEAN-Canada Cooperative Programme on Marine Science - Phase II, the system consists of two networks. An Awareness Network (AN) serves to inform on the occurrence of red tides in any member country. The Information Network (IN) provides member countries with materials useful for the understanding of red tides / harmful algal events.

The paper presents a review of the accomplishments of the network. Possible future directions to take are discussed.

RED TIDE MANAGEMENT IN BRUNEI DARUSSALAM

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Since the first occurrance of red tide in 1976, Brunei Darussalam has become vulnerable to *Pyrodinium* red tide. The event reoccurred in 1980 and twice in 1988 but there has been no report of its outbreak since then. Red tide however has become one of the major health problems and caused economic losses to the fisheries industry. Fortunately, the situation has improved after each event as a result of the various actions taken by the Department of Fisheries and other related government departments and institutions in the country.

A Red Tide Action Plan was formulated in 1989 under the ASEAN-USAID Coastal Resources Management Project which aimed at providing timely and adequate responses to safeguard public helath and to minimize economic losses during red tide occurrences.

In addition to these, public awareness campaigns on toxic red tides have also been conducted so as to increase public awareness and to eliminate unnecessary panic which can lead to economic losses. At the same time, it is intended to build up confidence in the steps taken by authorities during red tide occurrences.

The formation of various cooperative linkages between Brunei Darussalam and other countries has assisted the country to enhance its monitoring capability such as with its close communication with neighbouring Sabah, Malaysia and the formation of the Red Tide Information Network under the ASEAN-Canada Cooperative Programme on Marine Science - Phase II.

RED TIDE MANAGEMENT IN THE GULF OF THAILAND

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Abstract

Red tides have occurred in the Gulf of Thailand for many years and in recent years their incidence has become increasingly frequent. In the past, there was no serious impact on the marine environment or organisms but the more recent occurrences have caused serious problems.

The first and only outbreak of paralytic shellfish poisoning so far recorded in Thailand occurred at Pranburi, Prachuab Kirikhan in 1983. In this incident, 63 persons were hospitalized and one died after eating the green mussel (*Pema viridis*). *Alexandrium tamarense* was suspected to be the causative organism. In the same year, a large bloom of *Trichodesmium erythraeum* covering an area of about 7,000 km^2 caused extensive damage to fish farms along the east coast. Large blooms of *Noctiluca* year, in 1995, red tides were occurred 21 times. Most of the blooms, which were not the harmful algal bloom, have been occurred in the Upper Gulf of Thailand. The dominant algae specie that caused the algal bloom is *Noctiluca scintillans* and *Trichodesmium erythraeum*. It caused the damage of our natural coastal resources such as aquaculture areas, tourist area and benthic fauna.

In view of the negative impacts caused by harmful algal blooms, the government of Thailand has implemented and established various appropriate management measures and action plans such as building waste treatment plants and reducing nutrient discharge in the receiving water. Several agencies including the Pollution Control Department, Fisheries Department and the Chulalongkorn University are cooperating in monitoring surveys and several training workshops have been conducted forThai marine scientists with the support of the ASEAN-Canada Cooperative Programme on Marine Science.

Introduction

Red Tide phenomena have been noticed in the Gulf of Thailand for long time ago and the first scientific record of the bloom has been reported by Comdr. Sawang Charernpol in 1958 (Charernpol 1985). The number of the blooms have been recorded since 1991 (Table 1 to 5). In the past, red tide was regarded as a natural phenomenon and there was no serious impact of red tide on the marine environment or organism. According to the survey conducted by the Department of Fishery during 1981 and 1987 in the Gulf of Thailand, none of the red tide

occurrences was caused by organisms that produce toxins. Some of them were caused by *Tricodesmium erythraeum* and the others were caused by *Noctiluca*. However, there were four occurrences of red tide which caused serious problems during 1982 and 1992. The first red tide bloom occurred in the end of 1982 and second bloom occurred in the beginning of 1983 at Pranburi, Prachuab Kiri Khan Province which was the only recorded outbreak of paralytic shellfish poisoning. At that time 63 persons were hospitalized and one died after eating the green mussel (*Perna viridis*). *Alexandrium tamarense* was suspected to be the causative organisms.

Normally, red tide caused by *Tricodesmium* and *Noctiluca* have no harmful effect on fish in the sea. But, sometimes, red tides were driven by surface current and wind to the shore causing death of fish, both in culture ponds and along the seashore. On August, 1991, a *Noctiluca* bloom caused mortality of demersal fish in Sriracha, Cholburi Province and on August, 1992, the *Noctiluca* bloom occurred in Pattaya Bay causing death of fish and other marine organism. From 1991, the number of red tide occurrences have been increased and sometimes it caused harmful effect to marine fauna that had prolong exposure to the bloom and caused mass mortality of fishes due to anoxic condition and high ammonia concentration. In the future, if we do nothing about the red tide occurrence in the Gulf of Thailand, it might be the serious social and economic impact to our marine environment and natural coastal resources.

Present Status

Thailand lies in the tropical zone of southeast Asia. In the south, the country is bounded by the South China Sea and the Andaman Sea. Total length of coastline is 2,614 km. The total area of the Gulf is about 350,000 km^2 with a maximum depth of 84 m. and the Upper Gulf of Thailand have a coastline of 700 km. from Prachuab Kiri Khan Province to Cholburi Province covering 6 provinces (Figure 1). Thailand supports a population in excess of 60 million of which around 4 percent live along the coastal area. There are three monsoons in the gulf. The first one is the southeast monsoon (February to April) which is warm and dry, second is the Southwest monsoon (May to September) which brings heavy rains and the last one is the Northeast monsoon (October to January) which brings cooler strong wind and rough sea. The bottom sediment in the gulf are of three types : sediment clay, clayey sand and sandy clay. The finest grained sediment occurs along the western coast and extends across the basin floor of the gulf as a blanket of clay. For the Upper Gulf, the bottom sediment is clayey sand. In the Upper Gulf, the direction of the surface current which is driven by northeast monsoon is anticlockwise and in the southwest monsoon is clockwise.

Most of the red tide occurrences in the Gulf of Thailand, especially in the Upper Gulf of Thailand are caused by the excessive bloom of phytoplankton in the rainy season, particularly in the estuaries in the former. In the past, red tide occurrences were found near the river mouths and the number of occurrences were small, such as during December 1982. The red tide phenomena which occurred along the coastline of Samut Prakran Province and Samut Songkram Province were located on the Chao Phraya and Mae Klong rivers. In the recent years, due to the

results from the monitoring program under the ASEAN - Canada Cooperative Program on Marine Science, the frequency of occurrences have increased and were found along the coastline of the Upper Gulf (Lirdwitayaprasit et. al, 1993) and trend to increase year by year (Table 1-5 and Figure 2).

Economic and Social Impact

The main concern for social and economic impacts of red tide is damage to both coastal resources and coastal aquaculture. A major loss in aquaculture production was caused by the Tricodesmium erythraeum bloom, during May and June 1983. A large number of fish farms on the east coast were affected because anoxic conditions resulted from decomposition of this bluegreen algae. Estimated value of the fish lost clearing this incident is 26 million Baht. Sometimes it is impossible to estimate the value of the damage caused from red tides if the incident does not occur near an aquaculture area or the tourist area. However, other fish and marine organisms are still effected as with the heavy bloom of *Noctiluca* which occurred in August 1991 in Sriracha Bay and in August 1992 in Pattaya Bay. This problem is becoming serious because of the increasing amount of nutrients and organic pollutants from the river runoffs.

Land-Based Pollution Sources

We know that there are many conditions which influence the occurrence of red tide such as light, temperature, salinity and nutrients. Some conditions, such as light, temperature and salinity, we can not control because they are natural conditions. But, there are some conditions that we can control including nutrients and organic pollution. In general, there are nutrients in seawater but their concentration is controlled by the natural processes in the sea. As mentioned earlier, the nutrients in the sea are increasing year by year. This is the result of domestic waste loading which is discharged into the rivers. Sources of nutrients, or pollutants, in the marine environment are domestic wastes which have not been treated or partially treated (from big cities and the populated areas along the river bank and the coastline) before being discharged to the rivers. The major population centers of the Changwat bordering the Gulf of Thailand is shown in Table 6.

The larger source of pollution into the Gulf of Thailand is the Chao Phraya river which carry about 1.4 million m^3 /day of untreated wastewater from Bangkok to Samut Prakran Province. The lower Chao Phraya river suffers from very poor water quality with high level of BOD and contaminants and has a classification of 5 as defined in the Laws and Standards on Pollution Control in Thailand 2, 3 rd edition 1994. There are a number of other rivers that discharge into the Gulf of Thailand. Of these the Bangpakong, Tha Chin and Mae Klong river carry a lesser but still significant waste load from urban population centers.

Management Plan

Because of the severe environmental degradation in the last two decades, the Royal Thai Government has set the natural resources and environmental management at the first priority in the Seventh National Economic and Social Development Plan.

The Seventh National Economic and Social Development Plan (1991-1996) has three principal objectives, namely :

- ★ To sustain the country's economic growth at an appropriate level, with stability;
- ★ To promote more equitable income distribution and rural development;
- ★ To develop human resources, improve quality of life, and enhance the quality of the environment and natural resources.

Fundamentally, in the environmental area, the Plan focuses on five major areas of environmental management policy: natural resources management, environmental quality, energy and environment, industry and environment, and urbanization and environment. These are viewed as the central environmental concerns associated with continued economic growth. To achieve the targets set out in the Seventh Plan, a series of municipal waste treatment projects have been proposed. Bangkok currently has no large-scale waste water collection and treatment facilities functioning in the city are small treatment systems serving some national Housing Authority project, government centers and military facilities. For instance:

- The Bangkok Metropolitan Authority (BMW) has proposed a two-stages sewerage and treatment plant project, BMA 1 and BMA 2, worth more than 20,000 million baht (US$ 800 million) in total. The first stage (BMA1) will serve 10 densely-populated areas in inner Bangkok and provide a treatment capacity of 350,000 cu.m./d. For the second stage (BMA2), the authority planed to provide a project serving lightly-populated zone in the outer districts. With the completion of all of these projects, about 75 % of the domestic sewage and other waste water generated in Bangkok will be treated.

- The Ministry of Interior plans to provide centralized wastewater treatment facilities for the cities located along both sides of the Tao Fray river. An estimated investment cost 4,000 million baht (US$ 160 million).

- The Ministry of Science, Technology and Environment by the Pollution Control Department proposed the Development of an Action Plan to improve the Water Quality in the River Basin for the whole country (the northern region, the northeast region, the eastern region, the southern region and in the Gulf of Thailand and Andaman sea). These projects worth 200 million baht (US$ 8 million). The objectives are thus:

⇒ To prepare a Management Information System of water pollution sources in the areas,

⇒ To prepare a Geographic Information System of pollution sources and spatial information such as water quality, landuse etc.,

⇒ To prepare a Mathematical Model to forecast the change in river and sea water quality based on quantities and quality of pollution sources discharged to the river and to the sea, and the capacity of these rivers and marine to receive and assimilate the waste loads,

⇒To prepare a Phased Action Plan for the rehabilitation of river and sea water quality and a management plan to improve and upgrade river and sea water quality over a 20 year period and

⇒To prepare a detailed phased investment plan for wastewater management with the priority of each clearly defined and a programme for their implementation.

- The Ministry of Science, Technology and Environment proposed the wastewater action plan along the west coast of Phuket Island. This plan has provide the wastewater treatment plant for the west coast which is the recreation areas of island.

Monitoring Programme

Since 1992, the red tide monitoring programme was set up. The objectives are as follows :

- to monitor the red tide occurrences in the Upper Gulf of Thailand
- to collect the water quality and environment data while the bloom is occurred
- to warning through the local network to the owner of shrimp farms, the local authority and the people who lived nearby.

The output that we get from this programme will be used as a tool for handling the red tide problems. The red tide monitoring programme was first funding by the ASEAN-Canada Cooperative Programme on Marine Science Phase II. This funding was ending in 1996. From now on, the monitoring programme will be done by using the annual budget from our government.

Conclusion

Nitrogen and phosphorus are nutrients for the algae. Increasing nutrients in the water is one of the conditions for the blooming of algae. Due to the morphology and topography of the Upper Gulf of Thailand which is the semiclosed gulf, the larger amount of nutrient, which was resulted from the discharging of domestic waste through the four main rivers around the Upper Gulf, namely the Chao Phraya River, Tha Chin River, Bang Pa Kong River and Mae Klong River and direct discharging from the town which were located along the coastline, was accumulated in the gulf. Decreasing amount of nutrients in the marine water, by implementing the central treatment plants to

reduce the waste loads from domestic together with the use of water quality mathematical model and data from the monitoring programme, to the natural level should be the best way to limit the number of red tide occurrences and the size of the bloom.

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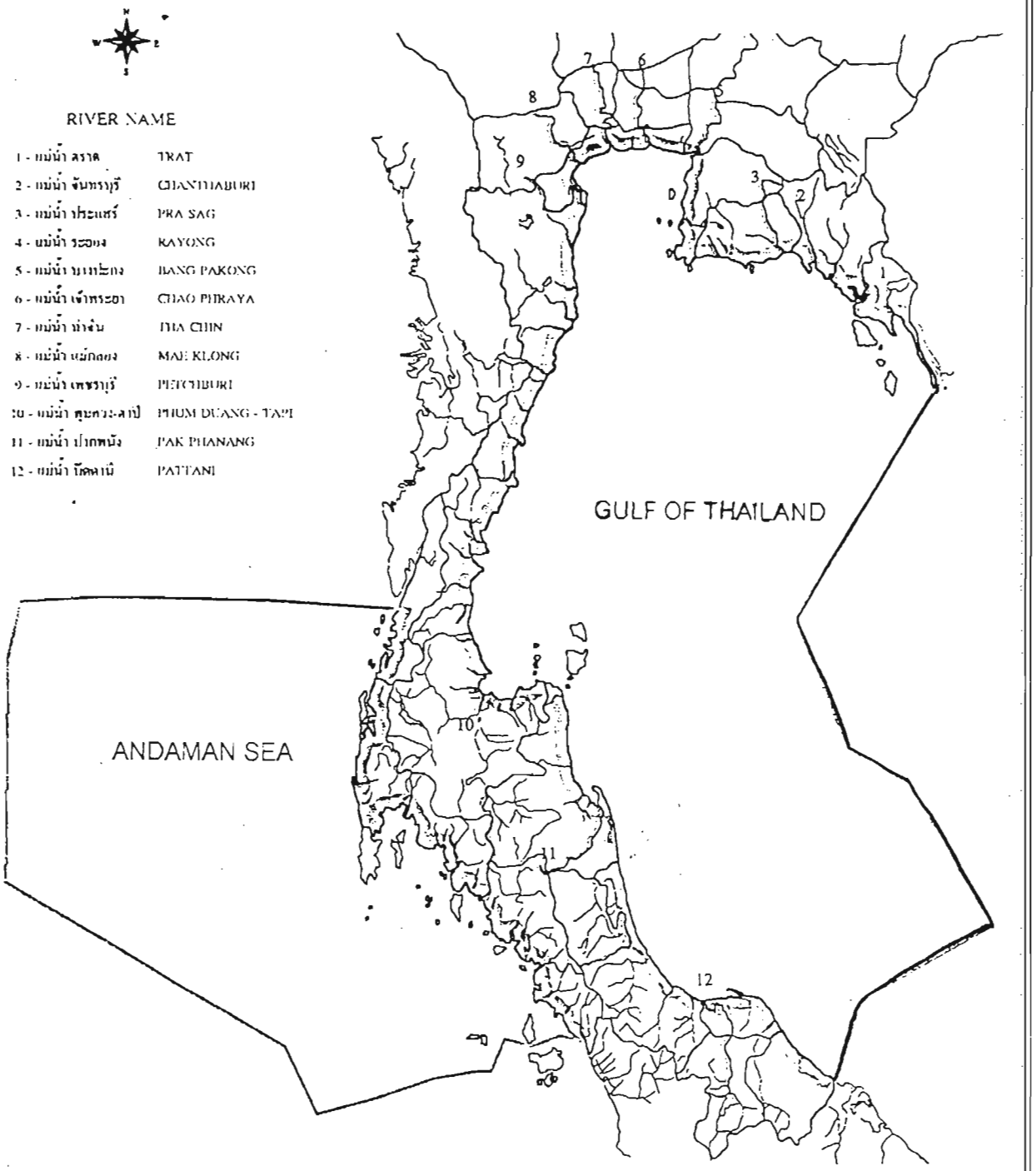

Figure 1 Gulf of Thailand and Andaman Sea

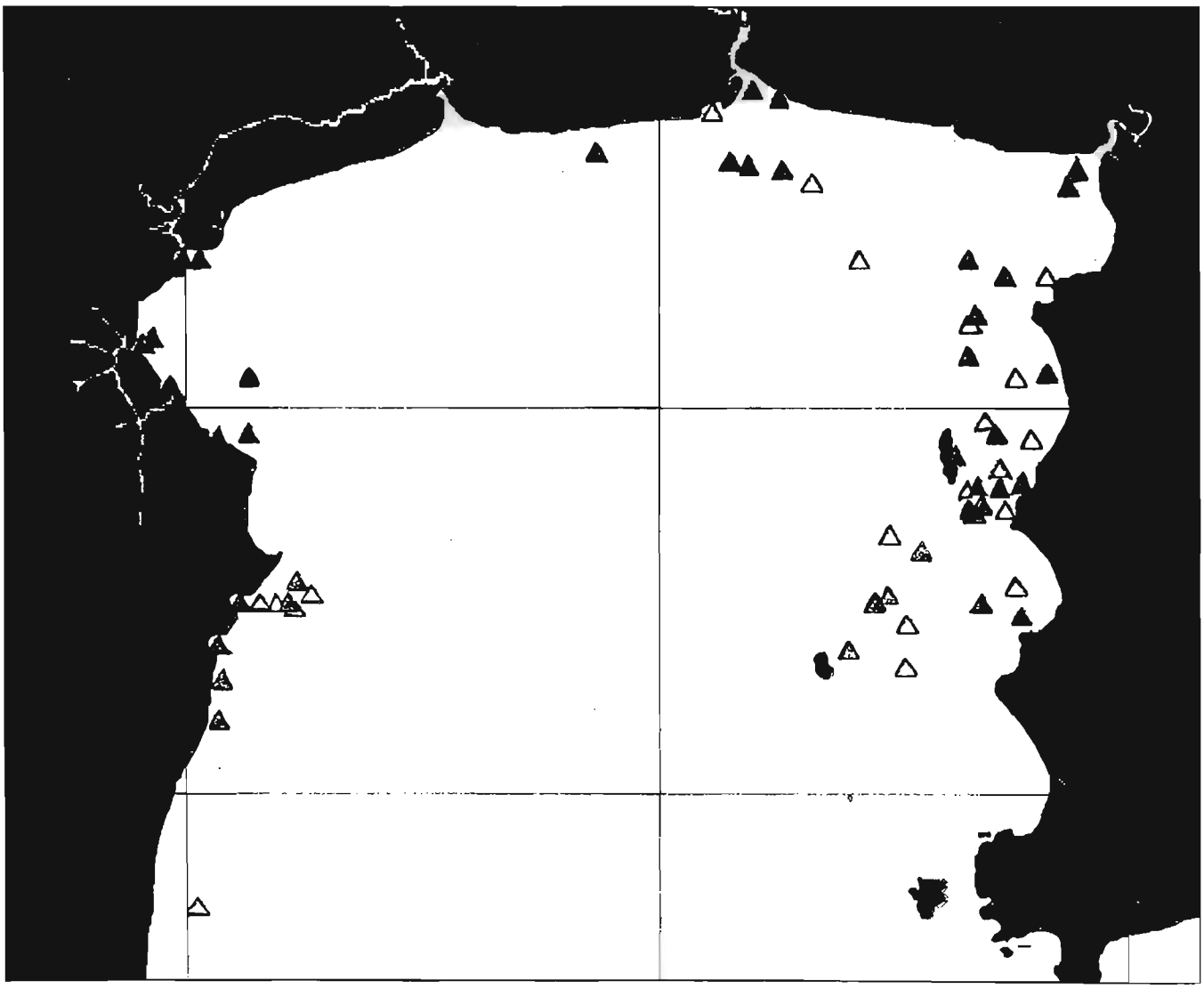

Fig. 2 : Location of Red Tide occurences during 1993-1995

▲ Year 1993

△ Year 1994

▲ Year 1995

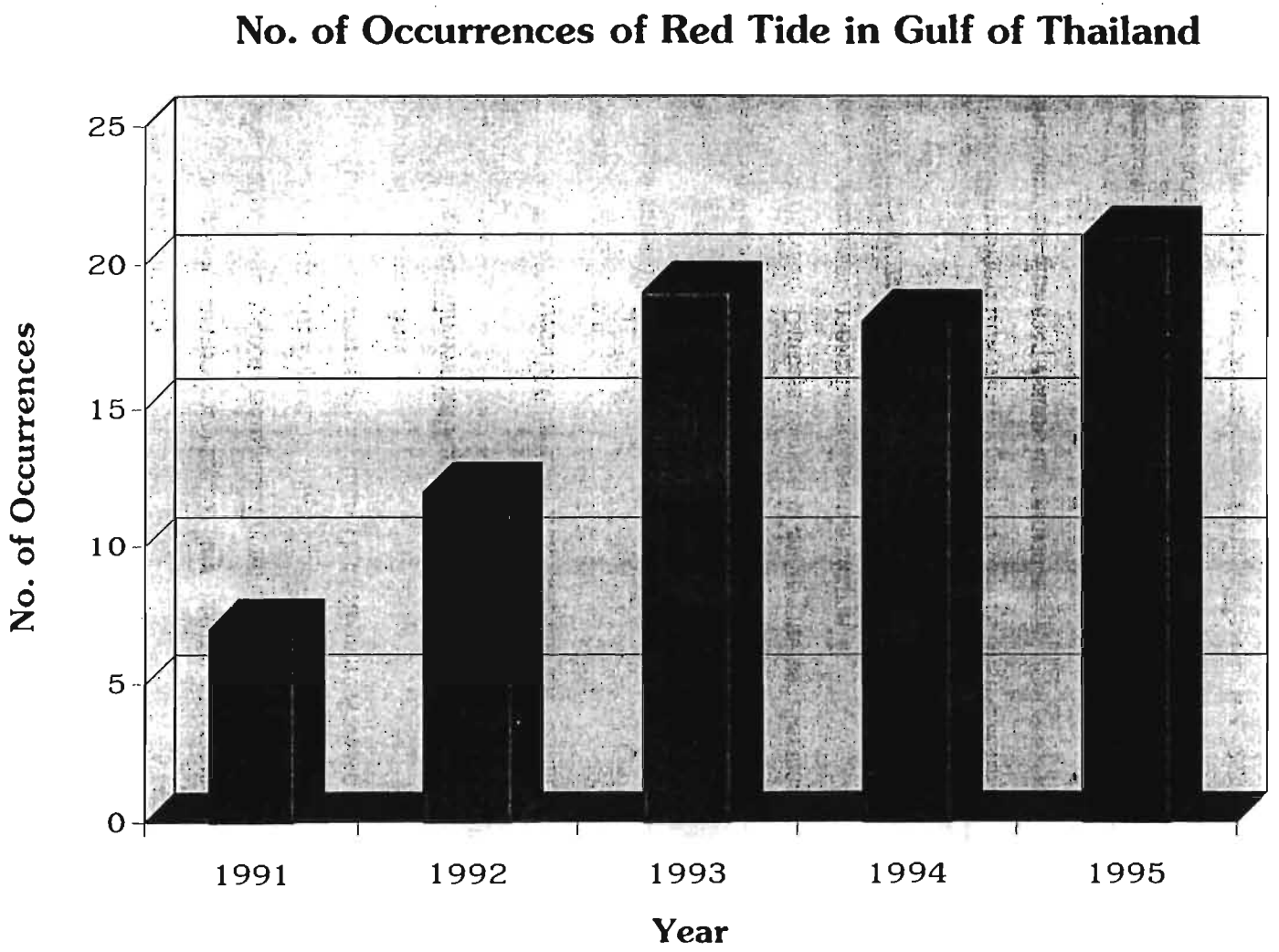
No. of Occurrences of Red Tide in Gulf of Thailand
No. of Occurrences
25
20
15
10
5
0
1991
1992
1993
1994
1995
Year

Table 1. Occurrences of red tide in the Gulf of Thailand in 1991.

Date	Water Colour	Affected Areas	Causative Organism
1. Jan, 16	Redish Brown	Tha Chin River Mouth (Samut Sakhon Province)	*Mesodinium rubrum* *Cochlodinium* sp.
2. Jan, 22	Green	Cha-Am (Phetchaburi Province)	*Noctiluca scintillans*
3. Feb, 7	Green	Cha-Am (Phetchaburi Province)	*Noctiluca scintillans*
4. Aug, 17-20	Green	Angsila - Laem Chabang (Cholburi Province)	*Noctiluca scintillans*
5. Sep, 14-17	Green	Ban Laem - Cha-Am (Chlburi Province)	*Noctiluca scintillans*
6. Nov, 2	Green	Muang Prachuap Khiri Khan (Prachuap Khiri Khan)	*Noctiluca scintillans*
7. Dec, 21	Green	Ta-Yang (Phetchaburi Province)	*Noctiluca scintillans*

massive fish kill

Table 2. Occurrences of red tide in the Gulf of Thailand in 1992.

Date	Water Colour	Affected Areas	Causative Organism
1. Jan, 11	Green	Ta-Yang (Phetchaburi Province)	*Noctiluca scintillans*
2. Jan, 31	Green	Ta-Yang (Phetchaburi Province)	*Noctiluca scintillans*
3. Feb, 15	Green	Hua Hin (Prachuap Khiri Khan)	*Noctiluca scintillans*
4. Feb, 22	Green	Hua Hin (Prachuap Khiri Khan)	*Noctiluca scintillans*
5. Mar, 18	Green	Chao Praya River Mouth (Samut Prakan Province)	*Noctiluca scintillans*
6. Jul, 1	Green	Bangsan (Cholburi Province)	*Noctiluca scintillans*
7. Jul, 8	Green	Banglamung (Cholburi Province)	*Noctiluca scintillans*
8. Aug, 15	Green	Bangsan - Phai Bay (Cholburi Province)	*Noctiluca scintillans*
9. Aug, 23	Green	Sichang - Sriracha (Cholburi Province)	*Noctiluca scintillans*
10. Oct, 4	Redish Brown	Cholburi Bay (Cholburi Province)	Water sample was not collected
11. Nov, 8	Green	Cha-Am (Phetchaburi Province)	*Noctiluca scintillans*
12. Nov, 22	Green	Ta-Yang (Phetchaburi Province)	*Noctiluca scintillans*

massive fish kill

Table 3. Occurrences of red tide in the Gulf of Thailand in 1993.

Date	Water Colour	Affected Areas	Causative Organism
1. Jan, 7	Green	Coastal areas of Phetchaburi Province	*Noctiluca scintillans*
2. Jan, 10	Green	Chao Praya River Mouth (Samut Prakan Province)	*Noctiluca scintillans*
3. Jan, 14	Redish Brown	Ban Leam (Phetchaburi Province)	*Cochlodinium sp.*
4. Jan, 15	Green	Ban Leam (Phetchaburi Province)	*Noctiluca scintillans*
5. Jan, 20	Green	Leam Pakbia - Hat Chao Samra (Phetchaburi Province)	*Noctiluca scintillans*
6. Jan, 24	Green	Mae Klong River Mouth (Samut Songkram Province)	*Noctiluca scintillans*
7. Feb, 6	Green	Leam Pakbia (Phetchaburi Province)	*Noctiluca scintillans*
8. Feb, 16-21	Green	Ban Bang Taboon (Phetchaburi Province)	*Noctiluca scintillans*
9. Mar, 18	Green	Phetchaburi River Mouth (Phetchaburi Province)	*Noctiluca scintillans*
10. Apr, 17	Brown	Ta-Yang (Phetchaburi Province)	*Trichodesmium erythraeum*
11. Apr, 23-27	Green Brown	Ban Leam (Phetchaburi Province)	*Noctiluca scintillans*
12. May, 1-5	Green	Mae Klong River Mouth (Samut Songkram Province)	*Noctiluca scintillans*
13. May, 8	Green	Sriracha (Cholburi Province)	Water sample was not collected
14. May, 20	Green	Tha Chin River Mouth (Samut Sakhon Province)	*Noctiluca scintillans*
15. Jun, 19-20	Green	Laem Chabang - Pattaya (Cholburi Province)	*Noctiluca scintillans*
16. Jul, 3	Green	Sichang - Laem Chabang (Cholburi Province)	*Noctiluca scintillans*
17. Jul, 18	Green	Sichang - Laem Chabang (Cholburi Province)	*Noctiluca scintillans*
18. Jul, 23	Green	Sichang - Sriracha (Cholburi Province)	*Noctiluca scintillans*
19. Jul, 28	Brown	Bang Prakong River Mouth (Cholburi Province)	Water sample was not collected

Table 4. Occurrences of red tide in the Gulf of Thailand in 1994.

Date	Water Colour	Affected Areas	Causative Organism
1. Jan, 2	Green	Laem Pakbia - Cha Am (Petchaburi Province)	*Noctiluca scintillans*
2. Jan, 8	Green	Laem Pakbia - Cha Am (Petchaburi Province)	*Noctiluca scintillans*
3. Jan, 29	Green	Bang Pra (Cholburi Province)	*Noctiluca scintillans*
4. Feb, 5	Green	Laem Pakbia - Cha Am (Petchaburi Province)	*Noctiluca scintillans*
5. Feb, 19	Green	Leam Pakbia - Bangkula (Phetchaburi Province)	*Noctiluca scintillans*
6. Apr, 7	Green	Bangsan - Ban Laem Chabang (Cholburi Province)	*Noctiluca scintillans*
7. Apr, 9	Green	Ban Pakklongkoa-Ban Bangpra (Cholburi Province)	*Noctiluca scintillans*
8. Apr, 17	Green	Sriracha - Sichang Island (Cholburi Province)	*Noctiluca scintillans*
9. Jun, 18-19	Green	Bangsan Bay - Sichang Island (Cholburi Province)	*Noctiluca scintillans*
10. Jun, 23	Green	Bangsan Bay - Banglamung (Cholburi Province)	*Noctiluca scintillans*
11. Jun, 26	Green	Bangpra - Sichang Island (Cholburi Province)	*Noctiluca scintillans*
12. Jun, 26	Green	Chao Praya River Mouth - Bang Prakong River Mouth	*Noctiluca scintillans*
13. Jul, 17	Green	Ban Huaprong - Ban Hat Won (Cholburi Province)	*Noctiluca scintillans*
14. Jul, 24	Green	Chao Praya River Mouth (Samut Prakran Province)	*Noctiluca scintillans*
15. Jul, 31	Green	Sichang Island - Phai Island (Cholburi Province)	*Noctiluca scintillans*
16. Aug, 14	Green	Cha Am - Hua Hin (Petchaburi Province - Prachuabkirikhan Province)	*Noctiluca scintillans*
17. Aug, 28	Green	Bangpo - Klongdan (Samut Prakran Province)	*Noctiluca scintillans*
18. Sep, 4	Green	Chao Praya River Mouth - Sichang Island (Samut Prakran Province - Cholburi Province)	*Noctiluca scintillans*

Table 5. Occurrences of red tide in the Gulf of Thailand in 1995.

Date	Water Colour	Affected Areas	Causative Organism
1. Mar, 11	Green	Ban Huaprong (Cholburi Province)	*Noctiluca scintillans*
2. Mar, 18-19	Green	Mahachai Bay (Samut Songkram Province)	*Noctiluca scintillans*
3. Mar, 18-19	Green	Sriracha (Cholburi Province)	*Noctiluca scintillans*
4. Apr, 20	Green	Phai Island (Cholburi Province)	*Noctiluca scintillans*
5. May, 6	Green	Bang Prakong River Mouth (Cholburi Province)	*Noctiluca scintillans*
6. May, 7	Green	Sichang Island - Phai Island (Cholburi Province)	*Noctiluca scintillans*
7. May, 20	Green	Bangsan Bay (Cholburi Province)	*Noctiluca scintillans*
8. May, 20	Green	Sriracha (Cholburi Province)	*Noctiluca scintillans*
9. May, 20	Green	Bang Prakong River Mouth (Cholburi Province)	*Noctiluca scintillans*
10. Jun, 10	Green	Bang Prakong River Mouth (Cholburi Province)	*Noctiluca scintillans*
11. Jul, 15	Green	Chao Praya River Mouth (Samut Prakran Province)	*Noctiluca scintillans*
12. Jul, 23	Green	Bangsan Bay - Pattaya (Cholburi Province)	*Noctiluca scintillans*
13. Jul, 23	Green	Sichang Island (Cholburi Province)	*Noctiluca scintillans*
14. Jul, 23	Green	Phai Island (Cholburi Province)	*Noctiluca scintillans*
15. Jul, 23	Green	Lan Island (Cholburi Province)	*Noctiluca scintillans*
16. Sep, 5	Green	Bangsan - Sichang Island (Cholburi Province)	*Noctiluca scintillans*
17. Sep, 10	Green	Chao Praya River Mouth (Samut Prakran Province)	*Noctiluca scintillans*
18. Sep, 10	Green	Tha Chin River Mouth (Samut Sakhon Province)	*Noctiluca scintillans*
19. Nov, 29	Green	Tha Chin River Mouth (Samut Sakhon Province)	*Noctiluca scintillans*
20. Nov, 29	Green	Chao Praya River Mouth (Samut Prakran Province)	*Noctiluca scintillans*
21. Nov, 29	Green	Bang Prakong River Mouth (Cholburi Province)	*Noctiluca scintillans*

Table 6 Total Population by Changwat along the coastline and number of tourist (1994)

Population along the coastline of the Gulf of Thailand

PROVINCES	Populations	No. of Tourist(1994)
SAMUT SONGKRAM	207,000	
PHETCHABURI	439,000	489,094
BANGKOK	8,661,000	
SAMUT PRAKAN	872,000	
CHACHOENGSAO	893,000	
CHOLBURI	928,000	2,362,615
RAYONG	438,000	1,039,951
CHANTHABURI	456,000	
TRAT	202,000	385,632
PRACHUAP KHIRIKHAN	451,000	367,545
CHUMPHON	416,000	
SURAT THANI	791,000	1,139,004
SONGKHLA	1,130,000	1,721,771
NAKHON SI THAMARAT	1,478,000	756,799
PAT TANI	1,541,000	
NARATHIWAT	577,000	454,905

Population along the coastline of the Andaman Sea

PROVINCES	Populations	No. of Tourist(1994)
RANONG	131,000	
KRABI	311,000	623,276
PHUKET	189,000	2,093,192
SATUN	231,000	

HARMFUL ALGAL BLOOM OCCURRENCES AND MONITORING STRATEGIES IN MALAYSIA

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INTRODUCTION

The occurrences of harmful algal blooms (HABs) are believed to be increasing all over the world since the 1970s (Anderson, 1989; Lam and Ho, 1989; Maclean, 1989; Park et al., 1989; Shumway, 1989 and Shumway, 1995); and yearly an estimated 2000 cases of human poisoning from HABs are recorded (Hallegraeff, 1993). Apart from human fatalities, HABs are known to cause huge economic losses both in the natural fisheries and in aquaculture (Corrales and Maclean, 1995; Okaichi, 1991; Shumway ,1990 and Smayda, 1991).

Owing to the increasing global reports of HABs and the economic impacts that could result from such events, this paper attempts to examine the status of HAB occurrences in Malaysia and their impacts on the natural fisheries and aquaculture. Monitoring methods that could be adopted to safeguard the safety of seafood are also discussed.

STATUS OF HARMFUL ALGAL BLOOMS IN MALAYSIA

East Malaysia

To date HABs in East Malaysia have been reported only from Sabah, and no occurrences have been documented from Sarawak. Red Tide occurrences were first reported in Sabah in January 1976. The causative organism causing paralytic shellfish poisoning (PSP) is *Pyrodinium bahamense* var. *compressum*. During this outbreak which lasted for 4 months, 202 cases of food poisoning with 7 fatalities (children ranging from age 4-11) were recorded (Roy, 1977). The bloom killed hydroids, sponges, molluscs, crustaceans, echinoderms, corals and fishes, probably by asphyxiation (White et al., 1984). The sea snail, *Oliva* sp. was found to be toxic and was responsible for 5 fatalities. Between 1976-1989 numerous blooms and over 300 paralytic shellfish poisoning cases and several deaths have been reported (Ting and Wong,1989). Since 1990, harmful algal blooms occur every year on the west coast of Sabah, but the last recorded bloom from the east coast was in 1988 (Ronnie Jamilus, personal communication). Apart from molluscs, such as *Perna viridis, Pteria* sp., *Atrina* sp., *Anadara* sp. and *Crassostrea* sp., planktivorous fish such as *Sardinella* sp. and *Decapterus* sp have also been reported to accumulate toxins. Many locations along the west coast are classified as high-risk areas and are monitored regularly, and shellfish have been reported to be toxic even when there is no visible plankton bloom.

Sabah supports an important *Sardinella* and *Decapterus* fishery, especially around the Kota Kinabalu and Papar areas on the west coast. In 1994 the *Sardinella* catch landed amounted to 13,306 tonnes worth RM 12,241,520 whilst the *Decapterus* landing was 12,954 tonnes worth RM 13,731,240 (Annual Fisheries Statistics, 1994). The State, however, supports a relatively small production of molluscs from aquaculture. For 1994, 8 tonnes of mussels, 5.5 tonnes oysters and 552.7 tonnes cockles valued at RM 3,185, RM 5,694 and RM 230,963 respectively were produced. Any red tide event would incur huge economic losses especially if the *Sardinella* and *Decapterus* fisheries are affected. However, the fish are safe for consumption after the removal of the gills and guts (Ting and Wong, 1989), but public ignorance and fear may cause the sale of fish to plummet during an outbreak.

Peninsular Malaysia

The first documented case of red tide occurrence in Peninsular Malaysia was in August 1978 when a bloom of *Noctiluca scintillans* in Telok Kumbar, Penang drove crabs and demersel fish to the shore, and almost 1000 tonnes were stranded and caught by fishermen (Choo, 1994). Between 1978-1990, a total of 8 red-tide cases caused either by *N. scintillans* or *Hornellia* sp. were reported (Choo, 1994). The first PSP poisoning was reported in November 1993 in Sebatu, in the district of Jasin, Melaka, where a family of 6 was hospitalised after a meal of mussels (*Perna viridis*). No visible discoloration of the sea was reported and the causative organism has not been confirmed, but is most probably either *Alexandrium tamiyavanichii* or *Gymnodinium catenatum*. Since 1993, no reports of HAB have been received.

Fishermen were reported to suffer losses during the 1978 bloom in Telok Kumbar where reduced fish catches after the bloom were reported (Jothy, 1984). The red-tide blooms in the Straits of Johor in 1983 and 1985 caused a loss of US$ 25 million to shrimp farms (Anderson et al., 1990). The HAB event in Melaka occurred in a mussel culture area and caused economic losses to the farmers. Before 1993, this area supported an industry comprising 47 mussel racks, each measuring 7.9m x 9.8m. Each rack could produce 6-9 tonnes of harvest a year, and the Jasin district could thus support a mussel industry of 282-423 tonnes worth RM 141,500 - RM 211,500 yearly. The district also exports 500-1000 strings of spat worth RM 3,750 - RM 7,500 annually. Thus the ban imposed on the movement of mussels for 8 months in 1994 caused a loss of about RM 145,000 - RM 219,000. Subsequent to the loss suffered in 1994, fewer fishermen opted for mussel culture, and in 1996 there are only 23 mussel racks in the Jasin area.

MONITORING PROGRAMMES

East Malaysia

In Sabah, due to the regular occurrences of HAB especially in the high-risk areas on the west coast, a comprehensive monitoring programme has been developed by the Department of

Fisheries to ensure the safety of seafood for consumption. Fortnightly, mouse bioassay tests for toxins in shellfish and plankton examinations are carried out; sampling frequencies may be increased during an outbreak. Whenever toxin levels exceed 80 μg /100g tissue or when the *Pyrodinium* cells reach a few thousand per litre, a public warning is issued to the area concerned (Wong and Ting, 1989).

Peninsular Malaysia

In Peninsular Malaysia HAB does not occur on a regular basis. Monthly monitoring of PSP toxins in mussels and oysters using the mouse bioassay method is carried out by the Fisheries Research Institute, Penang in two areas , that is the Jasin district in Melaka and the Telok Dalam/Telaga Nenas area in Perak. The former is an important mussel culture area, whilst the latter supports both a mussel and oyster culture industry.

DISCUSSION

The Department of Fisheries, Sabah has collected data on *Pyrodinium* blooms through their monitoring programme for the past 20 years, and efforts should now be directed at identifying triggers as well as developing models for the prediction of a *Pyrodinium* bloom event. The high-risk areas on the west coast do not support a very important mollusc industry, but support important *Sardinella* and *Decapterus* fisheries which occasionally could be affected by HABs. Even though these fish have been reported safe for consumption if properly cleaned and degutted, some countries, like Brunei and Singapore are known to have imposed bans on importing fish from Sabah during HAB occurrences. A good prediction model may help to regulate fishing intensity and choice of fishing sites during an HAB outbreak.

The occurrence of HAB in Peninsular Malaysia has so far been sporadic. Due to the constraints on trained manpower and inadequate funding, monitoring programmes should initially focus on areas where HAB are known to have occurred as well as in some important mollusc culture areas, especially oyster and mussel culture sites. Malaysia is the world's top producer of cockles (*Anadara granosa*), harvesting between 60-80 thousand tonnes annually and with almost 99% of the production coming from Peninsular Malaysia. Wong and Ting (1989) reported that although cockles have been found to accumulate toxins during a HAB event, the toxicity level is always significantly lower than in oysters. Cockles, which are benthic organisms feeding on bottom detritus, are unlikely to accumulate toxins from HAB cells, but may become toxic from cyst contamination. Emphasis should be given to toxin testing by mouse bioassay technique to ensure the safety of seafood, especially molluscs, for human consumption. To reduce sampling costs, culturists could be trained to collect samples and send them to authorised laboratories for toxin analyses.

ACKNOWLEDGEMENT

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ABSTRACTS

ORAL SESSION 3

PHYSIOLOGY AND BIOCHEMISTRY OF HARMFUL ALGAE

GROWTH REGULATION IN DINOFLAGELLATES: MECHANISMS LINKING CELL DIVISION TO THE DIURNAL CYCLE

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Dinoflagellate blooms are the source of toxins responsible for several disease syndromes associated with seafood consumption. In order to gain understanding of how environmental cues may trigger the rapid growth and reproduction of dinoflagellates that constitute a "bloom", we have undertaken the current project to identify molecular mechanisms which regulate the cell division cycle in dinoflagellates. We have previously identified the presence and mitotic activation of the eukaryotic cell cycle regulatory protein, CDC2 kinase in dinoflagellates, suggesting that dinoflagellates possess the typical cell cycle regulatory machinery present in higher eukaryotes. Our current work addresses mechanisms by which environmental cues may activate or inhibit the cell cycle regulatory machinery. Cell division in many dinoflagellate species is phased to the diurnal cycle, according to species-specific patterns in which division may occur during either the light phase or the dark phase of the diurnal cycle. Here we examine diurnal regulation of the cell cycle in two species of dinoflagellate, *Gambierdiscus toxicus* and *Amphidinium operculatum*. Both specie were grown in K medium at 26°C and a 16:8 LD cycle. Under these conditions, *G. toxicus* has a division rate of approximately 0.15 div./day, with cell division restricted to a three hour window late in the dark phase. Modulation of the LD cycle demonstrated that the onset of mitosis is linked to the light:dark transition, with mitosis occurring precisely six hours after the onset of dark. The signal which links cell division to the light:dark transition in *G. toxicus* is blue light dependent, such that the cessation of blue light permits the cell to proceed through the cell cycle to mitosis. This light-dependent signal occurs prior to the end of the S-phase, and may regulate S-phase entry. In contrast, under the same growth conditions, *A. operculatum* has a division rate of approximately 1 div./day, with synchronous cell division occurring during the light phase. S-phase begins approximately 5 hours after the onset of light and takes approximately 7 hours to complete. Mitosis follows at approximately 15 hours after the onset of light and is completed within three hours. Upon alteration of the LD cycle, S-phase entry continues to occur 5 h after the dark:light transition. Therefore, cell division is phased to the diurnal cycle by a signal emanating from the dark:light transition. These results suggest that the cell cycle in different dinoflagellate species may be phased to the diurnal cycle via different environmentally controlled signalling mechanisms.

INTERACTIONS BETWEEN NUTRITION, BEHAVIOR, AND TOXICITY IN *ALEXANDRIUM TAMARENSE*

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Toxic dinoflagellates of the genus *Alexandrium* have been implicated as causative organisms in Paralytic Shellfish Poisoning (PSP) in various regions of the world. Several studies concerning PSP producing dinoflagellates have revealed a positive relationship between nitrogen (N) availability and toxin content (Boyer et al. 1987, Anderson et al. 1990, Flynn et al. 1994). Such a relationship is very relevant in nature as toxic dinoflagellates typically inhabit nearshore areas (Ryther and Dunstan 1971) which are frequently limited by N and periodically N-stratified.

Several species of dinoflagellates are known to perform diel vertical migrations through gradients of nutrients and temperature in both natural (Eppley and Harrison 1975, Blasco 1978) and laboratory conditions (Eppley et al. 1968, Cullen and Horrigan 1981, Kamykowski 1981). Migrational behavior consequently enables dinoflagellates to utilize deep N-pools (Fraga et al. 1992) and confers some competitive advantage for phytoplankton in N-depleted surface waters (Holmes et al. 1967). It seems likely then, that the complex behavioral pattems exhibited by dinoflagellates have a great influence on cellular physiology and ecology, especially in nutrient-stratified waters, and in turn must effect the toxic properties as well. However, conventional batch-culturing systems can not accommodate the behavioral aspects of dinoflagellate ecology and therefore miss the effects of behavior on cellular physiology. As a result, a vertically stratified laboratory water column, where swimming behavior could influence photosynthesis and nutrition, was used in the present study to examine the effects of N availability on the toxicity of the dinoflagellate *Alexandrium tamarense*. These results were then compared with results obtained from batch and semi-continuous culture systems in which behavior is not a factor.

The experiments on batch and semi-continuous cultures produced results similar to what has been published previously, confirming a direct positive relationship between N availability and toxin content in this strain of *Alexandrium tamarense*. Variations in the allocation of N between cellular pools and toxin were observed during N-starvation, re-supply and under semi-continuous N-limitation. Lack of rapid mobilization of toxin during starvation or accumulation during resupply indicated that paralytic shellfish toxins likely function in a role other than the storage of N. In addition, the steady-state cultures, maintained at two contrasting rates of semi-continuous N supply, also demonstrated dramatically different cellular toxin profiles (i.e. changes in the relative abundance of the individual toxins). This is an interesting result as notable changes in toxin profile have not been reported for actively growing, non-axenic batch-cultures (Cembella et al. 1987,

Boyer et al. 1987, Flynn et al. 1994). It has been suggested that batch-culture experiments do not provide a N-stressed period of sufficient duration to allow for toxin profile changes. Therefore, because toxin profile changes occur over a longer time scale, they are much more pronounced in steady-state cells which remain viable for a longer period of time while enduring N-stress.

The particular focus of this work however, was investigated in a temperature-controlled, stratified laboratory water column contained in a 2.0m PVC tank (internal diameter of 0.29m). Stratification in the tank was provided by a thermal gradient produced by a running water bath. Sampling in the tank was performed four times daily (twice daily for the first week) by lowering a doubled silicone tube down the center of the water column. Profiles of fluorescence were obtained by pumping water from depth through a flow through fluorometer while a valve in the sampling line allowed discrete samples to be obtained at desired depths for cell counts, chlorophyll a concentration, fluorescence before and after the addition of the photosenthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), particulate C and N (CHN), inorganic macronutrient concentrations ($N0_3$-, $P0_4^{-3}$, NH_4+) and PSP toxin analysis. The 'tank' experiment ran for 24 days and consisted of five phases delimited by the nitrogen status of the medium.

Initially, when nitrate was replete throughout the tank, the highly toxic cells formed a thin surface layer which persisted throughout the 24 h light:dark cycle (Fig 1). When nitrate was depleted in the surface layer as a result of uptake by the cells, they began a nocturnal migration to the nitracline. During this phase the toxin content of the cells decreased gradually as the C:N of the cells increased, consistent with N limitation (Fig 2). The third phase saw the deep nitrate pool exhausted and deeper penetration of the cells during the dark period. The toxin content of the cells reached an equilibrium low level during this phase. To restore N-stratification in the water column, nitrate was added to the deep layer, initiating the fourth phase and prompting the cells to restrict nocturnal migration to the nitracline. During this phase toxicity of the cells increased. Finally, to bring the experiment full circle, N was added to the surface layer. During this fifth and final phase, cellular toxicity continued to rise as cells again formed a dense surface layer which persisted throughout the 24 h period.

Although it was previously demonstrated that a European strain of *Alexandrium tamarense* could migrate across steep pycnoclines (Rasmussen and Richardson 1989) and thermoclines (Santos and Carreto 1992), a marked diel pattern of vertical migration had yet to be confirmed in this PSP producing species. Like vertical migration in other flagellates, diel vertical migration (DVM) in *A. tamarense* was tightly correlated to the availability of N in the water column. In the presence of surface layer N, the cells did not undertake a nocturnal descent to the deep layer and as a result, did not initiate a DVM until surface layer N had been depleted. The culture subsequently utilized N in successive layers, increasing the depth of the nitracline and resulting in a progressively deeper nocturnal descent. Thus *A. tamarense* demonstrated a flexible pattern of DVM which enables it to continue to maximize its growth potential in an evolving nutrient regime.

A comparison of the toxin characteristics of *Alexandrium tamarense* between a

N-stratified regime and those of N-starved and N-limited cells indicates that although cells living in a N-stratified water column were less toxic relative to N-replete cells, they were able to maintain (Phase II) or attain (Phase IV) a moderate level toxicity through DVM. The N-limited semi-continuous cultures demonstrated that toxin profile changes do occur when cells are able to remain viable and adapt to a limiting supply of N. Although the N-starvation which occurred in the tank and batch cultures resulted in a much less pronounced toxin profile change, the N-stratified phase of the tank experiment did allow cells a short period to adjust to an increasingly limited N-supply and thus a definite trend in toxin profile changes is easily observed (STX % molar, Fig 2). It is becoming increasingly clear that although toxin profile changes do not correlate with cellular N status as closely as other indicators of N-stress such as C:N, chl per cell and toxin content, changes do occur if cells are given sufficient opportunity to adjust.

This study therefore suggests that toxicity in *A. tamarense* is closely related to nutritional status across various types of N-regimes and that toxic dinoflagellates inhabiting nitrogen depleted surface waters are likely capable of sustaining growth and a moderate level of toxicity through nocturnal migrations to deep N pools. Migrational behavior may therefore influence the contamination of shellfish by enhancing the ability of toxic dinoflagellates to sustain or promote growth in an area while maintaining or attaining a moderate level of toxicity.

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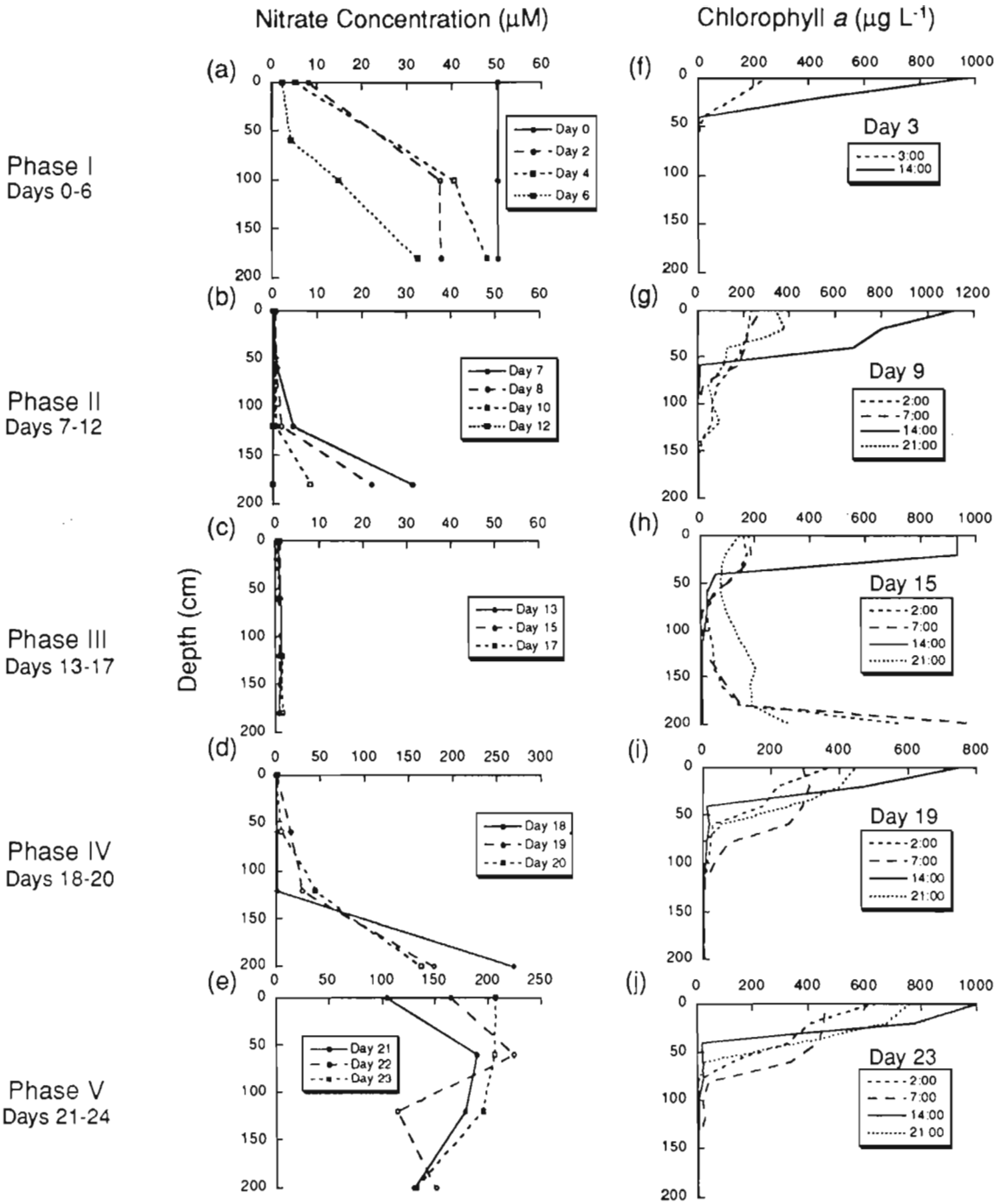

Figure 1. Relationship between the distributions of nitrate and chlorophyll in the laboratory water column throughout the five phases of the experiment. (a) to (e); Temporal shift in the nitrate concentration with depth (individual lines represent profiles taken at 14:00 of different days); (f) to (j); Profiles of cellular abundance displayed as chlorophyll *a* concentration (individual lines represent different points of the light:dark cycle of a single day). The five phases of the experiment are organized vertically.

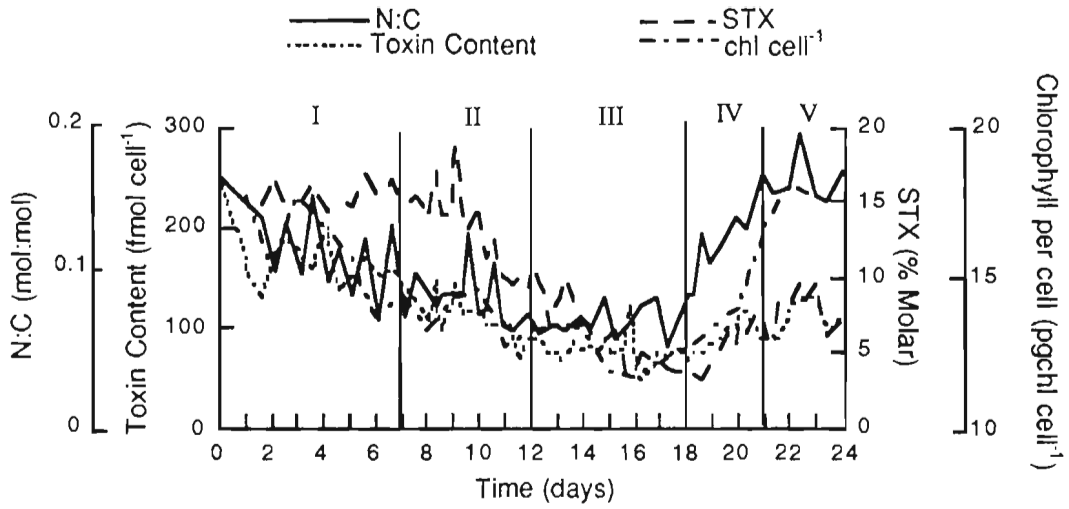

Figure 2. Temporal changes in the cellular composition of *Alexandrium tamarense* over the five phases of the tank experiment describing particularly the relationship between the N:C ratio and cellular toxin properties. The molar percentage of saxitoxin is used as a description of the toxin profile changes which occurred over the five phases. Also shown is the cellular chlorophyll *a* concentration as an additional indicator of cellular nitrogen status.

ASSESSMENT OF THE IMPORTANCE OF BACTERIA AS PSP PRODUCERS DURING A SEASONAL CYCLE IN THE ST. LAWRENCE ESTUARY

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We investigated the seasonal variations of PSP production by the natural bacterial community of the St. Lawrence Estuary, a region characterized by seasonal blooms of *Alexandrium* spp. Water was collected on a weekly basis from the pier of the Maurice Lamontagne Institute between May 31 and September 26, 1995. On each sampling day, 100 liters of water were sequentially filtered through 15, 5, and 0.22 μm 293-mm nylon filters. Material collected on the filters was suspended in 0.22 μm filtered seawater and split into three fractions: the first fraction was used for the determination of in situ PSP concentration, the second was incubated in duplicate in the dark for 21 to 68 h in a nutrient-replete and a phosphate-limited growth medium in order to determine its potential for PSP production, and the third fraction was used for bacterial and phytoplankton enumeration. We also determined by mouse bioassay and by HPLC the level of PSP toxins in blue mussels collected near the sampling station. The results presented here represent a preliminary assessment of the data from 7 of the 18 sampling dates.

In 1995, *A. tamarense* and *A. ostenfeldii* reached maximum concentrations of only 540 and 340 cells l^{-1}, respectively. In situ PSP toxins were only detected in the large size fraction (>15 μm) on June 13 (NeoSTX= 21 pM and STX= 6pM) and August 21 (STX= 1 pM). In both cases, *Alexandrium* spp. cells represent the most likely source of toxins. Results from the HPLC analyses of the mussel extracts revealed the presence of STX in all samples tested (maximum= 16 nmol/100 g; August 21), and GTX1 (maximum= 53nmol/100g) and NeoSTX (14 nmol/100g) on June 5 and August 21, respectively. The maximum concentrations of toxins measured in the mussels on June 5 and August 21 followed small increases of *A. ostenfeldii* and *A. tamarense*, respectively. Results from the mouse bioassays were always below detection limit (46 μg STX eq./100g).

No bacterial growth nor toxin production were observed in phosphate-limiting medium. On the other hand, dark incubation of material from each size fraction in nutrient-replete medium led to significant production of STX, NeoSTX, and GTX2+3. Toxin profiles varied from week to week. So far, no seasonal pattern has been observed. These results show that free and attached bacteria from the Gulf of St. Lawrence may produce PSP toxins. In 1995, there was no clear indication that bacteria contributed to the low level of PSP measured in the mussels. However, there does appear to be the potential for a bacterial contribution of PSP toxicity in the water column, including that associated with algal cells.

BACTERIA, DINOFLAGELLATES, AND PSP

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The production of paralytic shellfish toxins has historically been associated with dinoflagellates. However, evidence is increasing that bacteria can produce these toxins. In this paper, recent data supporting the production of paralytic shellfish toxins by bacteria will be persented, along with a description of the bacterial species invloved. In addition, the relationship between bacteria, dinoflagellates, and shellfish toxicity will be discussed.

AN UNEXPECTED TWIST IN DSP TOXIN BIOCHEMISTRY

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Since the characterization of the first diarrhetic shellfish poisoning (DSP) toxin in the late seventies, it was generally thought that the DSP toxins comprise a group of three lipid-soluble polyether compounds, okadaic acid, DTX-1, and DTX-2. These compounds are all powerful inhibitors of the eukaryotic serine / threonine phosphatases PP1 and PP2A, and this is believed to account for their toxicity. The DSP toxins are produced by species of dinoflagellates belonging to the genera *Prorocentrum* and *Dinophysis*. Our recent discovery of phosphatase-inactive water-soluble derivatives of the DSP toxins in cells of various toxin-producing dinoflagellates belonging to the genus *Prorocentrum*, adds a new and intriguing twist to the DSP toxin chronicles. Biosynthetic labelling studies reveal that these water-soluble compounds are the final biosynthetic products of the dinoflagellate and provide clues as to how the producing organism protects itself from these lethal toxins, and why okadaic acid is found.

ABSTRACTS

ORAL SESSION 4

OCCURRENCE OF ALGAL BLOOMS

INTERPRETATION OF SIX YEARS OF TOXIC PHYTOPLANKTON AND PSP MONITORING DATA FROM THE GULF OF ST. LAWRENCE

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Analyzing monitoring data is often discouraging. Changes in observation sites, types of analysis performed, and the personal, most often obscure the meaningful signals in the data set. Standardizing the data to facilitate comparisons, is generally difficult and tedious. However the rewards frequently justify the effort. Analysis of six years of phytoplankton data and 11 years of PSP shellfish-toxicity data collected along the entire south coast of Quebec, provides an accurate description of the geographical and seasonal pattern of PSP toxicity and *A. tamarense* in the region. In addition, it corroborates previous independent laboratory observations with field observations. For example, differences in the intoxication pattern of *Mya arenaria* and *Mytilus edulis* or relationships between PSP shellfish-toxicity and *A. tamarense* has been confirmed by the analysis of the data set. Furthermore it answers relevant question of PSP monitoring programs: What is the minimum cell concentration of *A. tamarense* at which the mussels PSP toxicity reaches the threshold of 80 μg/100g ? What is the lag time between the observation of *A. tamarense* in the water column and PSP toxins in shellfish? Finally it allows to redefine previous existing hypothesis and to evaluate the ongoing monitoring program.

HAB ORGANISMS IN INDONESIAN WATERS

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Abstract

Since the beginnings of the programme in 1991 several Red Tide incidents have been recorded, which occurred in East Flores, Jakarta Bay, Lampung, Kao Bay, and Ambon Bay. Not all incidents could be connected with Harmful Algal Blooms (HAB). Many more incidents in Indonesian waters, such as fish kills in the Bali Strait, East Flores waters, Jakarta Bay, Ujung Pandang and mass mortality of cultivated pearl oysters, could not be studied properly.

Identified HAB organisms are the dinoflagellates *Pyrodinium bahamense* var. *compressum*, *Dinophysis caudata*, *Dinophysis rotundata*, *Noctiluca scintillans*, *Prorocentrum lima, Gambierdiscus toxicus*, the cyanobacterium, *Trichodesmium thibautii*, and the diatom *Pseudonitzschia pungens*. Two other dinoflagellates, *Alexandrium* sp. and *Gymnodinium* sp. need further observations and identifications. *Pyrodinium bahamense* var. *compressum* and *Trichodesmium thibautii* are two HAB species identified for causing human health and fisheries' problems, while *Noctiluca scintillans* and *Gymnodinium* sp. are thought to be the species causing fish and shrimp kills in the Jakarta Bay.

Introduction

Indonesia only became aware of possible Red Tide problems in 1991. A small budget was allocated to study this phenomenon, following reports received from very remote places. PSP-like symptoms were suffered by coastal living people after consuming marine products. The reports came from East Flores, Ujung Pandang (South Sulawesi), and Sebatik Island (East Kalimantan). Following these reports sampling programs were conducted in these areas. The main object of the program was to find *Pyrodinium bahamense* var. *compressum*, the species reported causing PSP problems in South-East Asian Waters (Maclean, 1989a and b; Seliger, 1989; Jaafar et al.; 1989, Ting & Wong, 1989; Jara, 1993; and Cheong, 1993).

During 1991/1992 no Red Tide was reported from the sampling sites, but Red Tide due to blooming of *Trichodesmium thibautii* occurred in westem Jawa Sea. The organisms were drifted by currents to reach Lampung coastal areas, polluted shrimp ponds on the eastern Lampung coast, and killing shrimp reared in these ponds. The estimated loss was around US$.1.75 million

(Adnan 1992; Praseno & Adnan 1994).

This incident made us changed the strategy of Red Tide program, in which not only *Pyrodinium bahamense* var. *compressum* was studied, but also other potential HAB organisms should also be identified for possible negative impacts. To do so Indonesia has to come up with another strategy to cope with the problem. The vast size of the country, the unawareness, and the minimal number of scientists involved in the campaign were among the factors to be taken in consideration.

The Development of Monitoring System

Past cases of Red Tides were reported from several places in Indonesia. Adnan (1984) and Setiapermana (1993) reported on several incidents of Red Tide in Indonesian waters. These locations were a small village in East Flores (Lewotobi), a small island in South Sulawesi, and Sebatik Island in East Kalimantan, part of the island belongs to Indonesia and the other part to Malaysia.

As a maritime country, Indonesia has to develop a national monitoring system. The first step of developing this system was organize training courses, which were participated by fishery personnel as well as university staffs. The training programs were also supported by the ASEAN-Canada Cooperative Programme on Marine Sciences - Phase II (CPMS-II) and by the IOC/WESTPAC Program on Red Tides. Participants were trained on sampling methodology and identification of HAB organisms. As a result a preliminary monitoring network was established with RDCO-Jakarta acting as center with several links at locations with trained participants acting as respondents. The Laboratory of RDCO in Ambon is functioning as a center for east Indonesia. Presently the monitoring station covers 12 out of 27 Provinces, which are North Sumatera, Riau, Lampung, West Jawa, Jakarta, Central Jawa, West Nusatenggara, East Nusatenggara, South Sulawesi, East Kalimantan, the Moluccas, and Irian Jaya. Further training courses are planned to train people from other Provinces. Even with this effort Indonesia still has problems to monitor remote places, where Red Tides may occur undetected.

Identification of HAB Organisms

The Research and Development Centre for Oceanology of the Indonesian Institute of Sciences (RDCO-LIPI) has Plankton Laboratories at two locations, Jakarta and Ambon. These Laboratories are involved in the campaign of hunting HAB organisms. The Jakarta Laboratory covers the western part of Indonesia, while the Ambon Laboratory covers the eastern part of the country. identification of HAB organisms were made from samples obtained from the Jakarta Bay, Lampung, East Flores, Ujung Pandang in South Sulawesi, and Sebatik Island in East Kalimantan (covered by Jakarta Laboratory), and Kao Bay (Halmahera), Ambon Bay, Piru Bay and waters on the western part of north Irian Jaya (covered by Ambon Laboratory). Those

locations were chosen because of preceding reports on HAB affect on fisheries and human health. In addition several samples were obtained from locations where RDCO has ongoing research programs. Furthermore phytoplankton sampies were also received from fishery personnel, who have been trained at RDCO to monitor Red Tides in their respective working areas. For further identification of HAB organisms, samples were sent to experts, such as Dr. F.J.R. "Max" Taylor of the University of British Columbia, Canada and Dr. Y. Fukuyo of the University of Tokyo, Japan. Although plankton samples were received from various places, still Indonesian water has not been studied thoroughly, because of the vast size of the country. Incidents of Red Tide may occur in very remote places, where communication is still a problem. The lack of manpower and the minimal knowledge on the phenomenon are factors to be solved in the future. Table 1 shows potential HAB organisms found in those waters, with possible toxic or harmful species included in the list.

Impact of HAB on Fisheries and Human Health

The first HAB impact on fisheries in Indonesian waters was recorded in June-September 1991 where mass mortality of cultivated shrimp died in the brackish water ponds of the eastern coast of Lampung. The causative phytoplankter was identified as *Trichodesmium erythraeum* (Adnan, 1992). On December 8, 1993, fish kills occurred in the Jakarta Bay caused by high concentrations of ammonia. Noctiluca blooms, before the incident, was concluded to add up to the concentration of ammonia in the bay waters (Praseno, 1995). Praseno (1995) also reported mass mortality of cultivated shrimp in brackish water ponds of Kamal, west of Jakarta. The causative HAB organism was identified by Dr. F.J.R. "Max" Taylor as *Gymnodinium pulchellum*

The first HAB impact on human health was recorded by Wiadnyana et al. (1994) from Kao Bay, Halmahera Island in the eastern region of Indonesia. This incident was not the first incident, but previous incidents were not associated to HAB. The causative organism was identified as *Pyrodinium bahamense* var. *compressum*. This species has also caused problems in neighboring countries, such as the Philippines, Sabah-Malaysia, Brunei Darussalam, and Papua New Guinea (Maclean, 1989a and b; Seliger, 1989; Jaafar et a/.; 1989, Ting & Wong,1989; Jara, 1993; and Cheong,1993). As remedy local people use coconut juice, which seemed to be most helpful. *Pyrodinium bahamense* var. *compressum* also caused human health problems in Ambon Bay in 1994, where one child died and several others were treated (Wiadnyana et al. 1994). Many other fish kill, however, went by unreported, such as pearl oyster kills at mariculture sites in the Moluccas, Lombok, Bali, and Lampung Bay. Fish kill in the Bali Strait and shrip kill in several brackish water ponds at the northern coast of Jawa. Figure 1 shows HAB incidents in Indonesian waters. It should be noted that no HAB impact occurred in Bangka Strait, mainly because the small island, Marsigu, was unoccupied. *Pyrodinium* cells were dominating at samples taken from sea grass beds.

Further Plans

The Red Tide study in Indonesia will continue for several years. Beginning 1997 cultures of potentially toxic species will be conducted to test for possible harmful species. This will be followed by toxicity testing of marine products, especially those with commercial value. The study will also include ecology of phytoplankton blooms in estuaries. These steps are taken in parallel to the development of a National Red Tide Management System in Indonesia, with the involvement of relevant institutions.

Acknowledgement

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Table 1. List of potensial HAB organisms identified from Indonesian waters.

Nº	Phytoplankton species	Location
1	*Ceratium fusus*	Jakarta Bay, Bayur Bay-West Sumatera, Ujung Pandang, East Flores, East Kalimantan.
2	*Ceratium tripos*	Jakarta Bay, East Flores.
3	*Dinophysis acuminata*	Jakarta Bay, Kuala Tungkal-Jambi.
4	*Dinophysis acuta*	Jakarta Bay.
5	*Dinophysis caudata*	Jakarta Bay, Kuala Tungkal-Jambi, Lampung, East Flores.
6	*Dinophysis miles*	Jakarta Bay, Teluk Bayur-West Sumatera, East Flores.
7	*Dinophysis ovum*	East Flores, Jakarta Bay.
8	*Dinophysis rotundata*	Jakarta Bay.
9	*Gambierdiscus toxicus*	East Flores.
10	*Gonyaulax diegensis*	Jakarta Bay.
11	*Gonyaulax polyendra*	Ujung Pandang
12	*Gonyaulax polygramma*	Jakarta Bay.
13	*Gonyaulax sp.*	East Flores, Jakarta Bay.
14	*Gymnodinium pulchellum*	Kamal (Jakarta) shrimp ponds.
15	*Gymnodinium sp.*	East Flores.
16	*Noctiluca scintillans*	Jakarta Bay, East Kalimantan, Ambon Bay.
17	*Prorocentrum lima*	East Flores, Ujung Pandang, Jakarta Bay.
18	*Pseudonitzschia pungens*	Jakarta Bay.
19	*Pyrodinium bahamense* var. *compressum*	Kao Bay, Ambon Bay, Biak, Jakarta Bay, Marsegu Island (Bangka).
20	*Trichodesmium thiebautii*	Lampung, Jakarta Bay.

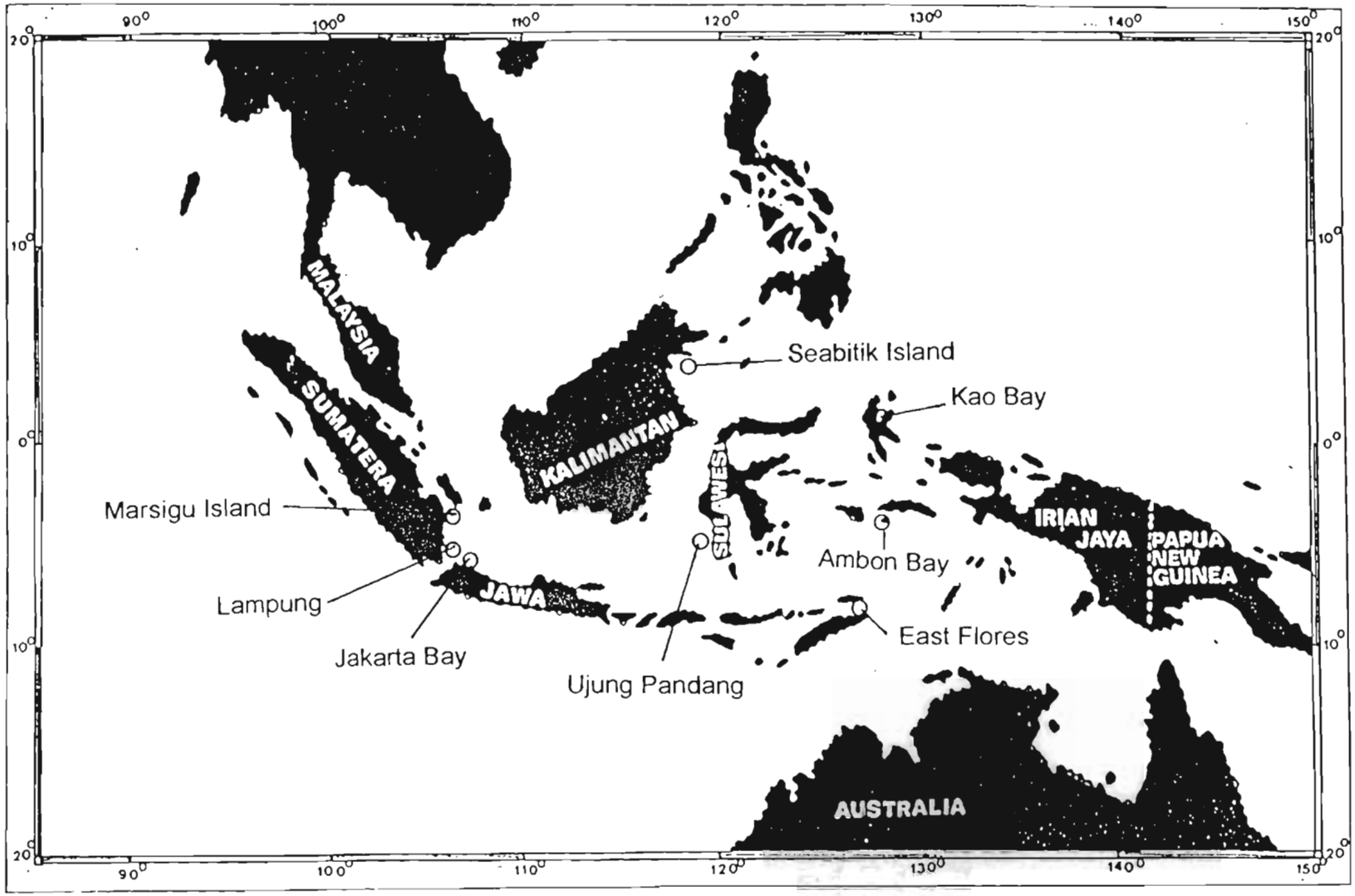

Figure 1. HAB occurences in Indonesian waters. ○ The Bloom at Marsigu Island, Bangka Strait, didnot cause negative impact.

OVERVIEW OF THE AUGUST 1996 RED TIDE EVENT IN THE ST. LAWRENCE: EFFECTS OF A STORM SURGE

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Between 19 and 21 July 1996, more than 225 mm of rain fell over the Saguenay River area in the Province of Québec. This unusual weather resulted in the flooding of several towns along the Saguenay River. In the St. Lawrence estuary, the heavy rain caused a drastic decrease in surface water salinity accompanied by a bloom of the toxic dinoflagellate *Alexandrium tamarense* on 29 July. In mid August, red tide concentrations of 3×10^6 cells l^{-1} of *A. tamarense* were measured in a patch located along the north shore of the Gaspé Peninsula. In the same area, mortalities of sand lance (*Ammodytes hexapterus*) and herring gulls (*Larus argentatus*) were reported. Domestic cats who had eaten dead fish on the beach also exhibited symptoms characteristic of paralytic shellfish poisoning (PSP). Results from HPLC analyses revealed concentrations as high as 360 µg STX eq/100 g in the dead sand lances. Levels of PSP toxins reached 110 and 48 µg STX eq/100 g in the intestines and brains, respectively, of the dead herring gulls. Concerns for the potential transfer of the toxins from the sand lance to commercial fish prompted the Department of Fisheries and Oceans to advise the population not to eat the livers of fish, e.g. cod, caught in this area of the St. Lawrence. An overview of this event will be presented.

SPATIAL, SEASONAL, AND YEARLY VARIANCE IN TOXIC BLOOM EVENTS ON THE COAST OF BRITISH COLUMBIA

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Although assessment of toxic phytoplankton populations in B. C. is limited, past bloom events have been estimated indirectly by monitoring levels of toxin in sentinel species. These sentinel species, filter-feeding bivalves, are cumulative recent-time indicators of toxic blooms that are unpredictable and often fleeting. Paralytic shellfish poison (PSP) elaborated by species of *Alexandrium* has been monitored for several decades in B. C. as a public safety measure. More recently amnesic shellfish poisoning (ASP), from species of *Pseudo-nitzschia*, has been monitored on the coast using mussels and razor clams as sentinel species. *Heterosigma carterae* is a lethal fish killer that is now constantly monitored at aquaculture sites in B. C. as a means of alleviating the drastic economic effects on farmed salmon. Toxicity records from sentinel species have demonstrated spatial, seasonal, and yearly variance in toxic bloom events over the past years and decades. Until satellite imagery is perfected to accomodate inclement weather conditions, toxicity monitoring of sentinel species will continue to provide time series data on toxic phytoplankton. These factors will be presented and discussed.

DOMOIC ACID ON THE WEST COAST OF THE UNITED STATES

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In 1991, domoic acid poisoning was recognized for the first time on the west coast of the United States when significant numbers of sea birds were found dead or dying along the beaches of Monterey Bay, California (Work et al, 1993). Examination of the stomach contents of the dead birds indicated they had been feeding on anchovies which were found by chemical analysis to contain high levels of domoic acid (>100 ppm). As a result of the outbreak, the California Fish and Game and California state Health Department issued an early warning to the coastal states of Washington and Oregon which began monitoring for the toxin. By December of 1991, domoic acid was found in razor clams (*Siliqua patula*) and Dungeness crab (*Cancer magister*) in Oregon and Washington state. At this time, little was known about the source and extent of the toxic occurrence as well as how long domoic acid would remain in the contaminated species. This lack of knowledge caused fisheries and health officials in the west coast states to close both razor clam harvesting and the Dungeness crab fishery, which prevented any confirmed serious human illnesses.

Razor Clams

Razor clams are an inter-tidal species found in sandy beaches, with extensive seaward reaches, on the west coast of the U.S. They inhabit the highly energetic "surf zones" and their diet consists predominantly of the surf diatoms *Attheya armatas*, *Asterionellopsis socialis*, and *A. glacialis* (Lewin and Norris, 1970). While some razor clams are found in northern California, recreational and commercial harvest occurs mainly in Oregon and Washington state. In Washington state, the harvest of razor clams is largely a recreational fishery but nevertheless is responsible for a large annual economic infusion (US $6-20 million) into the coastal areas. Because of the importance of this fishery, the Washington state Department of Fish and Wildlife (WDFW) monitors the razor clam population closely with a structured program which includes sampling at four major razor clam management areas. The Utilization Research Division of the National Marine Fisheries Service began working closely with the WDFW in order to monitor the progress of the domoic acid contamination within these areas. Since November 1991, we have received, during the razor clam digging season, samples collected regularly from the

WDFW management areas.

The average domoic acid levels in these razor clam samples are shown in Figure 1. These levels are a compilation of analyses done by our laboratory as well as the laboratories of the Washington state Department of Health and the U.S. Food and Drug Administration. The figure compares the overall average domoic acid level in razor clams at all WDFW management areas and domoic acid levels in clams at the Kalaloch beaches, located about 45 Km north of the WDFW beaches. The highest levels of domoic acid reported in razor clams occurred in the first week of December 1991 with the levels declining to below the closure level (20 ppm) by the summer of 1992. Levels of domoic acid at Kalaloch beach appeared to parallel values observed at all other beaches to the south until January of 1993, when a significant divergence occurred, i.e. Kalaloch beach razor clams continued to have much higher levels of domoic acid than the other sampling areas. In September of 1994 and event occurred at the Kalaloch beaches where razor clams contained domoic acid of 56 ppm and continued at levels of about 20 ppm well into 1995. The reason for the higher levels of domoic acid in razor clams at the Kalaloch beaches is not yet understood.

An attempt to identify a causative species of domoic acid production was made by microscopic analysis of water samples obtained from the same areas where clams were harvested. Examination of water samples has not revealed large numbers of *Pseudo-nitzschia* species from either the WDFW management areas or the Kalaloch beaches. The *Pseudo-nitzschia* species isolated from Twin Harbors and Kalaloch were analyzed by specific oligonucleotide probes (Scholin et al, 1996) resulting in identification of the samples as *P. pungens*, demonstrated by other laboratories to be presumably a non-toxic species.

Dungeness Crabs

Since domoic acid was observed in razor clams and anchovies, public health officials decided to analyze for domoic acid in other seafoods of commercial and recreational interest. Our laboratory has analyzed raw or uncooked Dungeness crabs taken from fishery sites along the Oregon and Washington coast. In December 1991, we found domoic acid levels ranging from 1 to 90 ppm in crabs taken from Grays Harbor, and levels ranging from 11 to 78 ppm from crabs taken inside Willapa Bay. A year later, in 1992, samples of Dungeness crab from the Oregon coast revealed lower concentrations of domoic acid in crab taken along the northern coastal areas (range: 0-15), while the southern sites had higher levels (range: 0-78 ppm, Wekell, et al, 1994). In feeding studies conducted in our laboratory, we found that domoic acid is retained in the live Dungeness crab soley in the hepatopancreas. During processing, i.e., cooking in boiling water, the domoic acid content of the hepatopancreas is reduced by 60% (Hatfield et al, 1995). Subsequent storage, either at refrigerated temperatures or freezing, permits the remaining water soluble domoic acid to diffuse into body tissues adjacent to the visceral cavity (<3 µg/g).

Current Work

The exact origin of domoic acid in Oregon and Washington waters is still unclear. While the presence of *P. australis* was confirmed in Monterey Bay in 1991, large numbers of any *Pseudo-nitzschia* species were absent from surf samples from Washington state beaches at times when high levels of domoic acid were observed in razor clams and Dungeness crabs during the same year. Intensive sampling by our laboratory in 1995, indicated that the predominant *Pseudo-nitzschia* species appears to be *P. pungens*. Isolates from the open coast at the Twin Harbors area and Point Whitney in Hood Canal, were identified as *P. pungens* by electron microscopy and molecular probes (Scholin et al, 1996). When these isolates were grown in a 12hr/12hr Light/Dark at 13∘C in f/2 medium, they readily produced domoic acid. Domoic acid production by these cultures was detected by both radioligand binding assay (Van Dolah et al, 1994) and the FMOC-HPLC method (Pocklington et al, 1989) resulting in values similar to those reported for *P. multiseries*, i.e., up to 150 femtograms/cell.

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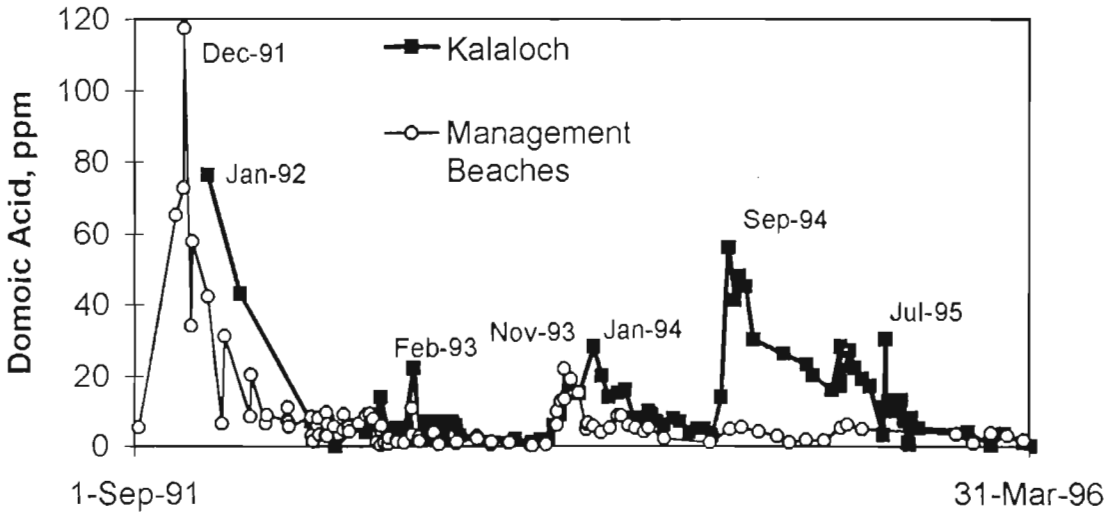
Figure 1. Domoic Acid Levels in Razor Clams at Kalaloch Beaches and WDFW Management Beaches



DOMOIC ACID IN MEXICO

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Abstract

For the last 20 years, several "red tides" have been observed in the Pacific coastline of México (Cortés Altamirano and Nuñez-Pastén, 1992; Sierra-Beltrán *et al.*, 1996a; Ochoa *et al.*, 1996). Both, toxic and non-toxic events appear to be a regular phenomenon in the zone and yet the country lacks of an effective systematic monitoring program. The extension of the Mexican coastline, the difficulties to access it, and the lack of human and material resources, have made it impossible to implement control measurements that could have prevented the number of human casualties experienced throughout this period (533 poisonings, 16 deceased). In addition to its human impact, one cannot neglect the environmental effect that this kind of events have shown provoking the death of underestimated numbers of fish, marine animals and sea-birds. A recent example, constitutes the corroboration of the presence of Domoic Acid in the Peninsula of Baja California that killed over 150 pelicans, and which adds to the list of marine biotoxins (PSP, DSP, CTX, TTX) already detected in this region.

Introduction

Mexican aborigines appear to have been familiar with the incidence of "red tides" along the coastline of the country before the conquest. According to reports from Alvar Nuñez Cabeza de Vaca on a XVI century edition (Shipwrecks), the Indians avoided the eating of shellfish after a visible "red tide" event as a means to prevent gastrointestinal diseases. The report makes reference to the death of four Spaniard soldiers who disregarded the recommendations of the Indians. Furthermore, with few exceptions, Indians usually eat the callus and not the viscera of the mollusks, thus avoiding exposure to pathogens and toxins accumulated in such tissues and reducing the risk of a disease.

Only recently the microorganism, or fish, associated with "red tides" , or poisoning events, in the Pacific coast of México have been identified (Table 1). Blasco (1977) and Turrubiates-Morales, (1992) describe *Gonyaulax polyhedra, Ceratium furca, Prorocentrum micans, Ceratium dens, Gonyaulax digitale and Gymnodinium Sanguineum,* as the main dinoflagellate components in "red tides" events observed in the West coast of the Baja California Peninsula. While *Prorocentrum sp., Alexandrium sp., Noctilluca scintillans* and *Phaeocystis sp.* are more typical in the Gulf of California (Sierra-Beltrán- et al, 1996a; PROFEPA, México, 1995). In addition, outbreaks of ciguatera and TTX poisoning have been registered in the Peninsula of Baja California in recent years in connection with consumption of contaminated fish

of *Serrinadae* and *Labridae* family (Lechuga-Deveze C., and Sierra-Beltrán, A, 1995), as well as with puffer fish (*Sphaeroides annulatus, S. Lobatus, Arothron meleagris, Lagocephalus laevigatus* and *Canthigaster punctatissima*; unpublished). *Mesodinium rubrum*, *Protoperidinium sp.* which are non toxic species, and *Gymnodinium catenatum*, as well as *Gonyalaux monilata*, associated with PSP (Paralyzing Shellfish Poisoning), have been frequently linked to harmful algal blooms in Mazatlán Bay (Cortés-Altamirano and Nuñez-Pastén, 1992), and in the South of the Pacific, at the states of Oaxaca (Saldate-Castañeda *et al.*, 1991) and Guerrero (Cortés-Altamirano *et al*, 1993), *Gymnodinium catenatum* and *Pyrodinium bahamense* have been notoriously associated with the larger number of human casualties so far reported in connection with a "red tide" phenomenon in México (Mee et al, 1986; Saldate-Catañeda et al, 1991; Colmenares and Barradas, 1996).

We may include now to the above list, a diatom, *Pseudonitzschia* sp., and the fish *Scomber japonicum*, mackerel, as the vehicles for Domoic acid, the toxin responsible of the Amnesic Shellfish Poisoning syndrome that became famous after the incident in Prince Edwards Island, Canada, in 1987. However, and as occurred in Monterey and in Santa Cruz Bay, California, US, on 1991 and 1993, this time the local pelican (*Pelecamus occidentalis*) community at Cabo San Lucas, México were the victims (Sierra-Beltrán *et al.*, 1996b).

Materials and Methods

Pelicans were collected from a site chosen apparently as cemetery by the sea-bird themselves since it is not the colony, nor the reproduction site, preferred by the pelicans. The mackerel fish came from catches carried out in the area were the pelicans were observed feeding. Both animals were dissected at the Marine Pathology Laboratory of this Center and extracts prepared from digestive tract and/or whole fish meal, as reported (Sierra-Beltrán *et al.*, 1996b). One dead pelican found in La Paz, Bay of normal causes, served as reference.

Mouse bioassay and HPLC analysis for Domoic acid was done as recommended by the IOC (International Oceanographic Commission, Training Course Report No. 29, Annex IV, pp. 5-6; and IN: Amnesic Shellfish Poisoning, ASP, HAB Publications Series, Vol. 1, Manual and Guides 31. UNESCO).

Results and Discussion

Following the events of human casualties in Eastern Canada on 1987 (Bird *et al*, 1988) attributed to the effect of a toxic aminoacid, namely Domoic acid, DA, and of the sea-bird mortality at Monterey Bay on 1991, (Work *et al.*, 1991, 1993), the most recent incident of DA outbreak causing a massive sea-bird mortality intoxication occurred off the Pacific coast of México, at Cabo San Lucas, in the tip of the Baja California Peninsula (Fig 1) on January 1996, as concluded from the results shown in Table 2 and Fig. 2. This is the first time that the sudden death

of pelicans in México is explained since the phenomenon seems to have been observed before in the Peninsula of Baja California, although then the causes remained obscure (Morales-Chávez *et al*, 1994).

Domoic acid is a excitatory tricarboxilic aminoacid which has been known for about 40 years and to which some insecticide and antihelminthic activity has been ascribed. More recently, the syndrome expressed by persons intoxicated with DA, which includes some mutagenic actions as well as gastrotoxicity, has been designated as Amnesic Shellfish Poisoning, ASP, mainly due to its effect on the hyppocampus that causes memory loss. Domoic acid, was named after a Japanese seaweed, *Chondria armata domoi*, from which was first isolated; later, it has been also found *in Chondria baileyana* and in *Alsidium corallinum* (for review see: Todd, 1990). Very recently has been reported in diatom species previously considered harmless such as *Pseudonitzschia pungens f. multiseries*, *P. pseudodelicatissima*, *P. australis*, *P. seriata*, *Nitzschia actydrophila*, and *Amphora coffaeiformis* l (for review see: Wright and Quilliam, 1995 and ref. 7).

No obvious algal bloom in the event registered in México early this year was observed. Yet, as in the cases of Monterey and Santa Cruz, CA, US, the pennate diatom *Pseudonitzschia* sp., is the suspected toxin source since it was found in the stomach content of contaminated mackerel fish, which were presumably eaten by the sea-birds affected. Also, the extract obtained from the pelican stomach showed a clear ASP syndrome in mice, with the characteristic scratching response.

At the time of the event, there were some local conditions that could have brought some upwelling into place, but this is only an appreciation resulting of climate charts and not from any direct observations or measurements. From such an incident we have learned that México is not a DA-free country anymore; that monitoring of DA in the human food chain should comprise the mackerel fish; and, also, that local resources to carry out a sound monitor program are limited.

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Table I.- Harmfull events occurred in the Pacific Coast of Mexico during the last 20 years.

TIME	PLACE	ORGANISM	EFFECTS	TOXIN DETERMINED	REF.
1976 / Nov	Acapulco, Gro.	Unknown	7 humans / 2 deaths	Not done	SSA
1979 / Apr	Mazatlan, Sin.	*Gymnodinium catenatum*	19 humans / 3 deaths	Sax	9, SSA
1984 / Jun	La Paz, B.C.S.	*Lutjanus sp.* fish	200 humans	Ciguatoxins	12
1985 / Nov	Acapulco, Gro.	Unknown	5 humans / 2 deaths	51µg Sax / g lioph. cells - 1720 µg Sax / 100 g shellfish	SSA
1989 / Dec	Salina Cruz and Huatulco, Oax.	*Pyrodinium bahamense var. compressum* and *G. catenatum*	99 humans / 3 deaths, several turtles and fish	380-570 µg Sax / 100 g shellfish	14
1991 / Nov	Bahía Concepción, B.C.S.	?	several tons of shellfish lost	Not done	@
1992 / Jan & Apr	Ojo de Liebre, B.C.S.	?	several tons of shellfish lost	Not done	@
1992 / Jun	Ensenada, B.C.	Unknown	110 pelicans	Not done	
1992 / Oct	Bahía Magdalena, B.C.S.	Dinoflagellate	dolphins, sea lions, sea birds, fish & turtles	Not done	@
1992 / Nov	Pto. Madero, Chis.	*Pyrodinium bahamense var. compressum*	2 humans / 1 deaths	45 mg Sax / 100 g shellfish	6
1993 / Apr	La Paz, B.C.S.	*Oscillatoria sp.*	none	Hepatotoxins	@
1993 / May	Bahía Concepción, B.C.S.	*Oscillatoria sp.*	none	No toxic	@
1993 / May	Alijos Rocks, B.C.S.	*Serranidae & Labridae* fish	7 humans	Ciguatoxins	8, @
1994 / Apr	Acapulco, Gro.	*Gymnodinium sp.* and *Gonyaulax sp.*	none	57-93 µg Sax / 100 g shellfish	SSA
1994 / Apr & Jun	La Paz, B.C.S.	*M. rubrum*	none	No toxic	@
1994 / Jun	San Hipolito, B.C.S.	*Gymnodinium sanguineum*	fish & sea birds	No PSP	@
1995 / Jun	Vizczino, B.C.S.	*Sphaeroides sp.*	2 humans / 2 deaths	Not done	SSA
1995 / Oct	La Paz, B.C.S.	?	Fish (Ballistidae)	Highly potent liposoluble toxin	@
1995 / Jan	San Felipe, B.C,S,	*Phaeocystis sp.* and *Noctiluca scintillans*	birds and sea mammals: whales, dolphins,seals, etc. (more than 900)	Not done	13
1995 / Dec	Acapulco, Gro.	*Pyrodinium bahamense var. compressum*, and *Gonyaulax catenella.*	192 humans / 3 deaths	598-3091 µg / 100 g shellfish	4, SSA
1996 / Jan	Atil, Son.	*Microcystis*/LPPB	fish	Mucus & scum	@
1996 / Jan	Cabo San Lucas, B.C.S.	*Pseudonitzschia sp.*	brown pelicans	Domoic acid	11, 16, @
1996 / Feb	Loreto, B.C.S.	*Noctiluca scintillans & Pseudonitzschia sp.*	none	No toxic	@
1996 / Feb	Sta. M. del Oro, Nay.	Cyanobacteria LPPB	fish	Oxygen depletion	@
1996 / Mar	Cabo San Lucas, B.C.S.	Cyanobacteria LPPB & *Chatonella sp.*?	bentonic fish	? Probably secondary infection.	@

SSA: Health Ministry @: Events assisted by CIBNOR

Table II. Results obtained with specimens collected at the onset of the event in Cabo San Lucas, B.C.S., Mexico. January, 1996.

Species/Locality	Organ/Tissue	Specimen Weight (g)	Mouse bioassay	HPLC Conc. (µg/g)
Pelecanus occidentalis/ Cabo San Lucas	Liver	38	Scratching, salivation,tail wiggling, incoordination	Traces
Pelecanus occidentalis/ Cabo San Lucas	Digestive tract contents	5	Scratching, salivation,tail wiggling, incoordination, diarrhoea, death.	37.17
Pelecanus occidentalis/ Cabo San Lucas	Liver	45	Scratching, incoordination	Traces
Pelecanus occidentalis/ Cabo San Lucas	Digestive tract contents	11	Scratching, incoordintion	Traces
Pelecanus occidentalis/ La Paz (Negative Control)	Digestive tract contents	2	Negative	Negative
Scomber japonicum/ Cabo San Lucas I	Digestive tract contents	3	Not done	142.85 @
Scomber japonicum/ Cabo San Lucas II	Digestive tract contents	15	Not done	Negative

@ This figure represent 4.76 µg / fish on average.

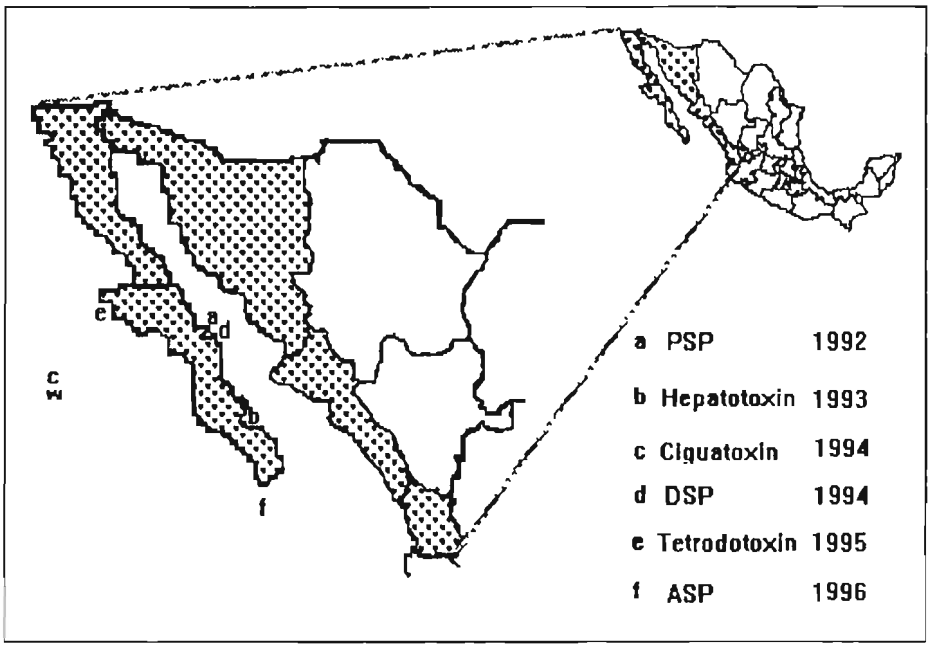

FIG.1.- Marine Biotoxins already detected at the NorthWest Pacific Coast of Mexico depicting the place and year of confirmation. Note the presence of DA at the tip of the Peninsula, a naturally recurring upwelling area.

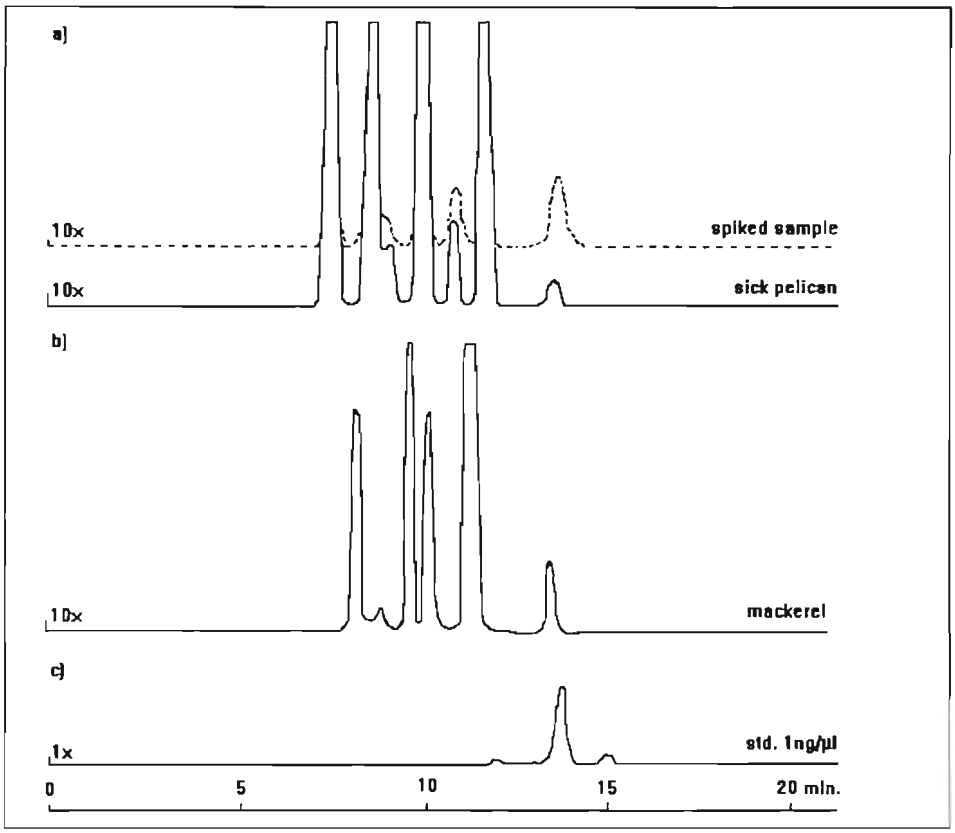

Fig. 2.- HPLC analysis was performed according to Quilliam's method (Ref. 7). Pure domoic acid was added to the sick pelican extract to confirm the authenticity of the peak obtained before spiking the sample. Negative control pelican as well as uncontaminated fish (sardine) did not show any peak corresponding to DA mobility (not shown). Gain: 1x and 10x absorbance at 242 nm. Retention time in minutes.

DIARRHETIC SHELLFISH POISONING TOXIN STUDIES IN SINGAPORE

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ABSTRACT

The purpose is to develop diagnostic and monitoring capabilities for the presence of diarrhetic shellfish poisoning (DSP) toxins in Singapore. DSP is a gastrointestinal illness caused by the consumption of shellfish contaminated by toxins produced by dinoflagellate species which have been ingested by the shellfish. It is a serious problem in Europe, Japan and North America. In Southeast Asia it has only been reported from the Gulf of Thailand. The disease may have not been detected in Singapore and the region because it is not fatal and the symptoms are easily mistaken for gastroenteritis caused by bacteria or viral contamination from shellfish. Okadaic acid and its analogues, the dinophysistoxins (DTX 1-4) are the main DSP toxins. They cause diarrhea, nausea, vomiting, abdominal pain and chills. It has been claimed to cause tumours and long-term memory loss. They are heat tolerant, thus will not be degraded by cooking the contaminated food. At the moment the methods developed to detect these DSP toxins are by using HPLC and Mass Spectroscopy which are expensive equipment not many could afford. One of the aim is to develop a biochemical test that is sensitive, cheaper and faster and can be used for bulk assays. It has to be relatively safe. The ^{32}P assay for DSP toxins was used and developed. The method relies on the inhibition of protein phosphatase (PP1 and PP2A) activities by the DSP toxins such as okadaic acid. The substrate for the PP2A was the ^{32}P-labeled glycogen phosphorylase-a. Pure and homogenous PP2A enzymes are expensive hence one of the adaptations was to use chicken brains as a source of PP2A. The other disadvantage of this assay method is the disposal of the radioactive materials. Therefore an alternative method which does not utilise isotopes was explored. This was a spectrophotometric assay which can detect the release of inorganic phosphate from para-nitrophenyl phosphate (*p*-NPP) by PP2A. We have modified the *p*-NPP assay into a 96 well titreplate format that can detect nM concentrations of homogeneous okadaic acid or about 0.04 µg/g of shellfish meat. This method, however, is not as specific and as sensitive as the radioisotope method. A cheaper source of PP2A was from the green mussel *Perna viridis* using a faster technique of preparation was tested. Further optimization is needed to improve the sensitivity. At the moment it is 50 fold less sensitive than the ^{32}P assay because of the slight yellow coloration of the PP2A extract.

Green mussels were collected from three locations in Singapore (once a month from Changi and Sungei Buloh and twice a month from Ponggol) and their digestive glands extracted for toxins which were then measured by a fluorescence HPLC after ADAM derivatization. Mussel samples were monitored from October 1995 to April 1996. No DSP toxins were detected so far. To prepare for future health regulations and strategies for protection of the public, the

uptake and depuration under artifical experimental conditions of the green mussel fed with a DSP toxin producing dinoflagellate, *Prorocentrum lima,* were studied. The asymptotic growth phase of this protozoan produces more toxin than at it exponential growth phase. Maximum toxic content was obtained after 12 hours of feeding and it depurates faster during lower salinities. Why this is so is not known. Gut clearance is a bit slower if DSP toxins were present. The data collected showed that within 48 hours all ingested DSP toxin produced by *P. lima* and concentrated within the digestive glands of the green mussel were completely evacuated. No DSP toxins were detected in the mantle nor the foot of the green mussel indicating that the toxins were not absorbed to other parts of its body. This showed that DSP toxins can be easily cleared just by placing the contaminated mussels in clean fresh seawater.

INTRODUCTION

This paper reviews the work done in Singapore on Diarrhetic Shellfish Poisoning (DSP) toxins. DSP is caused by the consumption of shellfish contaminated with toxins produced by certain dinoflagellates which are found in tropical coastal estuarine waters.The green mussel, *Perna viridis,* commonly consumed by the Singapore population, are cultured in the coastal waters of Singapore. Thus it is a precautionary measure to develop the capability to detect DSP in mussels produced in Singapore waters. The present Singapore studies cover three main objectives. The first was to develop assay methods for DSP toxins (mainly okadaic acid). Three assay methods, ^{32}P, *p*NPP assay and fluorescence high performance liquid chromatography methods were developed. The second objective was to monitor for DSP toxins in Singapore coastal waters using the mussels cultured in these waters. The third objective was to study the uptake and depuration behaviour of DSP toxins by the green mussel. A laboraory culture of the toxic dinoflagellate, *Prorocentrum lima*, was used as the source of DSP toxins.

ASSAYS FOR DSP TOXINS

^{32}P Assay

Okadaic acid and its analogues, the dinophysistoxins (DTX 1-4) are the major DSP toxins. These toxins inhibit three of the four known classes of serine/threonine protein phosphatases, PP1, PP2A and PP2B. Inhibition of protein phosphatases is thus used as a measure for the presence of DSP toxins. The ^{32}P assay method depended upon detecting the release of ^{32}P from ^{32}P-labelled glycogen phosphorylase-a by purified PP2A or PP1. PP1 and PP2A are expensive, unstable and time-consuming to purify. Sim and Mudge (1993) overcame these problems by using crude chicken brain extracts as a source of protein phosphatases for detecting and quantifying microcystins from freshwater cyanobacteria. We modified this microcystin assay of Sim and Mudge (1993) to detect the DSP toxins.

Okadaic acid (OACS-1) was purchased from the Marine Analytical Chemistry Standards

Program, Institute of Marine Biosciences, National Research Council of Canada. ^{32}P-ATP (10 mCi.ml^{-1}) was obtained from NEN and ^{32}P-labeled glycogen phosphorylase-a was prepared from glycogen phosphorylase-b essentially as described by Cohen et al. (1988) using the "Protein Phosphatase Assay System" from Gibco BRL. Approximately, 3 ml of a 3 mg.ml^{-1} solution of ^{32}P-glycogen phosphorylase-a was prepared and stored in 1 ml aliquots at 4°C and used within two weeks. This amount of ^{32}P-labeled substrate was sufficient for about 30 replicate assays. A crude mixture of protein phosphatases was prepared by homogenizing chicken forebrains from 3-5 day old chicks essentially as described by Sim and Mudge (1993).The homogenate was centrifuged at 500 x *g* for 10 minutes and the resulting supernatant diluted several fold to a final protein concentration of about 0.01 mg.ml^{-1}.

This amount of extract produced less than 30% dephosphorylation of the substrate which indicates that the reaction was linear with time and that the activity was proportional to enzyme concentration (Cohen et al., 1988). The protein concentration was estimated using BSA as the standard (Bradford,1976). The concentrated chicken brain extract was stored frozen in 1 ml aliquots at -20°C for up to 2 months. Extracts from the forebrain of one chick were sufficient for about 200 assays. Each assay (30 µl) contained 10 µl of diluted chicken brain extract, 10 µl of ^{32}P-labeled substrate and 10 µl of sample (okadaic acid or *P. lima* extracts) dissolved in buffer (400 mM Imidazole-HCl pH 7.6, 2 mM EDTA, 2% β mercaptoethanol and 20 mg.ml^{-1} BSA).

The assays were commenced by the addition of the labeled phosphorylase-a to the reaction vial, allowing about 30 seconds between vials. The reaction was allowed to continue for 10 minutes at 30°C and stopped by the addition of 90 µl of 20% (v/v) trichloroacetic acid. The vials were placed on ice for at least 10 minutes, then centrifuged at 1,200 x *g* for 10 minutes at 4°C. The radioactivity in 100 µl of clear supernatant from each vial was measured in a scintillation counter.

Culture of *Prorocentrum lima* and DSP Extraction

A New Caledonian strain (P6) of *Prorocentrum lima* was batch cultured in f_{10k} nutrient medium (Holmes et al., 1991) and cell extracts screened for DSP toxins using the ^{32}P assay. Stock cultures of *P. lima* were maintained in 250 ml Erlenmeyer flasks and mass cultures were grown in 2 litre Erlenmeyer flasks containing 800-1,000 ml of media. Cultures were grown at 24-27°C with a 12-12 light-dark photoperiod and 45-55 µmol.m^{-2}.s^{-1} of light from Sylvania Gro-Lux fluorescent tubes. Cultures were harvested after 6 weeks growth by vacuum filtration through Whatman GF/D glass fibre filters and quickly rinsed with 40 ml of de-ionised water to remove salt. Cells were disrupted in ice-cold methanol by sonication until microscopic observation revealed few remaining intact cells. The methanol extract was separated from the cell debris and glass fibres by centrifugation at 200 x *g* for 10 min. The methanol extract was filtered through Whatman GF/D filters, rinsed twice with 10-20 ml of methanol and the pooled extracts dried under reduced pressure using a rotary evaporator. The dried extract was weighed, dissolved in methanol in a 10 ml volumetric flask and stored at -20°C.

p-NPP assays

The need for radioisotopes can be a major impediment to the routine use of the above assay. Spectrophotometric assays have recently been developed which detect the release of inorganic phosphate from para-nitrophenyl phosphate (*p*-NPP) by PP2A (Takai et al., 1992; Simon and Vernoux, 1994). We therefore modified the *p*-NPP assay into a 96 well format which can detect nM concentrations of homogeneous okadaic acid or >0.04 µg of okadaic acid/g of shellfish meat.

Crude chicken brain extracts were initially used as a source of phosphatases for dephosphorylating *p*-NPP. Assays (90 µl) were carried out in triplicate in a 96 well microtitre plate. Each assay contained 60 µl of buffer (0.25 M sucrose, 5 mM Hepes pH 7.4), 10 µl of chicken brain extract and 10 µl of sample. Plates were incubated for 2 min at 37°C and then the reaction started by the addition of 10 µl of 5 mM *p*-NPP. The change in absorbance was measured at 405 nm every 30 s over 10 minutes using a Bio-Tek Ceres 900C microplate reader.

Assays using crude chicken brain extract were not reproducible, probably because of endogenous alkaline phosphatases in the extract. PP2A was therefore partially purified from the meat of local green mussels (*Perna viridis*). These mussels were shown to be free of okadaic acid and DTX-1 by fluorescence HPLC after derivatization of methanol extracts of the digestive tissue with 9-anthryldiazomethane (Lee et al., 1987; Marr et al., 1994). Mussels were dissected free of the digestive tissue and the meat (180 g) homogenized in 2.5 volumes of buffer-A (50 mM Tris pH 7.4, 2 mM EDTA and 2 mM DTT). All buffers and procedures except HPLC were carried out at 4°C. The homogenate was centrifuged at 15,000 x *g* for 15 min and the supernatant filtered through a Whatman GF/D filter. The protein was precipitated with 65% saturated ammonium sulfate and centrifuged at 15,000 x *g* for 20 min. The pellet was re-suspended in 100 ml of buffer-B (50 mM Tris pH 7.2, 0.1 mM EDTA, 1 mM DTT and 0.01% CHAPS) and the protein precipitated with 400 ml of ethanol. This was immediately centrifuged at 4,000 x *g* for 5 min and the pellet re-suspended in 200 ml of buffer-B. This volume was reduced by about half overnight in an Amicon ultrafiltration stirred cell (1,000 molecular weight cut-off). The remaining solution was centrifuged at 100,000 x *g* for 40 min and the supernatant chromatographed on a BioCad HPLC monitored at 215 and 280 nm. The supernatant (5 ml) was loaded onto a Bio-Rad Econopac (5 ml) Heparin cartridge in series with a Poros 20 HQ anion exchange column using buffer B at 1 $ml.min^{-1}$. After the 280 nm absorbance had returned to baseline (about 5 min) the Heparin cartridge was removed and the PP2A eluted from the anion exchange column at 4 $ml.min^{-1}$ using buffer-B for 1 min and then a linear gradient over 5 min to 1 M NaCl (in buffer-B) collecting 4 ml fractions. Fractions were assayed for activity (PP2A) at 405 nm using 5 mM *p*-NPP (PP1 is essentially inactive against *p*-NPP [MacKintosh, 1993]). The active fractions were pooled (20 ml) and stored in 1 ml aliquots at -80°C. These extracts were thawed and then diluted (by about half) with buffer-B before being used for assays. This amount of PP2A was sufficient for 1,300 single or 430 triplicate assays.

Kinetic assays were carried out in triplicate in a similar manner to those using crude

chicken brain extracts. The approximate volumes of the components used in the assay (total volume = 90 µl) were; 30 µl of PP2A extract, 40 µl of buffer C (40 mM Tris pH 8.1, 20 mM KCl, 30 mM $MgCl_2$ and 2 mM DTT) and 10 µl of sample (dissolved in 1% methanol or buffer). This was incubated for 2 min at 37°C and then the reaction started by the addition of 10 µl of 5 mM *p*-NPP and the change in absorbance measured at 30 s intervals over 10 min. The actual volume of PP2A extract used in the assay varied depending upon the enzyme activity to 5 mM *p*-NPP. PP2A extracts were diluted with buffer-C to produce control responses (100% activity) of 3-9 $mAU.min^{-1}$ over 10 min.These responses were linear over this period of time with $r^2 > 0.99$ in all cases. The reaction pH was approximately 8.0, which is near optimal for dephosphorylation of *p*-NPP by PP2A (Takai and Mieskes, 1991). Responses to 0.1 µM okadaic acid were considered maximal inhibition (0% activity) and values were adjusted accordingly as a percentage of control (100%) responses. The volume of methanol used in these assays had no effect on control responses.

Fluorescence High Performance Liguid Chromatography

Determination of DSP toxins by fluorescence high performance liquid chromatography (Lee et al., 1987) was also developed. To enhance the detection sensitivity of very small amounts (ng) of okadaic acid, a derivatization reaction with 9-anthyldiazomethane (ADAM) as a fluorescent labeling reagent was used. The methods described by Yoshida et al. (1988) and modified by Marr et al. (1994) were followed excepting that a 90 µl aliquot of sample extract or toxin standard and 10 µl of internal standard, deoxycholic acid (0.35 µg.100 µl-1 methanol; Pillet et al., 1995) were mixed with 900 µl of the ADAM reagent. After sample clean-up using Supelco solid phase extraction cartridges (Marr et al., 1994) the final fraction was dissolved in 200 µl of methanol for fluorescence HPLC.

Liquid chromatography analysis was performed on a Hewlett-Packard HP1050Ti HPLC with a HP1046A fluorescence detector. Separations were performed on a reverse-phase column (250 mm x 4 mm I.D.) packed with 5 µm LiChrospher-100RP18 (Merck, Phenomenex). Elution was performed at 26°C using a flow rate of 1.0 $ml.min^{-1}$, with 90% acetonitrile for 17 minutes, followed by a 1 minute gradient to 100% acetonitrile and a 10 minute wash with acetonitrile. The detector settings were 254 nm excitation, 412 nm emission protected by a 295 nm cut-off filter, and xenon lamp pulse frequency of 55 Hz.

MONITORING FOR DIARRHETIC SHELLFISH POISONING TOXINS IN GREEN MUSSELS

Mussels were collected once a month during low tides from Sunegi Buloh, and Changi and twice a month from a vendor who owns a mussel farm in Punggol from 18 October 1995 to 24 April 1996. These are locations where they are commonly found naturally as well as cultured. The digestive glands of the mussel were used for toxin extraction. The extraction procedure was similar to those used by Lee et al. (1987), Pleasance et al. (1992) and Marr et al. (1994).

Detection was by ADAM derivatization and fluorescence HPLC.

UPTAKE AND DEPURATION STUDY

DSP toxin in laboratory cultured *P. Lima.*

A ^{32}P protein phosphatase assay using chicken brain extracts demonstrated that the laboratory cultured *P. lima* did indeed produce inhibitory toxins which were subsequently confirmed by fluorescence HPLC to consist mainly of okadaic acid with no DTX-1 or -2. Maximum inhibition of the PP2A, the major protein phosphatase enzyme extracted from chicken brain, occurred at okadaic acid concentrations greater than 1 nM with an IC_{50} of about 0.1 nM. The limit for detection of okadaic acid using the fluorescence HPLC was 1 ng for the standard and 40 $ng.g^{-1}$ of the mussel tissue. The retention times for okadaic acid and deoxycholic acid (internal standard) were 9.4 ± 0.2 minutes (n = 35) and 18.5 ± 0.2 minutes (n = 64), respectively. No DTX-1 was detected from *P. lima* or any mussel extracts. Under these conditions DTX-1 elutes at approximately 13.9 minutes (Marr et al., 1994).

P. lima cell growth phase and toxin level.

During the 32-day culture period, *P. lima* cell populations grew exponentially up to day 24. Growth rate during the exponential phase was 0.2 $divisions.day^{-1}$ and the generation time, T_d = 4.8 days per division ($P<0.01$). This was followed by the stationary phase approximately 24 days after innoculation of the culture. The concentration of okadaic acid per cell increased significantly from an average of 2.6 ± 0.1 $pg.cell^{-1}$ (n = 3) during the exponential phase to 3.8 ± 0.4 $pg.cell^{-1}$ (n = 3) at the stationary phase ($P<0.05$).

Uptake and accumulation of DSP toxin in *P. viridis* digestive glands

P. lima was batch cultured up to the stationary phase and fed to green mussels, *P. viridis*, to examine DSP toxin accumulation and depuration. A preliminary experiment was first conducted to confirm that the green mussel *P. viridis* would feed on the toxic *P. lima* cells. The maximum toxin content in the digestive gland was 12 hours after the start of feeding with *P. lima*. Mean okadaic acid concentration in the digestive glands peaked at 0.19 ± 0.09 $\mu g.g^{-1}$ digestive gland (n = 3).

Depuration of *P. lima* DSP toxins in *P. viridis* at two salinity levels.

At the higher salinity of 30-35 ppt the mean okadaic acid concentration accumulated by the mussels was 0.28 ± 0.06 $\mu g.g^{-1}$ digestive gland (n = 3). When the mussels were transferred to clean seawater, the concentration of okadaic acid declined to undetectable levels 48 hours after the transfer to clean water (60 hours after feeding). A linear decline model suggests a daily rate of decontamination of 0.12 ± 0.03 μg okadaic $acid.g^{-1}.day^{-1}$ (n = 5). At the lower salinity of 20-22 ppt the accumulated mean okadaic acid concentration was 0.23 ± 0.07 $\mu g.g^{-1}$ digestive gland (n =

3). The mussels depurated at a daily rate of 0.23 ± 0.06 µg okadaic acid^{-1}.g^{-1}.day^{-1} (n = 3), which was nearly twice the rate at salinity 30-32 ppt.

Tissues from the mantle and foot of mussels were also screened for DSP toxins. No okadaic acid or DTX-1 was detected in these organs during the feeding and the depuration periods. Thus it appears that within the time period studied, the toxins were confined to the digestive glands of the mussel and that the toxins were not adsorbed by the mussel.

RESULTS AND DISCUSSION

The fluorescent HPLC method was used as a reference for the ^{32}P and *p*-NPP assay techniques. The 96 well, *p*-NPP assay developed in this work is proposed for future use in the rapid screening of DSP toxins from many shellfish samples per day. The major advantages of this assay are that it is simple, rapid, requires minimal sample clean-up, does not require radioisotopes and has a detection limit at least five-fold lower than the regulatory limit for DSP toxins. The major disadvantage of the assay is its relatively low sensitivity to DSP toxins from crude extracts compared to homogeneous toxins.

Twenty five green mussel samples were collected during the period of monitoring and no DSP toxins were detected in twenty four samples except for one sample from Punggol collected on 31 October 1995 which was positive but was unconfirmed. The presence of DSP organisms in Singapore waters thus need further investigation.

The depuration rate for this mussel species (0.12 - 0.23 µg okadaic acid.g^{-1}.day^{-1}), *P. viridis*, was slower than that for Mediterranean mussels (0.8 µg okadaic acid.g^{-1}.day^{-1}) contaminated with *Dinophysis spp.* (Marcaillou-Le Baut et al., 1993). Haamer et al. (1990), also observed faster depuration (0.6 µg okadaic acid.g^{-1}.day^{-1}) for Mediterranean mussels. Water flow, temperature, aeration and feeding are all factors which may affect the depuration process (Novaczek et al., 1992). *P. viridis* may be capable of depurating faster in the wild. In France, DSP contaminated mussels depurated faster when placed in outdoor ponds than those in laboratory conditions (Marcaillou-Le Baut et al., 1993). Depuration under natural conditions was not attempted in this study because of the danger of introducing a foreign strain of *P. lima* into Singapore waters.

Mussels transferred to a lower salinity (18 to 20 ppt) for depuration seemed to show a faster rate of decontamination. A study using *Mytilus edulis* contaminated with the amnesic shellfish poisoning toxin, domoic acid, also showed faster clearance of the toxins from the digestive glands as compared with the mussels transferred to seawater of normal salinity (Novaczek et al., 1992). It is unlikely that the faster decontamination observed in *P. viridis* was due to higher metabolism of the mussel since a sudden change in salinity would normally depress the metabolism of the mussels (Hawkins and Bayne, 1992). The effect of salinity stress on depuration of toxins is not well understood. *P. viridis* is an estuarine species that survives (as an

adult) in salinities between 12 and 45 ppt (Tan, 1973). Thus this may be the reason why depuration rate was faster at lower salinities.

No toxins were detected in the mantle and foot of *P. viridis* within the 60 hours of analysis. This shows that okadaic acid was not transported to these organs and that it tended to concentrate only in the digestive gland. *P. viridis* depurate okadaic acid possibly through gut clearance of the ingested dinoflagellates. Most of the depuration is completed in less than 48 hours. This study provides background information for local shellfish farmers, marketers and regulatory authorities. Since *P. viridis* is a local delicacy which is cheap and commonly consumed, monitoring of DSP toxins in these shellfish is important.

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ABSTRACTS

ORAL SESSION 5

TOXICOLOGY

NEUROTOXICITY OF REPEATED EXPOSURES TO DOMOIC ACID

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Domoic acid (DA), the causative agent of amnesic shellfish poisoning, is a demonstrated environmental neurotoxin to humans. Clinical evaluation of individuals exposed to DA in 1987 revealed decreased performance on delayed visual-spatial and verbal recall tests. Generally, brain nuclear groups are believed to absorb substantial injury prior to the observation of overt symptomatic effects. In this regard, low levels of toxicity may be silent until a threshold level of damage occurs, either through additional environmental exposures and/or biological events of the aging process. We have utilized a pulsed exposure protocol in laboratory mice to determine if DA induces cumulative adverse effects. The lowest observable effect level for DA was determined to be 0.5 mg/kg using c-fos as a biomarker of effect. Single doses (0.25 to 4.0 mg/kg) of DA were administered and serum levels determined to increase proportionally. DA was cleared rapidly (>95% in 2h) from the serum and excreted largely in the urine. A pulse exposure protocol was established in which animals received four doses, one every other day. DA clearance from the serum did not change between the first and fourth exposure indicating that prior exposure to DA did not alter the animal's ability to metabolize or clear the toxin. Symptomatology was quantified by the method of Tasker, *et. al.* (1991) in two strains of mice, the outbred ICR and the inbred DBA, a strain sensitive to induced seizures. DA given between 0.5 and 2.0 mg/kg caused a dose dependent increase in toxicity score in both strains with consistently higher scores in the DBA mice. However, the toxicity score did not change with the second, third, or fourth dose in either strain of mice. These results indicate that repeated doses of DA cause no apparent further symptomatic toxicity. Chronic toxicity was examined in the DBA mice using a battery of behavioral tests based on modifications of the Morris water maze. Significant impairment of working memory was observed at 1.0 and 2.0 mg/kg; however, the animals that received four exposures showed better performance than the animals receiving a single exposure. One possible interpretation of this data is that additional damage caused by the repeated exposures counteracted the poor test performance of the first exposure. Neurodegeneration in selected animals from the bahavioral study will be analyzed by cupric silver histochemistry to relate working memory performance in single and repeated exposures to regional neuronal damage.

FEEDING INHIBITION OF THE ROTIFER *BRACHIONUS PLICATILIS* CAUSED BY DOMOIC ACID

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Rotifers fed at satiation levels with toxic *Pseudo-nitzschia multiseries* demonstrated a high rate of toxin accumulation to a maximum of 76 μg/g at 27 h exposure. Continued feeding caused a marked decline to a minimum of 18.5 μg/g during the next 48 h of feeding after which assimilation reoccurred for the next 21 h of exposure. This toxin retention curve indicated either feeding inhibition or a metabolic equilibrium change of toxin retention in the tissue. Examination of fatty acids in the rotifer tissue during intoxification illustrated a retention of dietary algal fatty acids in the rotifer that emulated the toxin retention and indicated feeding inhibition. Corroboration of feeding inhibition was demonstrated by eradication of fecundity in rotifers fed toxic *Pseudo-nitzschia multiseries* relative to that provided from feeding rotifers the non-toxic *Pseudo-nitzschia pungens*, although both forms of the alga had similar nutritional values.

RETENTION AND PHYSIOLOGICAL EFFECTS OF DOMOIC ACID IN SOME BRITISH COLUMBIA BIVALVES FED CULTURED *PSEUDO-NITZSCHIA MULTISERIES*

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Pacific bivalves exposed to toxic *Pseudo-nitzschia multiseries* were the Pacific oyster (*Crassostrea gigas*), the Manila or Japanese littleneck clam (*Venerupis philipparum*), the California mussel (*Mytilus californianus*), and the spiny scallop (*Chlamys hastata*). Initial exposure to the alga resulted in rapid accumulation of toxin in the tissue. With continued exposure, toxin levels peaked then declined to provide either sinusoidal or steady state retention curves. In general, levels attained by the bivalves reflected the cellular domoic acid content of the alga. Complete closure of the oyster occurred from high toxin accumulation and physiological response to the toxin was demonstrated by changes to blood chemistry and haemocyte activity. No adverse physiological effects have been noted for either the clam or mussel. Motor responses in the scallop were severely diminished on exposure to the alga and mortalities occurred with high toxin inclusion. With clearance of toxin at lower levels of inclusion the reflex response in scallops recovered with loss in body burden of domoic acid. Exponential rates of clearance were demonstrated in all bivalves examined.

KINETICS OF DIARRHETIC SHELLFISH TOXINS IN THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*

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INTRODUCTION

Diarrhetic shellfish poisoning (DSP) occurs worldwide and is considered a serious global threat to the development of commercial shellfish industries, as well as posing a significant risk to public health. Bivalve molluscs, including scallops, can acquire and sequester DSP toxins by filtering toxic dinoflagellates from either the water column or from benthic seston. Dinoflagellates implicated as DSP toxin producers include several planktonic *Dinophysis* spp. and a few benthic/epi-benthic species of *Prorocentrum* (Lee et al., 1989). The severe gastroenteritis syndrome characteristically associated with the consumption of DSP-contaminated shellfish occurs due to the protein phosphatase inhibition activity of the DSP toxin complex, including okadaic acid (OA) and its dinophysistoxin derivatives (DTX 1-3) (Yasumoto et al., 1985). Recent studies have used LC-MS to identify naturally-occurring esters of OA in *P. lima*, which can be rapidly transformed to OA via enzyme-linked hydrolytic reactions (Quilliam et al., 1996).

The relation of DSP toxin incidence in bivalve molluscs to planktonic blooms of *Dinophysis* spp. has been frequently documented, however attempts to describe DSP toxin kinetics in shellfish have been rare. The present study investigated the dynamics and metabolic fate of DSP toxins in the bay scallop, *Argopecten irradians*, when fed cells of the epibenthic dinoflagellate *Prorocentrum lima*, a known producer of DSP toxins, in controlled laboratory microcosms. Toxin kinetic parameters determined include uptake rates, compartmentalization, biotransformation pathways and detoxification times.

METHODS

Pre-reproductive adult bay scallops (mean shell ht. = 39mm) were fed a DSP-toxigenic strain of *Prorocentrum lima* (mean cellular toxin content = 10.0 pg OAeq. $cell^{-1}$), isolated from Mahone Bay, NS (Jackson et al., 1993). Scallops were exposed to cells of *P. lima* harvested in late-exponential phase and continuously metered into an 80L recirculating aquarium tank at a rate adjusted to yield a relatively constant cell concentration of 10^5 cells L^{-1}. Clearance rates and absorption efficiencies of scallops were determined in flow-through feeding chambers to examine the effects of prolonged DSP toxin exposure on physiological feeding parameters. Following two weeks of toxin exposure, scallops were depurated for three weeks on a diet of the non-toxic

diatom *Thalassiosira weissflogii*.

Scallops were frequently removed during both the toxin exposure and depuration periods for toxin analysis. Five tissue pools were excised to examine anatomical compartmentalization toxins within scallops: viscera; gonad; gills; mantle; and adductor muscle. Tissue and algal extracts were analyzed by liquid chromatography combined with ion-spray mass spectrometry (LC-MS) for OA, DTX-1 and OA-esters. Biotransformation pathways of DSP toxins were also examined in vitro, by incubating purified DSP toxins with scallop viscera homegenates in the presence and absence of ruptured *Prorocentrum lima* cells.

RESULTS and DISCUSSION

Bay scallops ingested DSP-toxigenic *Prorocentrum lima* cells at relatively constant rates (1.6×10^6 cells d^{-1} g^{-1} wet wt.) throughout the two week exposure period. No mortalities occurred during the toxin exposure period, nor was feeding behaviour inhibition observed (eg. - valve closure, clapping). At concentrations of approximately 100 cells mL^{-1}, bay scallop clearance rates were similar when exposed to *P. lima* compared to equivalent biovolume cell concentrations of *Thalassiosira weissflogii*, a non-toxic diatom and proven suitable food source for bay scallops. Pseudofeces production and feeding inhibition behaviour was only observed at very high cell concentrations of *P. lima* (> 400 mL^{-1}). The efficiency of organic matter absorption during passage of cells through the gut decreased significantly from 80% to 62% when scallops were changed from a diet of *T. weissflogii* to toxic *P. lima*. Absorption efficiency remained relatively constant throughout the toxin exposure period, during which time a high abundance of viable *P. lima* cells were observed by microscopy in fecal ribbons of scallops. After one week of depuration, clearance rates had increased significantly while absorption efficiencies remained at levels observed during toxin exposure.

Uninhibited ingestion of toxic *P. lima* cells resulted in a rapid toxin increase in scallop tissues during initial toxin exposure (Fig. 1). After only 18 hours of exposure to *P. lima*, toxin levels in viscera tissue had exceeded commonly accepted DSP regulatory levels (0.2 μg^{-1} DSP toxin g-1 viscera). However, after two weeks of exposure, total toxin reatined in scallop tissues was very low (< 1%) compared to the amount of toxin ingested over the same period. Most of the total toxin body burden in the scallops was contained in the viscera (76%), however a significant portion was also associated with gonadal tissue (12%) which only comprised 5% of the total tissue wet weight in the pre-reproductive scallops. Toxin levels were relatively low in gill, mantle and adductor muscle tissues.

Rapid release of toxins within the first two days of depuration from all tissues except for viscera indicated that toxins were not tightly bound to these tissues. In the case of gonadal tissue, the relatively high DSP toxin levels observed during the uptake phase was most likely derived from recently ingested *P. lima* cells in the gut contents of the intestinal loop which passes through the gonads. These results suggest that although gonadal tissue can become unfit for human

consumption during exposure to DSP-toxigenic microalgae, this compartment can also be detoxified very rapidly by evacuation of gut contents via fecal deposition during depuration. Detoxification of visceral tissue was biphasic, comprised of a rapid release of 30% of the total toxin load within the first 16 hours of depuration, followed by a slower loss of remaining toxin at a rate of 8.4% d^{-1}.

Toxin composition of scallop tissues was significantly different from that of ingested *P. lima* cells. While DTX-4, a water-soluble ester derivative of OA (Quilliam et al., 1996), was a major DSP-toxin component in *P. lima* cell extracts, this compound was not detected in scallop tissues when analyzed by negative ion-spray LC-MS. Moreover, the greater proportion of OA and OA-diol ester derivatives in scallop tissues compared to *P. lima* extracts suggested that DTX-4 is rapidly hydrolyzed to OA-diol ester and OA via enzyme-linked pathways upon digestion of *P. lima* cells within the gut of scallops. *In vitro* incubations of purified DTX-4 with scallop viscera homogenates indicated that hydrolysis of DTX-4 to OA-diol ester and OA occurred very slowly in the presence of only scallop viscera enzymes. However, in the presence of ruptured *P. lima* cell contents, hydrolysis of DTX-4 to OA-diol ester was very rapid, followed by a slower conversion of OA-diol ester to OA. Thus, it appears that *in vivo* bioconversions of DSP toxins within scallops occurs as a result of the activity of endogenous *P. lima* esterases liberated upon disruption of *P. lima* cells by digestive processes within the scallop gut.

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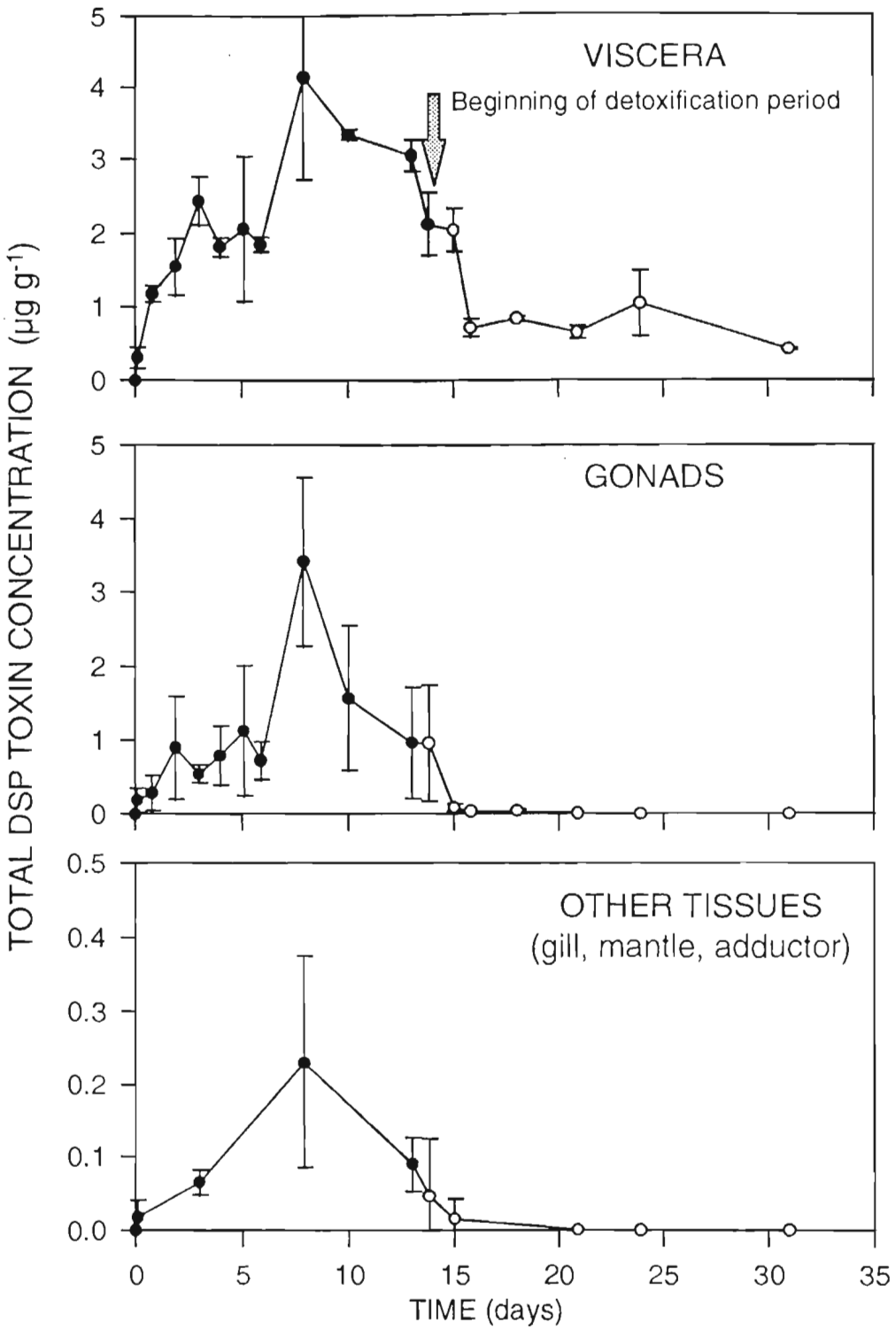

Fig. 1. Temporal pattern of DSP toxin uptake (solid circles) and loss (empty circles) in *Argopecten irradians* tissues during two week exposure to *Prorocentrum lima*, followed by a 3 week detoxification period. Error bars represent ± 1 standard deviation (n = 3).

ABSTRACTS

ORAL SESSION 6

OVERVIEW OF RESEARCH PROGRAMS ON HARMFUL ALGAL BLOOMS

REVIEW OF PHYCOTOXIN RESEARCH IN WESTERN WASHINGTON

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While Washington state has a long history of harmful algal blooms (based primarily on the presence of toxins in shellfish), research on phycotoxins and the organisms that produce them has not kept up with the problems associated with these blooms even though they are a significant and increasing threat to human health and fisheries resources. HAB problems in western Washington are related to paralytic shellfish poisoning (PSP), known here since the early 1940s and caused by members of the dinoflagellate genus *Alexandrium*; large mortalities of pen-reared salmonids, a problem since the early 1960s and caused by the diatoms *Chaetoceros convolutus, C. concavicornis,* and possibly other species and the raphidophyte flagellate *Heterosigma akashiwo*; and domoic acid poisoning (DAP), present here since 1991 and probably caused by diatoms in the genus *Pseudo-nitzschia*. Diarrhetic shellfish poisoning (DSP) has not been detected here, but at least four species of the dinoflagellate genus *Dinophysis,* known to produce DSP in Europe and Japan, are present and sometimes relatively abundant in local waters.

A number of local state and federal agencies and organizations are working together to learn more about HAB problems in western Washington waters. Much of the cooperation among these groups stems from 1991 when domoic acid first occurred on the Washington coast. At that time, a series of meetings quickly identified the responsible state and federal agency personnel, university scientists, local Native tribes, and shellfish growers who had a stake in the problem. Discussions with scientists and shellfish growers from eastern Canada provided much useful information and general support. Additional shellfish, Dungeness crab, and phytoplankton monitoring began and, as a result, no substantiated accounts of human illnesses were identified. The recreational razor clam harvest was most severely impacted and the commercial Dungeness crab fishery was closed for a short time. Oysters never became toxic and there were no closures of the economically important coastal oyster industry. Unfortunately, east coast news media reported that "shellfish" were toxic and the public assumed that included oysters. Economic losses to the already depressed coastal area were near $20 million.

Agencies and organizations currently working on HAB problems in western Washington include:

1. Washington Department of Health (WDH). This agency has monitored toxins in shellfish since the 1940s. Sampling and testing has often been seasonal and/or sporadic, but in the last few years, mussel cages have been used at 33 sites. Mussels from the cages are harvested and tested on more or less regular schedules, especially during the spring and summer, but not always with the same frequency. Phytoplankton monitoring was done at 20 of these sites in 1993-94 and continues on a regular basis at 8 sites and at others when problems arise. One bonus of this phytoplankton monitoring program has been obtaining information on the distribution and relative abundance of all phytoplankton species in western Washington waters, not just the potential toxin producing species.

2. Washington Department of Fish and Wildlife (WDFW). This agency is responsible for the razor clam fishery and monitors their populations at a series of management areas on Pacific coast beaches. They have provided razor clams to WDH and the National Marine Fisheries Service for domoic acid testing and to the School of Oceanography for use in toxin depuration investigations. They collect phytoplankton samples for the School of Oceanography at the same time that razor clams are harvested for toxin analyses.

3. National Marine Fisheries Service (NMFS). This agency tests shellfish and crabs for domoic acid, using a variety of methods (HPLC, FMOC, receptor binding assay). Their main objective is to develop a model for domoic acid in razor clams and Dungeness crabs that will include seasonal domoic acid levels, clam-to-clam variability, and location of possible geographic hot spots. NMFS, with School of Oceanography personnel, hopes to identify the causative organism(s) and learn where they come from and how the razor clams and Dungeness crabs become intoxicated with domoic acid. Culture studies with *Pseudo-nitzschia* spp. are in progress to determine environmental conditions that affect domoic acid production. Dungeness crabs have been studied for their ability to take up and depurate domoic acid.

4. U.S. Food and Drug Administration (FDA). This agency monitors state activities through the Interstate Shellfish Sanitation Commission. Individual states set their own regulations, while FDA oversees interstate commerce.

5. School of Oceanography, University of Washington (SO). Personnel continue to collect samples and monitor for harmful phytoplankton species in order to identify the source of domoic acid in razor clams and Dungeness crabs; environmental data also are collected and analyzed. Phytoplankton samples from the WDH monitoring program, Native tribes, fish growers, and others who collect samples are analyzed for species present and their relative abundances. They operate a phytoplankton telephone hotline to obtain and provide information on phytoplankton blooms. A list of people from state and federal agencies, universities, Native tribes, shellfish and finfish growers, and interested local citizens is maintained in order to obtain and disperse information concerning algal blooms. Cultures of some toxic species are maintained. Laboratory studies with NMFS

determined how long razor clams retain domoic acid. Training programs for phytoplankton identification were run in Washington, Oregon, California, and Alaska.

6. School of Fisheries, University of Washington (SF). Investigators are studying *Chaetoceros* spp. and *Heterosigma akashiwo* that kill both pen-reared and wild fish. Investigations have concentrated on environmental conditions that affect blooms, whether a toxin(s) is produced, and how fish are killed.

7. Native tribes. Several coastal Native tribes do their own phytoplankton monitoring or provide samples to the School of Oceanography.

8. Other agencies (e.g., Washington Department of Ecology), shellfish growers, local port captains, and private individuals frequently call us when blooms occur at their sites, or provide us with samples. A rapid response group composed of university personnel (SO, SF) is available to collect and identify phytoplankton and determine environmental conditions at the time of a bloom.

9. Olympic Coast National Marine Sanctuary (NOAA). This organization provides ship time to obtain phytoplankton and other samples on the Washington coast. Sampling must be done within the Sanctuary, but that is where much of the domoic acid problem occurs. This participation is important because in the past, there has been no opportunity to obtain samples from offshore and it is thought that both PSP and DAP toxins in coastal shellfish come from offshore populations of *Alexandrium* and *Pseudo-nitzschia,* but there has been no proof.

10. Other issues/management problems
a. Subsistence fishery: this fishery exists along much of the Pacific coast of Washington and involves several self-governing Native tribes that are not subject to state regulations. They set their own rules and obtain shellfish testing through negotiation with the WDH.
b. Non-traditional shellfish harvest: this is primarily a problem with immigrant groups who harvest gastropods and other shellfish that are not regulated by the state. The harvest is increasing and is a growing concern for resource managers. Communication problems increase the risk because standard closure notices for PSP and DAP may not be understood, or, in the case of non-traditional shellfish, may not be posted where harvesting occurs.

REVIEW OF PHYCOTOXIN RESEARCH IN BRITISH COLUMBIA

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I will briefly review current research on harmful algal blooms and phycotoxin research in British Columbia. In the Department of Fisheries and Oceans, research is being carried out in the Aquaculture Division at the Pacific Biological Station on uptake, depuration and physiological effects of toxins, in particular domoic acid, in both commercially harvested shellfish as well as other marine organisms (J.N.C. Whyte). At the Institute of Ocean Sciences, studies in the Ocean Science and Productivity Division focus on processes associated with the development of blooms and on pathways of toxins through the marine foodweb (J.R. Forbes). As well, regular satellite monitoring is carried out to recognize high phytoplankton concentrations on the continental shelf and offshore waters (J.F.R. Gower).

In the University of Victoria, research is conducted on the physiology of toxin production in *Heterosigma*, and on its toxic effects on fish (E.A. Black, L. Hobson). At the University of British Columbia, research in the Department of Oceanography focuses primarily on taxonomy and systematics, as well as phytoplankton community dynamics, in particular harmful species, in areas where the salmon aquaculture industry is established (F.J.R. Taylor). In the Department of Food Sciences, studies are being done on biochemical indicators of PSP toxicity, in efforts to develop alternative testing methods (D.D. Kitts, D. S. Smith). At the Institute for Aquaculture Research at Simon Fraser University, research is continuing on modes of action of fish-killing phytoplankton, and on remedial measures, in particular development of feeds containing mitigating agents (L.J. Albright).

THE MARINE BIOTOXIN PROGRAM OF THE SOUTHEAST FISHERIES SCIENCE CENTER CHARLESTON LABORATORY

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The Marine Biotoxins Program conducts critical research and provides scientific guidance to promote the effective management of fisheries, public health, and ecosystem health on issues related to marine biotoxins and harmful algae. The Charleston Laboratory played a key role in development of the U. S. National Plan for Marine Biotoxins and Harmful Algae and the Marine Biotoxins Program developed upon commencement of the National Plan. The Program serves to foster the implementation of the National Plan through its research efforts, participation in the Interagency Panel on National Marine Biotoxins Initiatives and joint support with NSF of the U. S. Office for Marine Biotoxins and Harmful Algae. The Program provides U. S. Representation to the Joint IOC/FAO Intergovernmental Panel on Harmful Algal Blooms and serves on its aquatic Toxin Working Group. The Marine Biotoxins Program provides a complete coverage of marine biotoxins and harmful algae with expertise in all the major classes of seafood toxins that spans causative organisms, chemical structure, detection methods, and hazard identification.

ASPECTS OF PHYCOTOXIN RESEARCH AT THE NRC INSTITUTE FOR MARINE BIOSCIENCES

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Phycotoxin research is, and will continue to be, an important area of research at the Institute for Marine Biosciences (IMB). A multidisciplinary approach, involving marine biology, bioorganic and analytical chemistry, including NMR spectroscopy and mass spectroscopy, is focussed on four major research areas: (1) Studies on the physiology and growth of toxic algal species, including the identification of new toxic species, and modelling the kinetics of phycotoxin uptake and depuration in bivalve molluscs. (2) The isolation and characterization of new marine toxins and their derivatives, and how these toxins are biosynthesized in microalgae. (3) The development of new methods, mainly based on LC or CE coupled to mass spectrometry, for the detection of known toxins as well as for any new toxins as they are discovered, and the preparation and certification of analytical standards and reference materials that are distributed through the MACSP program. (4) The large scale purification and chemical inter-conversions of PSP toxins, and in cooperation with industry, investigations into new detection methods for PSP toxins. A brief overview of these research activities will be presented.

HARMFUL ALGAL BLOOM RESEARCH IN ATLANTIC CANADA

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Department of Fisheries and Oceans research in eastern Canada is directed from four institutes - Institute Maurice Lamontagne (Quebec), Gulf Fisheries Center (New Brunswick), St. Andrews Biological Station (New Brunswick) and Bedford Institute of Oceanography (Halifax). Areas of research include: phytoplankton monitoring and population dynamics; oceanography; phycotoxin production; toxin uptake, storage and depuration; fate and effect of toxins on the food web and improving methods for detecting toxins. A synopsis will be presented.

HARMFUL MARINE ALGAE RESEARCH IN NEWFOUNDLAND - PAST, PRESENT, AND FUTURE

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Detection of the effects of harmful algae on shellfish aquaculture in Newfoundland before 1994 had been limited to monitoring toxins in shellfish tissue by the Inspection Branch of the Department of Fisheries and Oceans. Preliminary investigations to detect harmful marine algae have been conducted in only two Bays in Newfoundland over a three year period 1988 - 1991 (McKenzie et al. 1991. Occurrence of microplankton species in the water column and shellfish toxins in tissues of the giant scallop, Placopecten magellanicus, at two sites in Newfoundland. Can. Tech. Rep. of Fish. and Aquat. Sci. No. 1799 p.15).

During the winter of 1992, several aquaculture sites were closed when high levels of Paralytic Shellfish Poisoning (PSP) were detected by DFO Inspection. Examination of the stomachs of the toxic mussels collected in the winter of 1992 revealed that the cause of the high toxicity in the mussels was due to the presence of resting cysts of the toxic dinoflagellate *Alexandrium fundyense* which had been resuspended from the marine sediments at the aquaculture sites (Schwinghamer, P., M. Hawryluk, C. Powell and C.H. McKenzie 1994. Winter occurrence of PSP in inshore Newfoundland waters is caused by resuspended hypnozygotes of *Alexandrium fundyense*. Aquaculture 122:171-179). In order to determine the extent of this toxic dinoflagellate 'seed population' at aquaculture sites, preliminary work funded by the Provincial Department of Fisheries was conducted to detect these cysts or seeds at 13 coastal Newfoundland aquaculture sites. (McKenzie, 1993 &1994. Report on the Occurrence and Abundance of *Alexandrium* Cysts in Marine Sediments at Seven Aquaculture sites in Newfoundland. Part I and Part II. Ocean Sciences Centre and Department of Fisheries Report). The results of these reports indicate that these cysts are found throughout the Province, occurring in numbers ranging from only a few cysts cm^{-2} to > 1000 cysts cm^{-2}. Only two sites contained enough cysts to prevent the its use as an aquaculture site and these sites have been permanently closed to shellfish aquaculture. One site containing over 9.1 tonnes of mussels was permanently closed to shellfish aquaculture, with the grower losing his investment because the shellfish were never below toxic limits and could not be transferred to another site. Study of the distribution of the cysts within this highly contaminated site suggested that sediment type, water depth, wind force, currents and site geography were possible factors in their accumulation.

In the fall of 1994 a research project with scientists at the Ocean Sciences Centre and Department of Fisheries and Oceans studying the management of bivalve aquaculture to minimize exposure to paralytic shellfish poisoning was funded through a three year National Science and Engineering Research Council strategic grant. This is not a monitoring program. The objectives

of this study are to determine desirable aquaculture site characteristics, to develop methods to detect harmful cells and cysts, to determine a time-frame for the safe harvest of bivalves after exposure to PSP and to develop methods to determine the risk of transferring toxic cysts and vegetative cells during the transportation of bivalves either as seed or product to processing plants.

The second field season of this project began in June of this year and the emphasis is on the movement and transportation of the cysts and vegetative cells through spat and product transportation between sites and processing plants.

So far in Newfoundland there have been only seasonal periodic closures of sites due to PSP. Several other species of harmful algae have been detected throughout Newfoundland with only a minor impact on the industry so far. Many areas of Bonavista Bay were closed in October 1993 due to high concentrations of Diarrhetic Shellfish Poisoning (DSP). Large numbers of *Dinophysis norvegica* (>40,000 cells per mussel) were detected in the stomachs of toxic mussels. (McKenzie et al 1995. A *Dinophysis norvegica* bloom and its implications in the occurrence of a diarrhetic shellfish poisoning episode in mussels from Newfoundland during the late autumn in 1993. Proceedings of the Fourth Canadian Workshop on Harmful Marine Algae. May 3-5, 1994. Sidney, B.C. Canadian Technical Report of Fisheries and Aquatic Sciences 2016.)
No commercial aquaculture sites were effected by this closure. There has been some signs of Amnesiatic Shellfish Poisoning (ASP) caused by domoic acid producing diatoms species of *Pseudonitzschia* throughout the province but all detected cases have been well below closure limits. The detection of these toxins have all been through testing of the shellfish themselves. No algae monitoring program is in place in Newfoundland. Other Canadian Provinces, Japan and European countries where the aquaculture industry is taken very seriously have had algae monitoring programs in place for several years in an attempt to have a early warning system to prevent harvesting and transportation of toxic shellfish. As the mussel and scallop aquaculture industry continues to grow in Newfoundland, the need for more information will continue to grow to aid in the management of this industry to the risk of exposure to harmful algal toxins and to aid in site selection, holding capacity and general site management. Newfoundland has no algae monitoring program. The gap in information caused by the lack of such a program will continue to become more costly and more dangerous to the industry and consumers as production increases and more shellfish and spat are prepared for harvest and transport between sites.

MARINE BIOTOXIN SHELLFISH MONITORING IN CANADA'S NEW FOOD INSPECTION AGENCY

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In the Federal Budget of February, 1995 the Government directed Fisheries and Oceans (DFO), Agriculture and Agri-foods Canada (AAFC) and Health Canada (HC) to "...work co-operately on measures, including possible changes in organizational structure, to improve the effectiveness and cost efficiency of the federal component of the Canadian food inspection system...". In May, 1995 the Office of Food Inspection Systems (OFIS) was established to review organizational options with the 3 federal departments, industry provinces and other stakeholders. During the March, 1996 Budget the Federal Government announced its intention to form a single food inspection agency from portions of DFO, AAFC and HC. A total of approximately 4550 employees (4000 - AAFC, 400 - DFO and 150 - HC) will be transferred from the three departments to the new agency. DFO's Inspection Directorate is currently responsible for Canada's shellfish biotoxin monitoring under the Canadian Shellfish Sanitation Program and it is anticipated that that responsibility, along with the trained staff, will be transferred to the food agency by April of 1997. It is expected that the agency will continue to provide the same level of safety of molluscan shellfish.

ABSTRACTS

POSTER SESSION

RECENT ASPECTS OF THE DISTRIBUTION AND OCCURRENCE OF TOXIC (PSP AND DSP) DINOFLAGELLATES IN JAPAN

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Systematic research and monitoring network on shellfish poisoning in Japan has been administered by the Japan Fisheries Agency sine 1978. In 1995, almost 170 stations for toxic phytoplankton were set along the Japanese coast. Based on the results from recent investigations, we represent the present aspects of the distribution and occurrence of toxic dinoflagellates in Japan.

The main species responsible for PSP in Japan are *Alexandrium tamarense* and *A. catenella*. *A. tamarense* which has been mainly distributed in the northern part of Japan is extending its distribution southward. Recently, it has been found in temperate Hiroshima Bay, and cultured oyster and short-necked clam have been contaminated by PSP since 1992. Meanwhile *A. catenella* is widely distributed and has been found even in the islands affected by Tsushima Warm Current at the west of Kyushu Island. *A. cohoticula* already confirmed its toxin production capability and was recorded in Sagami Bay in 1988 (Ogata *et. al.* 1990), but it did not cause PSP. The occurrence of *Gymnodinium catenatum* has been recorded in the coastal waters of Kyushu Island and Seto Inland Sea. However, PSP caused by this species was first reported in Senzaki Bay, Japan Sea coast of western Honshu Island in 1986 (Ikeda *et. al.* 1989). We estimate that this species would be a causative organism of PSP events in the coastal waters of western Kyushu Island and Gokasyo Bay in 1996.

The main species responsible for DSP in Japan are *Dinophysis fortii* and *D. acuminata*. *D. fortii* is distributed along the northern Japanese coast. DSP of scallops caused by this species has been recorded only in the northern part of Japan. Despite the wide distribution of *D. acuminata* around the Japanese coast, DSP caused by this species has been recorded only in the northern part of Japan. While DSP was first recorded in Seto Inland Sea in this Spring, the causative organism has not been confirmed.

The further outbreak and expansion of PSP by *G. catenatum* and DSP caused by *D. acuminata* in the coastal waters of Japan shall be predicted with fear.

DISTRIBUTION OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM OSTENFELDII* IN THE GULF OF ST. LAWRENCE, CANADA

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On the Canadian east coast, the toxic dinoflagellate genus *Alexandrium* is generally represented by the species *A. tamarense* Lebour (= *A. excavatum, Gonyaulax tamarensis, Protogonyaulax tamarensis*) and *A. fundyense* Balech. A detailed examination of the thecal structure of *Alexandrium* species from the St. Lawrence estuary and gulf in 1994-1995 revealed the presence of a third toxic species: *A. ostenfeldii* (Paulsen) Balech and Tangen. *A. tamarense* and *A. ostenfeldii* have also been discriminated on the basis of their maximum width which is in average 33 ± 4 μm and 46 ± 5 μm, respectively. Both species exhibited similar spatial and temporal distributions during those two years. When *Alexandrium* was abundant (> 1000 cells l^{-1}), *A. ostenfeldii* represented up to 46% (minimum 0%, average 11%) of the total *Alexandrium* population. Since the two species are known to have different toxin composition and total toxicity, the presence of *A. ostenfeldii* in the phytoplankton community may thus significantly alter the pattern of PSP toxins accumulation and detoxification in shellfish.

MONITORING ALGAL BLOOMS WITH PASSIVE OPTICAL SENSORS

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Environmental and economic impacts of harmful algal blooms (HABs) have increased in recent decades, concurrent with the escalating influence of human activities on estuarinand coastal ecosystems. Consequently, it is imperative to know the degree to which present trends of HABs and human activities are linked, and if current trends will lead to unacceptable environmental degradation in particular coastal regions. Essential to this effort will be reliable, quantitative, and practical means to describe the frequency of blooms and long-term trends in bloom dynamics. The development of monitoring systems will also be important for the protection of aquaculture sites.

Relatively simple passive optical sensors, deployed in situ, can be used to detect algal blooms in coastal waters. A Tethered Spectral Radiometer Buoy (TSRB) measures upwelling radiance (ocean color, $Lu(\lambda)$) in seven wavebands (six channels corresponding to the SeaWiFS ocean color satellite, plus 683 nm for chlorophyll fluorescence), as well as downwelling irradiance at 490 nm (Ed(490)), to yield estimates of spectral reflectance (Cullen et al. 1994). It measures the ratio of green:blue (e.g. Lu(555):Lu(443)), an index of chlorophyll used in satellite imagery, and also the fluorescence of chlorophyll near 683 nm, another indicator of phytoplankton. The measurements are normalized to downwelling irradiance, so differences in brightness can be distinguished and interpreted. Thus, the radiometer can clearly distinguish changes in ocean color associated with the presence of phytoplankton (Fig 1). A dinoflagellate bloom in Bedford Basin, Nova Scotia, a diatom bloom off the coast of Oregon, and an onshore-offshore gradient in chlorophyll were easily detected with a TSRB, not only because of characteristic shifts in ocean color, but also because of the prominent signal from solar-stimulated fluorescence of chlorophyll.

A bloom of *Gonyaulax digitale* developed in a subsurface layer during the summer of 1993 in Bedford Basin, Nova Scotia. Afternoon winds eroded the thermocline and entrained the bloom into the mixed layer. Measurements from the TSRB readily distinguished the bloom and showed that the water turned red when the bloom was transported to the surface (Fig 2). It would be possible to develop automated systems to recognize these kinds of changes. Vertical distribution of the phytoplankton was also detected with a simple measure of water clarity: penetration of light at 490 nm, as determined with paired sensors for Ed(490) separated by 1m.

During a diatom bloom in the plume of the Columbia River, variability of surface chlorophyll a was very well described by both the green:blue radiance ratio and the fluorescence signal as measured by the TSRB (Fig 3). However, in coastal waters, the influence of dissolved organic materials and suspended sediments limit the utility of the 443:555 ratio, which is used for

the open ocean. Instead, the 490:555 ratio is used to estimate chlorophyll for coastal waters (Fig 4), but there are still regional differences, and some large departures from the CZCS algorithm for coastal waters (Gordon et al., 1988). It is clear that terrestrial runoff severely restricts the use of simple ratios to estimate chlorophyll; further research will be needed to characterize the fluorescence signal as a measure of chlorophyll .

Passive optical sensors have potential in a wide variety of applications. The capability to obtain critical observations prior to and during the initiation of blooms makes such an instrument a useful research tool in the study of the oceanography and ecology of harmful algal blooms. Also, because passive optical sensors measure radiometric quantities, data are appropriate for regional comparisons and for long-term monitoring and assessment. Although passive optical sensors are unlikely to distinguish toxic from non-toxic blooms, they are very well suited as early warning systems and monitors of long-term trends in coastal eutrophication and bloom dynamics. Autonomous moored sensing systems can contact the user if pre-defined conditions are encountered. Thus, radiometer buoys may also be useful as early warning systems for aquaculture operators.

While much useful information can be obtained from passive optical sensors, there are inevitably some disadvantages which need to be addressed. For instance, fouling can necessitate periodic calibration and maintenance, and further development of robust interpretations of optical measurements is needed. Also, relatively narrow spatial coverage requires a large array of instruments supported by airborne imagers for synoptic information. The advantages however, include a low relative cost and low power consumption making them particularly appropriate for use on moorings. In addition, data can be collected on cloudy days, when airborne color sensors are useless.

In conclusion, the pressing need to characterize, understand, and manage our changing coastal environments requires the development of practical technologies for measuring biological and physical variability in coastal waters. Relatively simple optical measurements provide a great deal of information on biological variability in coastal environments, including the capability of detecting aigal blooms. Although toxicity cannot be assessed, we suggest that passive optical measurements provide reliable, quantitative, and practical means to describe the frequency of blooms and long-term trends in bloom dynamics.

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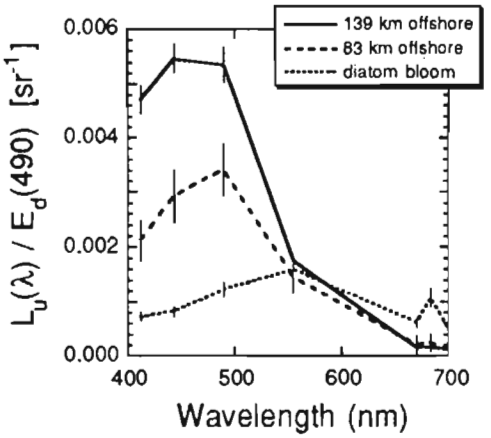

Fig. 1. Measures of ocean color (upwelling radiance at wavelength λ (nm), $L_u(\lambda)$, normalized to downwelling irradiance at 490 nm, $E_d(490)$, as recorded with a tethered spectral radiometer buoy (described by Cullen et al., 1994) in coastal waters off the coast of Oregon. Mean ± s.d. for data recorded at 1 s^{-1} and averaged over deployments of about one hour: (—) 139 km offshore; (- - -) 83 km offshore; and (···) during a diatom bloom in the plume of the Columbia River, about 8 km offshore. Patterns are very similar to those that stimulated interest in remote sensing of ocean color (Hovis et al. 1980)(from Cullen *et al.* 1996 in press).

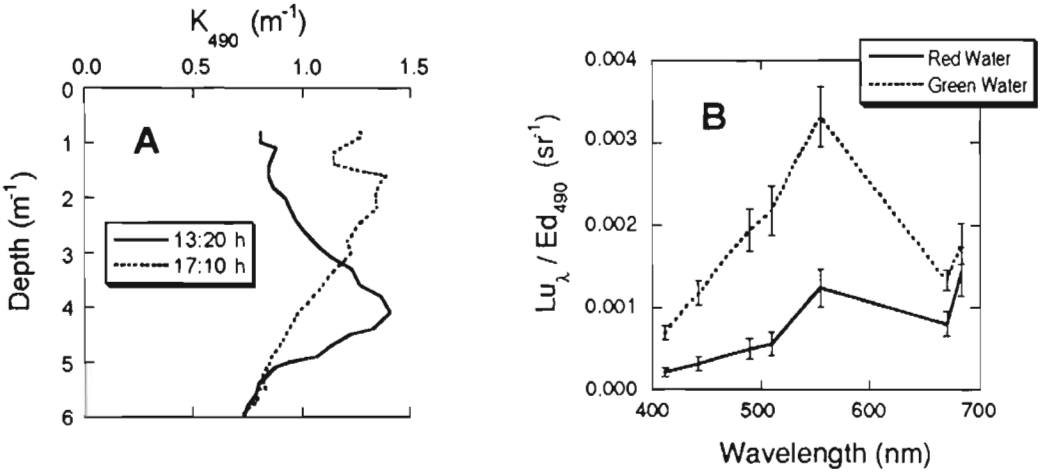

Fig. 2. Detection of the dinoflagellate bloom with passive optical measurements, Aug. 18, 1993. A. Diffuse attenuation of downwelling irradiance at 490 nm at 1320 h (subsurface peak) and 1710 h (after wind mixing). B. Spectra of $R(\lambda)_{TSRB}$ during a period of patchy entrainment of the dinoflagellates into the mixed layer: the red water spectrum is the average for 1345 - 1350 h, and the green water spectrum is for 1354 - 1359 h. Error bars are standard deviations (from Cullen *et al.* 1996 in press).

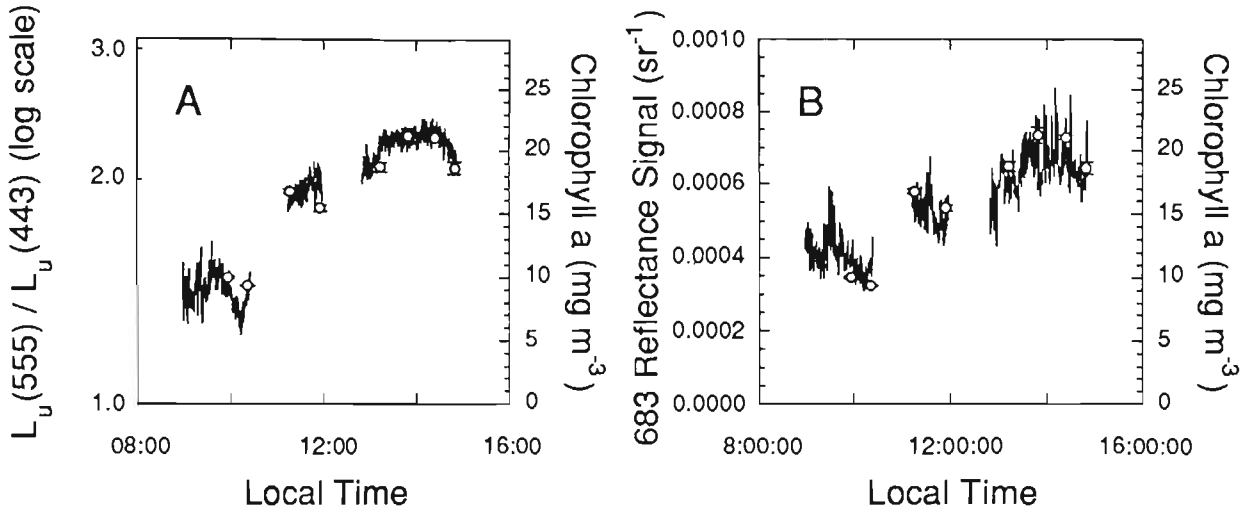

Fig. 3. Measurements from the TSRB related to Chl at the surface about 8 km off the coast of Oregon in the plume of the Columbia River, 20 Sept. 1994 (60-sec medians of data recorded at 1 s^{-1}). A. The ratio of $L_u(555)$ to $L_u(443)$, plotted on a logarithmic scale. B. Upwelling radiance at 683 nm, normalized to $E_d(490)$ and corrected for reflectance of solar irradiance using a linear base-line correction derived from R_{TSRB} at 670 and 700 nm (cf. Neville and Gower 1977). Chlorophyll (open circles: mean ± s.e. of triplicates) was measured fluorometrically on samples collected with a bucket, filtered, and extracted with a mixture of DMSO:acetone. The phytoplankton assemblage was dominated by a non-toxic form of *Pseudo-nitzschia*; the increase of chlorophyll during the day could have been due to advection as well as net growth of the phytoplankton (from Cullen *et al.* 1996 in press).

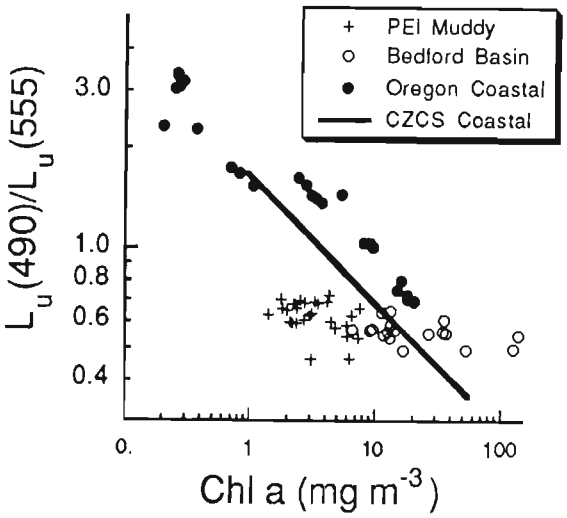

Fig. 4. Relationships between Chl at the surface and ocean color as measured with a TSRB ($L_u(490):L_u(555)$): observations from muddy waters off Prince Edward Island (crosses); Bedford Basin, Nova Scotia (open circles); and coastal waters off Oregon (filled circles). An algorithm similar to one used for CZCS data from coastal waters (Gordon et al. 1988) is represented with the line: Chl = $3.64 \cdot (L_u(490):L_u(555))^{-2.62}$. Here, 490 and 555 nm are used rather than Gordon's 500 and 560 nm, and L_u as measured with the TSRB is used rather than normalized water-leaving radiance (from Cullen *et al.* 1996 in press).

PHYTOPLANKTON MONITORING IN THE SOUTHWEST BAY OF FUNDY, NB

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A phytoplankton monitoring programme was initiated in 1987 in the southwestern Bay of Fundy with three main purposes: to establish baseline data for phytoplankton populations in the local area, to determine whether the rapidly expanding salmonid aquaculture industry was having an impact on the environment, and to act as an early warning to regulatory agencies and industry of potential harmful algal species. Although 17 locations were initially sampled, the programme has gradually been scaled down to four sites that continue to be monitored today.

Samples are collected weekly during June through September, biweekly during May and October, and monthly during the colder months of November through March. Parameters measured include temperature, salinity, nutrients, chlorophyll a, and phytoplankton distribution and abundance. Methods for sampling have been described previously. [1,2].

Salinities vary from 28.6‰ in spring to 35.0‰ in late summer. Temperatures vary from a low of -0.5°C in March to a high in September of 16°C. Although phytoplankton are present year round, when water temperatures increase in early May to 4°C, cell densities rise significantly and continue until October.

To date more than 200 species of diatoms, dinoflagellates, ciliates and smaller zooplankton have been observed. Dominant organisms observed in the plankton, and times when they tend to dominate, are: *Chaetoceros debilis* and *Thalassiosira* spp., May and June; *Alexandrium fundyense*, July; *Pseudo-nitzschia pseudodelicatissima*, August; *Mesodinium rubrum*, September; diatoms such as *Ditylum brightwelli*., *C. debilis*, or *Skeletonema costatum* in October; and *P. pseudodelicatissima* and *Cylindrotheca closterium* from November to April.

Species present that produce toxins and are responsible for shellfish areas being closed to harvesting are *A. fundyense* (PSP), and *P. pseudodelicatissima* (ASP). Most shellfish harvesting areas are closed to harvesting due to unacceptable levels of PSP toxins annually and generally during the summer. Since 1987, highest concentrations of *A. fundyense* (6.36×10^4 cells L^{-1}) were observed in Lime Kiln Bay in 1993. Blooms of *A. fundyense* tend to occur from June to August and peak in mid-July. Although *P. pseudodelicatissima* cells have been observed annually and may be observed year-round, it tends to bloom in June and again from August to October with peaks mid-June and late August. Concentrations exceeding 1.0×10^6 cells L^{-1} were present during the summers of 1988 and 1995 resulting in levels of domoic acid in shellfish exceeding the regulatory level for harvesting.

The abundance of *A. fundyense* and *P. pseudodelicatissima* follow erratic patterns during sampling periods, often with swings in the data on about a weekly basis, suggestive of a tidal influence. Differences in concentration from year to year must have been related to factors other than temperature or salinity, which showed similar values and patterns of change during the nine summers.

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HARMFUL EFFECTS OF CERTAIN DIATOMS ON THE REPRODUCTIVE SUCCESS OF COPEPODS

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It is well known that blooms of toxic dinoflagellates can produce massive kills of fish species. On the other hand, diatoms, which also constitute a major component of the phytoplankton community, are rarely reported as being toxic for marine organisms and are traditionally considered as appropriate food for herbivorous species. However, the importance of diatoms as a high-quality food source for copepods has recently been questioned. In particular, the studies of Poulet, Ianora and co-workers[1-7] on the planktonic copepods *Calanus helgolandicus*, *Temora stylifera* and *Centropages typicus* have shown that up to 100% egg mortality could be induced when adult females were fed with certain diatom species. These experimental results are consistent with the hypothesis that these diatoms contain a toxin that blocks embryogenesis and serve as deterrents to the growth and survival of copepod populations. We present here supporting evidence of harmful effects of a common diatom species, *Thalassiosira nordenskioldii*, on the reproductive success of the planktonic copepod *Calanus finmarchicus*, one of the dominant members of the zooplankton community of the lower St. Lawrence Estuary in eastern Canada.

The feeding/egg production experiments were performed with female *Calanus finmarchicus* collected between 20 and 22 June 1995 at a single station located in the lower St. Lawrence Estuary (48° 40'N, 68° 35' W). The collection was done with a 1 m diameter net (333 μm mesh size) towed from 250 m to the surface. The net contents were immediately transferred into 4 L glass jars filled with surface seawater and transported to the laboratory within 1 h of sampling. Within 5 h of arrival, healthy-looking females were sorted from the catch using a dissecting microscope. Prior to the experiments, the females were held for 5-7 d in 4 liter containers filled with 0,2 μm filtered seawater, at a temperature of 5 to 6° C and a salinity of 28 to 30 parts per mil.

The reproductive response to various food treatments was quantified by incubating 7 females in 1 L egg separation containers, as described in Runge[8]. The containers consisted of Plexiglas cylinders closed off at the bottom end with a 571 μm mesh nitex screen and immersed into 2 l glass beakers containing 0,2 μm filtered sea water enriched with phytoplankton. Phytoplankton species used included the centric diatom *Thalassiosira nordenskioldii* (Tnord) and the dinoflagellate *Prorocentrum micans* (Prom), which were tested separately. Initial cell concentration was 10 000 cells mL^{-1} for Tnord and 1000 for Prom. Four replicate containers per

food treatment were used and the experiment lasted 23 days. Every day the animals were transferred to new containers with fresh medium and egg production was recorded. In order to determine egg viability, each batch of eggs was then transferred with a repeating micropipette to Erlenmeyer flasks containing 125 mL filtered sea water (0,2 μm) and incubated for 72 h. Egg viability was determined from the proportion of unhatched eggs and unhealthy (i.e. dead or deformed) nauplii at the end of the incubation relative to the initial number of eggs. All procedures were conducted at a temperature of 5 to 6° C, a salinity of 28 to 30 parts per mil and under a 14 h light : 10h dark cycle.

Phytoplankton for these experiments came from unialgal cultures (isolates supplied by the Center for culture of marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME; Clone designation : CCMP995 for Tnord; CCMP693 for Prom) maintained in exponential growth phase in batch cultures (19 liters), with the use of natural seawater (filtered at 0,2 μm) enriched with f/2 medium[9]. The cultures were maintained at a temperature of 15 to 16° C, a salinity of 28 to 30 parts per mil and under a 16-hour light : 8-hour dark cycle. Air filtered at 0,2 μm was continuously bubbled through the cultures to supply CO_2 and maintain algal suspension.

Daily fecundity and egg viability for female *Calanus finmarchicus* fed ad libidum on a dinoflagellate (Prom) or a diatom (Tnord) are illustrated in Figure 1. Females had different reproductive responses depending on the diet. With Prom, egg viability remained extremely high (>70%) and stable throughout the 23 d period of investigation. Fecundity was very low at the beginning of the experiment, but increased steadily, attaining rates up to 60 egg d^{-1} after 11 d (daily mean fecundity: 44,2 eggs $females^{-1}$ d^{-1}). With Tnord, fecundity was somewhat lower at the beginning of the experiment, but was comparable to the Prom-fed females after the first 14 d (daily mean fecundity: 43,1 eggs $females^{-1}$ d^{-1}). Egg viability, on the other hand, was initially high with Tnord, but, after ca 10 d of incubation, the females began producing abnormal eggs which failed to hatch or hatched into unhealthy nauplii. After 20 days, >70% of the eggs remained unhatched. When all data were pooled, a comparison among diets indicated significant differences between Tnord and Prom for total egg production and egg viability (Table 1). A lower viable egg production rate was observed in the Tnord treatment.

A close examination by microscopic techniques of abnormal clutches induced by Tnord revealed that eggs underwent strikingly abnormal development. Abnormal eggs were characterized by a darker color, the presence of globular cytoplasm, and by scattered, irregular, asymmetrical globules corresponding to nuclei. These structural anomalies reflected an abnormal cell division during mitosis. In many cases, egg development was arrested at the morula or gastrula stages and death occurred shortly after. Among unhealthy nauplii, most of them presented strong anatomical anomalies. Their bodies were asymmetrical and crumpled. The 3 pairs of appendages were also asymmetrical, shortened and abnormal in segmentation. The number and length of setae were unusual. In most cases, deformed nauplii were found dead. Some could swim, but swimming behavior was aberrant in comparison to normal nauplii. Because of the asymmetrical length of the left and right pairs of appendices the direction of the trajectory was

twisted rather than linear, as in the normal case. Such anomalies were never observed with the dinoflagellate Prom.

Further experiments (not shown) showed that production of abnormal eggs and weak, deformed nauplii could also be induced by another diatom of the genus *Navicula* (isolated from St. Lawrence Estuary waters), but not by the two flagellates *Isochrysis galbana* and *Pavlova lutheri* (from culture collections at Bigelow). A similar arrest of embryonic development has recently been reported for 3 other copepod species (*Calanus helgocentricus*, *Centropages typicus*, and *Temora stylifera*) fed ad libitum with 3 diatom species, *Chaetoceros curvisetum*, *Phaeodactylum tricornutum*, and *Thalassiosira rotula* [1-7].

One of the major causes of unsuccessful hatching with a diatom diet may be due to anti-mitotic agents contained in diatom cells, as suggested by Poulet et al.[6], or originating from virus or bacteria associated with these diatoms. Although this cannot be ascertained until the inhibitory chemical compounds can be identified, the results of our study support this hypothesis. By ingesting Tnord, *Calanus finmarchicus* could accumulate anti-mitotic agents in gonads during vitellogenesis. The suggestion that these inhibitors are accumulated in gonads is inferred from the initial high egg viability observed with Tnord. This was followed by a progressive diminution in hatching success on successive days. If the accumulation of anti-mitotic agents was insufficient to block egg development, some embryos would hatch into deformed nauplii. Another possible cause for abnormal egg hatching and naupliar development may be related to nutritional characteristics of the food. However, some of our recent findings indicate a reduction in hatching rate in *C. finmarchicus* even when females were exposed to mixture of *Thalassiosira* and good food items, suggesting that blocking of embryonic development is chemically mediated. The seaweed literature gives a number of examples of various noxious compounds that serve as chemical defenses and feeding deterrent for predator populations[10]. We cannot exclude this possibility in phytoplankton.

In addition to 3 diatom species (*Chaetoceros curvisetum*, *Phaeodactylum tricornutum* and *Thalassiosira rotula*) for which some deleterious effects to copepod reproduction have been demonstrated[1-7], this laboratory study confirmed that *Thalassiosira nordenskioldii* could induce reproductive failure in copepods. This diatom species is very common in the St. Lawrence Estuary and regularly dominates the biomass during phytoplankton blooms[11-12], reaching concentrations comparable to those used in our test (10^3 cells ml^{-1}). Due to the dynamic physical environment of the lower St. Lawrence Estuary, diatoms, including *T. nordenskioldii*, maintain high standing stocks throughout the summer months[11-12]. Based on our experimental evidence from controlled feeding studies, under such conditions *Thalassiosira* could be ingested in quantities high enough to induce anomalies in eggs. Alternatively, copepods could reduce the effects by shifting their diet to non-toxic food items. The effective role of the inhibitory effect of certain diatom species upon egg development of *Calanus* in the St. Lawrence ecosystem remains to be determined. Studies are now being conducted to identify the mode of action of these diatoms and their impacts in the region's ecosystem.

Acknowledgements

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Table 1. Calanus finmarchicus. Summary of results (mean, SD, and statistical tests between diets) of feeding/egg production experiments with females fed either a dinoflagellate (Prorocentum micans) or a diatom (Thalassiosira nordenskioldii). N= number of replicate experiments for each diet.

Biological response	Type of food		Significance of statistical test
	Prorocentrum micans (n=4)	Thalassiosira nordenskioldii (n=4)	
Total egg production (eggs*female^{-1})	796.8 (21.7)	627.2 (64.2)	F=25.03*
Egg viability (%)	78.0 (4.3)	44.0 (3.5)	F=97.36*

* Significance at 99%

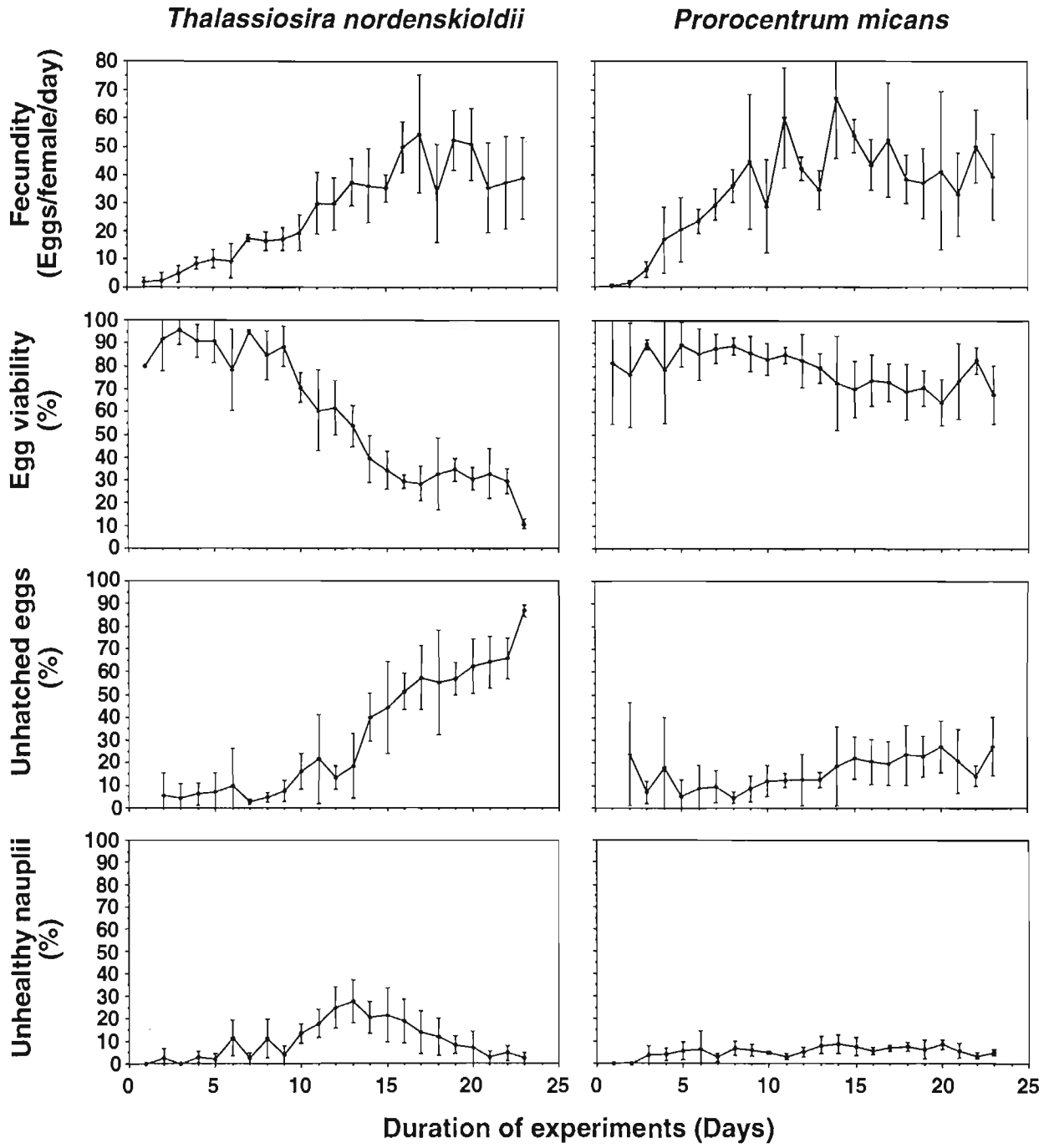

Figure 1. Mean daily fecundity and egg viability with standard deviations for female Calanus finmarchicus fed ad libidum on a dinoflagellate (Prorocentrum micans) or a diatom (Thalassiosira nordenskioldii). Nonviable eggs were further classified into unhatched eggs and unhealthy nauplii. Results are means of 4 replicate experiments for each diet.

ALEXANDRIUM CYSTS IN COASTAL COLD OCEAN SEDIMENT AND THEIR ROLE IN BIVALVE AQUACULTURE MANAGEMENT

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During the winter of 1992, several aquaculture sites were closed when high levels (515 μg saxitoxin per 100g mussel tissue) of PSP toxicity were detected in harvested mussels (*Mytilis edulis*) by the Canadian Department of Fisheries and Oceans Inspection Branch. Examination of the stomachs of the toxic mussels revealed that the cause of the high toxicity was due to the presence of hypnozygotes or resting cysts of the toxic dinoflagellate *Alexandrium fundyense* which had been resuspended from the marine sediment at the aquaculture site (Schwinghamer et al. 1994). Subsequent sediment surveys revealed that at one former aquaculture site the *Alexandrium* cyst concentration was 1130 cysts per cm^2 (McKenzie 1993, 1994). The bottom sediment of this cove consisted of fine silt and clay over a meter deep. The site with the highest cyst concentrations has been permanently closed to shellfish aquaculture.

This coastal area is a major centre of bivalve aquaculture in Newfoundland. A multidisciplinary study (biology, chemistry, physics) is being conducted to determine factors which influence the distribution of these cysts. This study includes water, sediment, sediment trap and bivalve analyses as well as an investigation of the circulation patterns in the region. This study is being conducted to develop aquaculture management practices which would minimize the risk of PSP contamination.

Sediment samples were collected throughout the research area in Notre Dame Bay, Newfoundland. Ocean Sciences Centre SCUBA divers collected bottom sediment cores by hand. Samples were collected with a modified 50 cc centrifuge tubes. Processing of the sediment for analysis included cleaning and concentrating the *Alexandrium* cysts, using the density gradient method of Schwinghamer et al. (1991). Abundance of *Alexandrium* cysts was determined microscopically using a Zeiss Inverted microscope.

Seabird CTD samples were collected throughout the research area and at the four aquaculture sites. Chlorophyll α analysis was conducted on whole water samples and sediment trap samples using a Turner Designs Model 10 Fluorometer. Underwater video was taken using a Sony Handicam and a DV 2 underwater camera.

The results of a sediment survey conducted in late November 1994 indicate that the highest cyst concentration is confined to the previously closed site (Site 4). Maximum cyst abundance was determined to be 2.2×10^4 cysts cm^{-2}. Site 3 which was separated into three

growing areas was found to have very few cysts detected (5.7×10^1) at Site 3:1 or Site 3:3, but had 3.5×10^2 cysts cm^2 at Site 3:2. This suggests that outside the barrier islands of Site 4 that the cysts are being dispersed northward toward Site 3:2. However, no cyst concentrations were found to be above 10^3 cysts cm^{-2} outside of Site 4. Seabird CTD data collected during the November field trip indicate the presence of a small fall bloom within the research area. Highest chlorophyll levels, 3.5 μg/L, were found in the more protected southwestern area of the tickle. The northern sites had chlorophyll levels of 0.5 to 1.0 μg/L. The northern sites are much more exposed to Notre Dame Bay and the North Atlantic Ocean. The neck of the tickle north of Site 1 was found to have very strong tidal currents and cycles. Site 1 is quite open and relatively deep (ca. 30 m) and subject to strong flushing. The bottom of Site 1 is primarily rock and coarse gravel. It is also prone to severe ice damage to the mussel lines in the early spring. Site 2 is relatively protected and contains many mussel lines. It is ca. 10 -25 m deep and has a coarse gravel/sand bottom. Site 3 is also protected, but has a relatively shallow, silty area to the east of the site which could retain cysts if they were introduced into the site.

Site 4 is our primary site of interest. It is a former mussel aquaculture site which has been permanently closed to aquaculture due to its high, year-round PSP levels. This site is very protected with two barrier islands which separate this cove from the tickle. This very sheltered area would appear to have been an excellent choice for an aquaculture site, however the sediment at the site indicates the fundamental problem with the site with regards to the accumulation of PSP contaminated cysts. The sediment at this site is made up of over 1 meter deep silt. There is little to no flushing of the system and any toxic cysts present, whether resuspended or from a vegetative bloom, remain and increase the toxicity of the site. The cyst concentration in November of 1993 was 1.1×10^3 cm^{-2} by November 1994 the highest concentration was 2.2×10^4 cm^{-2}. The highest concentrations of cysts were found along the shallow northeastern edge of the site. We believe that wind and currents caused this concentration of cysts in this area. Winds from the southwest are unusually strong and prolonged in this region. With such a large accumulation of cysts in a relatively shallow area, resuspension and mussel contamination was an almost constant problem when this was an operating shellfish site. The presence of a fall phytoplankton increase is demonstrated by the water and sediment trap chlorophyll contents. Sediment traps were left at the site for two days with two cylinders poisoned and two unpoisoned. During the two days in November the trap collected 26.928 μg/L chlorophyll α. Such an increase suggests significant resuspension at the site. High winds were in fact a problem during the recovery of the sediment traps on the second day.

Conclusions

It is important to have site selection criteria during the selection of a potential aquaculture site to minimize the risk fo exposure to cysts of PSP containing *Alexandrium*.

Suggested criteria include:

1. Sediment type as an indication of system flushing and potential for resuspension.
2. Depth as an indication of resuspension activity as well as potential temperature and salinity stratification.
3. The use of sediment traps to determine the sedimentation rates of phytoplankton and resuspended cysts.
4. Sediment testing to determine existence of potential exposure to toxic cysts.
5. Bottom visual survey which might suggest the environmental health of a site.

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The authors would like to acknowledge and thank the growers who allowed their sites to be used for this study. We also thank the OSC dive team, as well as Mike Riehl and Jeanette Wells for their excellent technical assistance. Funding for this study was provided by an NSERC Strategic Grant to R.J. Thompson et al.

VIABILITY AND GERMINATION OF *ALEXANDRIUM* HYPNOZYGOTES EGESTED IN *MYTILUS EDULIS* FAECES

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To determine the effects of passage through the bivalve gut upon the viability and germination of *Alexandrium* sediment resting stages, eight mussels (*Mytilus edulis*) were removed from a permanently closed aquaculture site in Newfoundland in July 1996. Faecal pellets were collected and the contents examined microscopically for identification and enumeration. While *Scrippsiella* cysts were abundant, few viable "classical" *Alexandrium* hypnozygotes were found. More numerous were cysts of the same size and shape as *Alexandrium* hypnozygotes, containing red accumulation bodies, which could not be accurately identified as the remainder of the cell contents were concealed by globulous material coating the outer surface. When examined under blue-light excitation, the chlorophyll *a* of these cysts autofluoresced. This occurs only during the season of the year when germination takes place, July and August in Newfoundland waters. Following incubation and excystment, the motile stage of these cells was identified as *Alexandrium fundyense*. Although the rate of germination was low, these cysts remained viable after egestion, indicating the resistance of the multilayered resting stage to the bivalve acidic gut, extracellular digestion and mechanical action of the crystalline style. This resistance could have serious implications for current aquaculture practices, as the transport of contaminated bivalves among sites may result in the transfer of viable *Alexandrium* hypnozygotes, capable of germinating and seeding a toxic dinoflagellate bloom in a previously uncontaminated site.

DOES UPTAKE OF *ALEXANDRIUM FUNDYENSE* CYSTS CONTRIBUTE TO THE LEVELS OF PSP TOXINS FOUND IN THE GIANT SCALLOP, *PLACOPECTEN MAGELLANICUS?*

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Scallops, *Placopectin magellanicus*, in most areas of the Bay of Fundy, New Brunswick, have year round paralytic shellfish poisoning (PSP) toxin concentrations greater than the regulatory concentration of 80 mg STX eq 100 g-1 wet wt. Scallops (mean shell height of 10.7 cm), age 3-5 years, were collected by SCUBA and individually tagged near Parker Island, Bay of Fundy. Half were hung 2 m below the low tide water level and the remainder were placed on bottom below. Monthly samples of scallop, water and sediment were collected.

In October, 1993, mean concentrations of PSP toxins in the digestive glands and mantles were 3 205 and 1 018 mg STX eq 100 g^{-1} wet wt, respectively. Eight months later (June, 1994), surface and bottom PSP concentrations in digestive glands had declined to 504 and 682 mg STX eq 100 g^{-1} wet wt whereas those in mantles had declined to 802 and 681 mg STX eq 100 g^{-1} wet wt. During July, 1994, *A. fundyense* concentrations of 320 cells L^{-1} were observed at Parker Island and 14 200 cells L^{-1} offshore. Subsequently, toxin concentrations in surface and bottom scallop digestive glands increased to12 720 and 11 408 mg STX eq 100 g^{-1} wet wt whereas those from mantles increased to 2 126 and 1 748 mg STX eq 100 g^{-1} wet wt, respectively. PSP concentrations in October, 1994 were similar to those measured in October, 1993.

There were no statistically significant differences in uptake and depuration profiles of PSP toxins held at the surface compared to those on bottom suggesting that *A. fundyense* cyst concentrations in the sediment (45 cysts cm^{-3}) did not contribute significantly to the year round presence of toxins. It appears to be due to the accumulation and slow depuration of toxins from the *A. fundyense* summer bloom.

DOMOIC ACID PRODUCTION AND CELL DIVISION BY *PSEUDO-NITZSCHIA MULTISERIES* IN RELATION TO A LIGHT:DARK CYCLE IN SILICATE-LIMITED CHEMOSTAT CULTURE

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The pennate diatom *Pseudo-nitzschia multiseries* is capable of producing the neurotoxin domoic acid (DA) while growing in silicate-limited continuous culture. This seems contrary to the observed lack of toxin production by continuously-dividing cells during exponential growth in batch culture. However, cells in chemostat culture are limited by the concentration of silicate, a situation similar to that experienced by non-dividing cells during stationary phase in batch culture. Production of DA may also occur only during a specific part of the cell division cycle, for example after completion of mitosis.

Experiments were carried out to investigate DA production in relation to the cell cycle. This becomes easier to study if the cells are grown in a chemostat illuminated on a light:dark cycle to produce a synchronized culture. Knowledge of the relationship between DA production and cell division will help to interpret toxin production in the field.

Pseudo-nitzschia multiseries strain KP-82 was grown in a chemostat vessel made from a 2.8 L Fernbach flask containing 1.5 L of f/2 medium. Silicate was the limiting nutrient. Illumination (ca. 360 $\mu E\ m^{-2}\ s^{-1}$) was provided by circular fluorescent lamps on a 12:12 h L:D cycle. The flow rate of the medium into the chemostat vessel allowed a cellular growth rate of 0.5 d^{-1} (= 0.7 divisions d^{-1}).

Domoic acid in the sonicated cells (separated from the medium) was measured with an HPLC using the high sensitivity FMOC derivatization method. Cell numbers were determined by counting triplicate 5 µL aliquots of culture, under a light microscope. Extracted cellular DNA was measured according to Berdalet and Dortch (1991; Mar. Ecol. Progr. Ser. 73: 295-305) using Hoechst 33342. Chlorophyll *a* and silicate concentrations were measured using standard methods.

A partially-synchronized culture was obtained with the 12:12 h light:dark cycle. Cell division occurred during the first 8 hours of the light period, as seen by the increase in cell number (Fig. 1) and chlorophyll *a* concentration (Fig. 2). Cell biomass then decreased during darkness due to washout. Cellular DA followed an inverse pattern, i.e., it decreased during the first 8 hours of the light period and started increasing ca. 4 hours before darkness (Fig. 3).

The period of cell number increase coincided with a depletion of Si from the growth medium, as expected (Fig. 4). Silicon began accumulating again in the chemostat during the dark period, in the absence of cell division, because fresh growth medium continued to be pumped into the chemostat vessel without being utilized. Changes in cellular DNA were more difficult to interpret, but it tended to decrease during the light period and increase in darkness (Fig. 5).

The inverse relation between the periods of cell division and DA production is consistent with batch culture studies, which show production of DA by non-dividing cells during stationary phase. The timing of DA production and cell division also suggests that toxin is produced during a specific part of the cell cycle when the cells are not dividing.

The period of decrease in cellular DNA may be interpreted as corresponding to the interval following mitosis and cytokinesis in an idealized diatom cell cycle (Fig. 6). Synthesis of DA appears to begin after the cells have completed mitosis but prior to DNA replication; this may therefore be during the "G1" phase of the cell cycle. Our results cannot specify this cell cycle period with any more confidence. This may best be accomplished with studies using, e.g., tritiated adenine, or the expression of the PCNA using Western blotting.

The period ca. 8 h into the light phase (when DA synthesis begins) also corresponds to a minimum in silicate concentration in the growth medium, when the cells have depleted Si as a result of frustule formation during cell growth. There may therefore be a link between Si depletion and the initiation of DA synthesis in chemostat culture, as also appears to occur in stationary phase batch culture.

In stationary phase batch culture, cells may remain "stuck" in a specific part of the cell cycle due, for example, to the lack of Si. These cells continue to produce DA. In a Si-limited chemostat, toxin production begins only when the cells have depleted the Si content of the medium and are not dividing. The timing of cell division, occurring during the majority of the light period when DA is not produced, is also consistent with the hypothesis that there is a competition for energy between primary metabolism (e.g., C and Si uptake) and secondary metabolism (DA production).

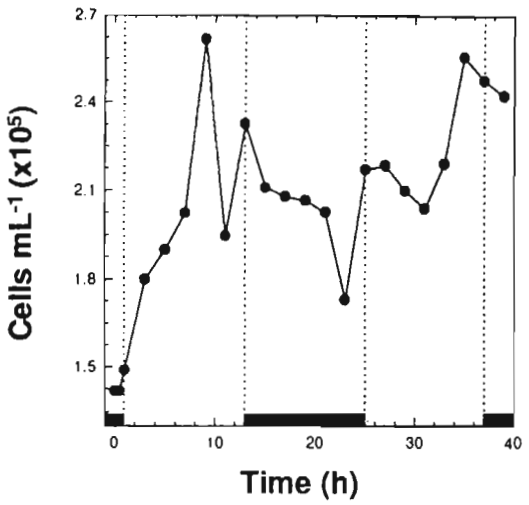

Fig. 1 Concentration of *P. multiseries* cells in a chemostat during a light:dark cycle. The dark period is shown by the horizontal bars.

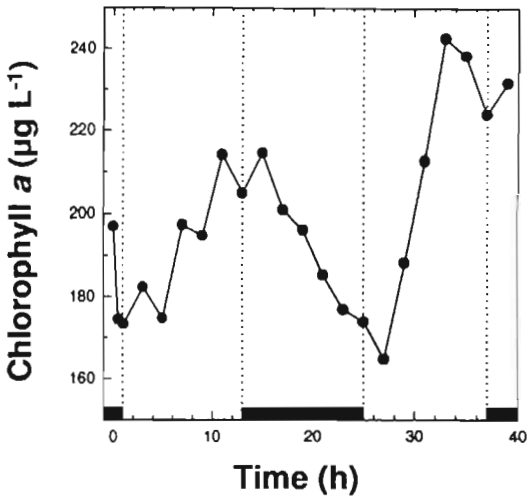

Fig. 2 Concentration of cellular chlorophyll *a* in a chemostat during a light:dark cycle. The dark period is shown by the horizontal bars.

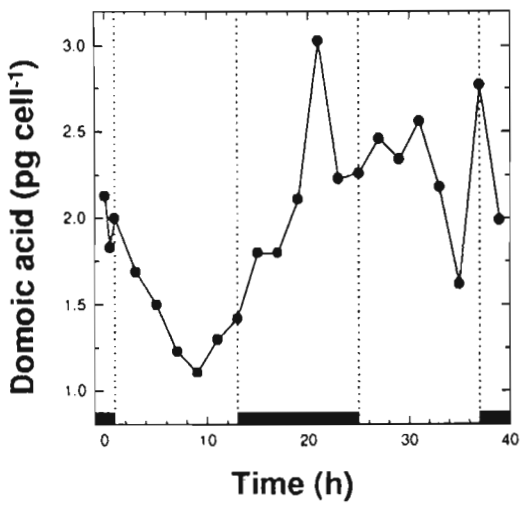

Fig. 3 Cellular domoic acid in a chemostat during a light:dark cycle. The dark period is shown by the horizontal bars.

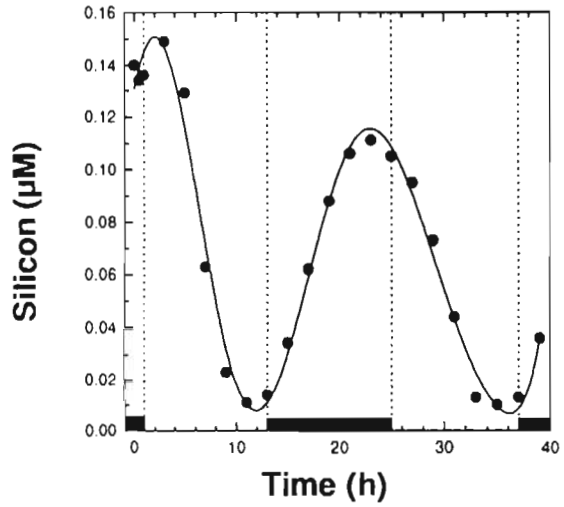

Fig. 4 Concentration of soluble silicon in a chemostat during a light:dark cycle. The dark period is shown by the horizontal bars.

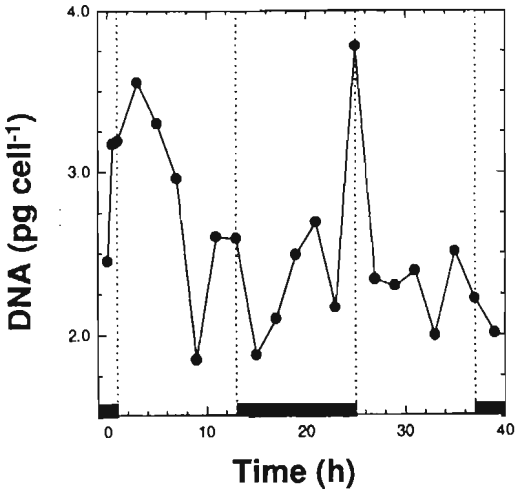

Fig. 5 Concentration of cellular DNA in a chemostat during a light:dark cycle.

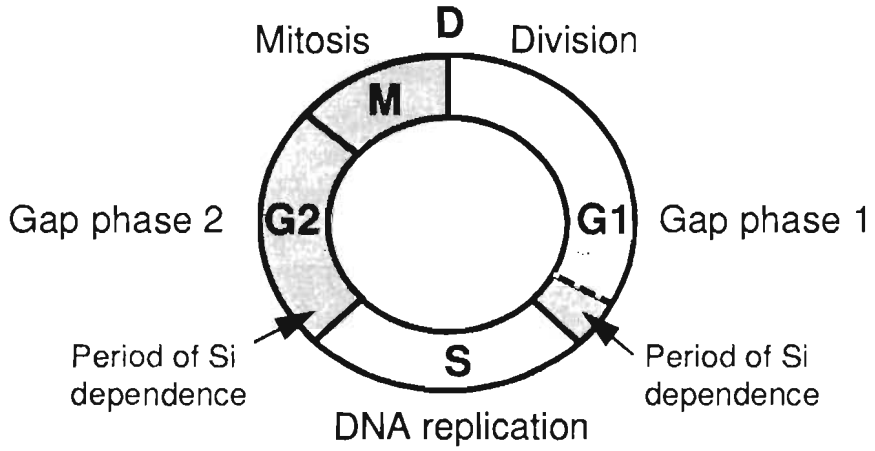

Fig. 6 Idealized cell division cycle in diatoms (after Brzezinski, 1992; J. Plank. Res. 14: 1511-1539).

A MONITORING PROGRAM FOR HARMFUL ALGAL BLOOMS

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Washington state has a long history of harmful algal blooms as indicated by the presence of toxic shellfish and mortalities of pen-reared salmonids. While selected areas are monitored for toxins in shellfish by the Washington Department of Health (WDH; PSP since 1957, domoic acid since 1991), little information is available on the temporal and spatial distribution of the causative algal species. Further, the species that kill fish, *Heterosigma akashiwo, Chaetoceros convolutus* and *C. concavicornis*, are not now known to produce toxins so the best way to determine their presence before fish die is by routine phytoplankton monitoring that can also detect the presence of toxin producers, often before the shellfish become toxic.

In 1990, in response to requests from local fish growers, a system was designed so the growers could monitor their own sites. This consisted of training for sample collection and phytoplankton identification, a picture guide to the harmful and common nonharmful species in local waters based on the published literature, and rapid field support when a bloom occurred. A phytoplankton hotline was started for participants to call in their results, which are summarized so that anyone calling the hotline can obtain the information.

During the two-day workshops, participants were introduced to the phytoplankton with a lecture describing the major taxonomic groups, e.g., diatoms, dinoflagellates, and flagellates, and illustrated with 35 mm slides and overheads showing pertinent morphological characteristics. Emphasis was on potentially toxic species found in local waters, but other common and frequently abundant and/or rare species were also illustrated. Following a brief review on how to use a microscope, time was spent looking at samples and cultures provided by us and by the participants. On the second day, there were lectures and demonstrations on how to collect phytoplankton and how to analyze the samples quantitatively. Students had the opportunity to set up samples for and use an inverted microscope, but because most of the participants usually use standard microscopes, counting slides (e.g., Palmer-Maloney, Sedgewick-Rafter, and haemocytometers) were demonstrated and were available for testing.

When domoic acid first occurred in Washington in the fall of 1991, the program was expanded to include shellfish growers, personnel from state (Agriculture, Ecology, Fish and Wildlife, Health, Sea Grant), and federal agencies (Department of the Interior, Food and Drug Administration, National Marine Fisheries Service), local Native tribes,

and the general public. An additional 45 people were trained and the field guide expanded. Workshops were also given in California, Oregon, and Alaska.

The usual protocol for fish and shellfish growers is to collect at their home sites daily or as frequently as deemed necessary for a particular site. Samples are analyzed at the sites (species identifications, relative abundances) and results telephoned to our hotline number daily or weekly. State and federal agency personnel usually collect at a variety of sites and bring the samples to us at the University of Washington for analysis. A relatively regular sampling and analysis schedule is in place for the Washington Department of Health Office of Shellfish Programs and a recent one-year project at 20 of the 33 sites monitored for shellfish toxins by WDH helped predict domoic acid in Hood Canal mussels in the fall of 1994. Collections by other agencies are often done only when there are kills of either wild or penned fish and when blooms are apparent. We (University of Washington School of Oceanography personnel) also sample at a number of sites, usually on an irregular basis, and also report our findings on the telephone hotline. University personnel (Oceanography, Fisheries) are available to do intensive sampling (plankton and environmental parameters) and/or sample analyses when a harmful event occurs. We are usually able to respond within two-three hours after receiving a call for help depending on the site and its distance from the University.

We have responded to or provided information on a variety of blooms, including:

1. *Heterosigma akashiwo*: A bloom in central Puget Sound in July 1990 killed Atlantic salmon at one of the fish farms. Two UW people spent a day sampling and analyzing the samples to determine the source of the bloom and to locate a bloom-free area where the grower could tow his pens. A bloom in an embayment in southern Puget Sound killed wild fish in late September 1994. Up to five UW personnel collected phytoplankton and environmental samples over a 4-day period to determine the responsible organism and environmental conditions.

2. *Rhizosolenia setigera*: A yellow-brown mat of this diatom occurred in southern Hood Canal in August-September 1991 and lasted about 6 weeks. Local home owners had never seen anything like this and it apparently has not happened again.

3. *Pseudo-nitzschia australis*, *P. multiseries*: A bloom in Hood Canal in November-December 1994 was found during the WDH phytoplankton monitoring. Domoic acid occurred in mussels (ca. 10 µg/g) and in phytoplankton (ca. 14 µg/g).

4. *Ceratium/Prorocentrum* bloom: Blooms occurred off the open Pacific Coast in August 1994 and August-October 1995 and were found during routine phytoplankton monitoring. Razor clams on coast beaches and oysters in coastal bays turned pink. Growers could not sell their oysters because consumers thought they were toxic (they were quite safe). Calls came from oyster growers, a Japanese food distributor, and local Native tribes.

5. Unidentified green flagellate: A yellowish-green cloud in Blaine Harbor (near the Canadian border) was observed by the harbor master in August 1996. He collected a sample from the cloud and we collected additional samples the following day. The samples were kept alive and brought back to our laboratory for identification. The bloom disappeared overnight and was no longer visible when we arrived. No fish or bird distress was noted. We could not positively identify the flagellate (possibly a *Chlamydomonas*) and the diatoms in our samples were species common to Puget Sound. This is a good example of the transient nature of many of the blooms about which we receive calls.

In summary, our phytoplankton monitoring program uses a multi-agency cooperative approach that also includes community involvement.

The project took several years to get going even after the training workshops. It was difficult to get people, even agency personnel who routinely do monitoring work, to collect samples and/or call us when something unusual was seen. The usual excuse was "I didn't have anything to collect in." Even when people did sample for us, they did not always collect the same set of data each time.

We are available to go into the field when someone calls about a fish kill or other unusual incident, or at least suggest ways for them to collect and get the samples to us.

Unfortunately, the program as originally conceived seems to be winding down. Grower and agency personnel have changed; fewer growers are collecting samples, in part due to market-driven consolidation, and only one (a fish grower) continues to make regular calls to the telephone hotline. Moreover, agency priorities have changed in response to budget limitations.

A real bonus from this program is that we are gradually obtaining useful information not only on the toxic species, but on all species in western Washington waters. This includes species present, relative abundances, and temporal and spatial distributions.

Looking to the future, we are deploying unattended in situ sensors that monitor and record environmental data (air and surface water temperatures, incident radiation [PAR], wind speed and direction, rainfall, and surface conductivity). One is in place in central Puget Sound (National Marine Fisheries Service facility at Manchester, WA) and the second will be put near the mouth of the Grays Harbor estuary on the open Pacific coast. Although these sensors will not collect phytoplankton, they will provide a consistent set of environmental data. We will collect phytoplankton samples regularly at these and other sites and will continue to receive samples from others as their time and resources permit.

BIOPHYSICAL PROCESSES ASSOCIATED WITH PHYTOPLANKTON DYNAMICS IN THE SOUTHERN GULF ISLANDS, BRITISH COLUMBIA

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From 1992 to 1994 we studied four sites in the southern Gulf Islands and Strait of Georgia, to extend our knowledge of oceanographic processes controlling the development of algal blooms in the area. Sampling was carried out weekly in spring and summer; less frequently during the rest of the year.

The four sites were selected for widely differing oceanographic regimes: Saanich Inlet is a normally quiescent fjord, with limited freshwater input at the head, mainly in the winter, and limited exchange with outside waters over a sill at the mouth. It is subject to semi-urban development pressures, with sections closed to shellfish harvesting due to sewage contamination from septic seepage and agricultural runoff, and regular summer closures due to PSP. Swanson Channel, between North Pender and Moresby Islands, is in an area of strong tidal mixing, where oceanic water moving in from Juan de Fuca Strait is drawn to the surface. The Active Pass station, actually in the Strait of Georgia, two nautical miles north of the east end of the pass, was chosen as representative of waters outside the Fraser River plume, although it was frequently affected by the plume in summer; the Georgia Strait station, a further two miles north, was normally within plume waters,

We will show some preliminary analyses, including temporal variability in temperature, salinity and transmissivity over the three year period. Data from Saanich Inlet show a poor correlation between the concentration of toxic *Alexandrium* spp. in the water column and the appearance of PSP toxins in nearby shellfish, indicating that monitoring of these taxa is not of practical assistance in this location with respect to seafood product safety monitoring. Finally, we will present data on seasonal variability in the occurrence of *Heterosigma carterae*, *Chaetoceros convolutum* and *C. concavicorne*, which can cause mortality of net-penned fish in aquaculture operations.

HARMFUL *CHAETOCEROS* SPP. AND *HETEROSIGMA* SPP. ON THE CONTINENTAL SHELF WEST OF VANCOUVER ISLAND

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The diatoms *Chaetoceros convolutum* and *C. concavicorne*, and the raphidophyte *Heterosigma carterae* have caused substantial economic loss to British Columbia salmon aquaculture operations, through killing of net-penned fish. The industry has in recent years moved a number of farms to inlets on the west coast of Vancouver Island, in order among other things to reduce their losses due to these harmful algae. However, they remain susceptible to blooms occurring both internally within the inlets where the farms are located, and from blooms advected from offshore locations on the continental shelf. Review of phytoplankton taxonomic data from the continental shelf off the west coast of Vancouver Island (based on 639 samples collected between 1980 and 1989) shows that these harmful species occur frequently throughout the area.

H. carterae occurs in about 7% of samples collected in May, 20% in June, dips to 13% in July, thereafter rising to 23% in August and 40% in September. The majority of our samples contained low concentrations (<1000 cells.L^{-1}). However during July 1985 a bloom was encountered on La Perouse Bank off the southwest coast of the island, with peak cell concentration of 3.9×10^7 cells.L^{-1}). *Chaetoceros convolutum* and *C. concavicorne* (counts combined in these samples) showed increasing frequency throughout the spring summer and fall, from a low of 15% in April to 85% in September, before dropping back to 28% in October. These too normally showed low concentrations, with only 5 of 252 positive samples exceeding 1000 cells.L^{-1}. The maximum recorded, 34,600 cells.L^{-1}, was in Barkley Sound on southwest Vancouver Island in June 1989. Typically, fish farms in inner coastal waters have suffered losses from *H. carterae* in mid-late summer and from *C. convolutum / concavicorne* in late fall.

I have used discriminant analysis to assess whether there are distinctive water properties associated with the presence and concentration of these taxa. Water properties included in the analysis of the phytoplankton samples are depth, temperature, salinity, chlorophyll, nitrate+nitrite, orthophosphate and reactive silicate. On the basis of the analysis, presence of *H. carterae* can be predicted correctly in 75% of samples, and absence in 61%, incorporating depth, temperature, salinity, orthophosphate and reactive silicate. This improves to 74% and 73% in samples containing >5,000 cells.L^{-1}. *For C. convolutum + C. concavicorne* samples containing these can be correctly distinguished in 62% of cases, and absence correctly predicted in 84%, incorporating depth, salinity, chlorophyll nitrate+nitrite and reactive silicate in the analysis. Removing nutrients from these analyses marginally reduces the predictive power of the analysis. However, further development of this approach, incorporating such variables as light and water column stability,

and incorporating some site-specific data, may provide the basis for more directed and efficient monitoring programs for fish farms, by monitoring easily measured environmental variables and reducing the detailed microscope analyses.

COMPARISON OF THREE GREEN-FLUORESCING DNA STAINS IN FLOW CYTOMETRIC STUDIES OF GROWTH RATES AND CELL CYCLE OF DINOFLAGELLATES AND DIATOMS

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Quantification of cellular DNA content throughout the cell cycle has been used to estimate *in situ* growth rates of phytoplankton. However, interference of chlorophyll auto-fluorescence with DNA stains that emit in the yellow/orange region of the spectrum has been a drawback in the use of flow cytometers equipped with argon lasers (488 nm - blue excitation). Extraction of pigments is not always satisfactory for some species, such as the toxigenic benthic dinoflagellate, *Prorocentrum lima*. We explored the use of three recently introduced green-fluorescent stains, TOTO-1, SYTOX and PicoGreen, and applied them to the harmful dinoflagellates *P. lima, P. minimum, P. micans, Alexandrium tamarense, A. fundyense, Scrippsiella trochoidea,* and diatoms *Pseudo-nitzschia multiseries* and *Ps. pungens*.

TOTO-1 and PicoGreen have been considered as the best DNA stains for marine prokaryotes (Li et al., 1995; Marie et al., 1996). Our results showed that all three stains are equally good for the two *Pseudo-nitzschia* species, but TOTO-1 is not satisfactory for the dinoflagellates tested. The saturation concentrations of the stains are 0.5 - 4.5 μM for TOTO-1, 2 - 10 μM for SYTOX, and dilutions of 60 to 125 fold for PicoGreen as supplied by Molecular Probes Inc. (concentration is not released by the manufacturer) (Fig. 1). For individual stain, no significant difference was found among the species tested.

Addition of cell membrane permeants (Triton-X-100 or Nonidet P40) one or two minutes before the addition of preservative (1% paraformaldehyde - PFA) is better than at the time of staining. For the dinoflagellates, it is critical to add the cell membrane permeant *before* preservation by PFA. Addition of methanol or ethanol (50% of final concentration) within 2 hours of preservation improves substantially the permeability of dinoflagellate cell membrane. However, caution should be taken when applying methanol or ethanol to diatoms since the cells tend to form clumps and attempts to disaggregate will damage the integrity of the cells.

Generally, the order of preference of the three stains is SYTOX > PicoGreen > TOTO-1. SYTOX can be used for all 8 species and the optimal stain concentration does not change significantly with the size and concentration of cells. For permeablized cells, the staining is rapid and stable during a period indicated in Table 1.

Since all these stains are specific to dsDNA, application of RNase is not necessary, which is consistent with the work of Li et al (1995) and Marie et al (1996). The results with *Pseudo-nitzschia*

multiseries show no significant difference in fluorescence of stained cells before and after the treatment of RNase for >2 hours at room temperature; similar results were found with *Prorocentrum lima*.

Studies of *in situ* growth rates and cell cycles in relation to toxin production are in progress.

Table 1. Time needed for optimal DNA staining (at room temperature in darkness, stain concentrations were at saturation levels) for flow cytometry of each species.

TAXA	Minimum (min)	Best within (h)	Maximum acceptable (h)
Prorocentrum lima	10	5	12
P. micans	10	5	12
P. minimum	5	5	12
Alexandrium tamarense	10	5	12
A. fundyense	10	5	12
Scrippsiella trochoidea	10	5	12
Pseudo-nitzschia pungens	1	1.5	12
Ps. multiseries	1	1.5	12

Note: Cells stained by SYTOX and kept at room temperature in darkness can last for more than two weeks with only minor fading in fluorescence. No antifading reagent was added.

References:

Li, W.K.W., J.F. Jellett and P.M. Dickie, 1995. DNA distributions in planktonic bacteria stained with TOTO or TO-PRO. *Limnol. Oceanogr. 40:* 1485-1495.

Marie, D., D. Vaulot and F. Partensky, 1996. Application of the novel DNA stains YOYO-1, YO-PRO-1 and PicoGreen for flow cytometric analysis of marine prokaryotes. *Appl. Environ. Microbiol. 62:* 1649-1655.

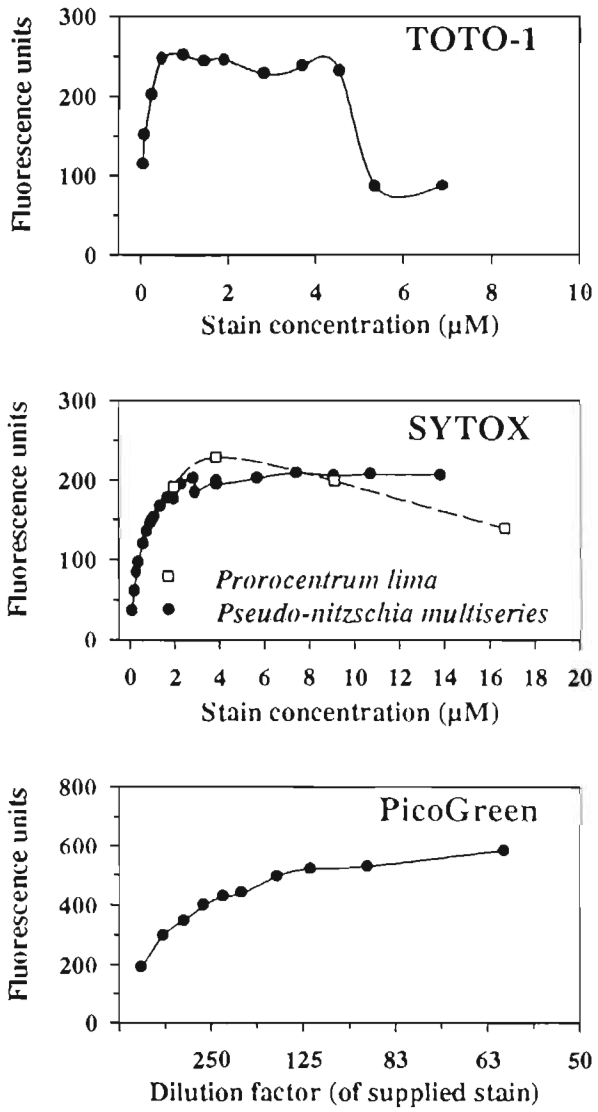

Fig. 1. *Pseudo-nitzschia multiseries* and *Prorocentrum lima*. Relationships between stain concentrations and fluorescence intensities of DNA-stained cells. Each datum point is the mean of 4 replicates.

PHYTOPLANKTON AND POTENTIALLY HARMFUL SPECIES IN THE COASTAL WATERS OF KHANH HOA PROVINCE, CENTRAL VIETNAM

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Phytoplankton surveys were implemented from March to December, 1995 in the coastal waters of Khanh Hoa province where aquaculture enterprises are being developed.

Two hundred and three phytoplankton species were identified as follows: Cyanophyceae (6 species), Bacillariophyceae (150 species), Dinophyceae (45 species), and Dictyochophyceae (1 species). Of these, 23 species are potentially harmful (approximately 11% of the species total recorded).

Outbreaks of some species, such as *Coscinodiscus* sp. and *Noctiluca scintillans* are considered to be important and can be related to growing eutrophication.

The distributions and cell densities of *Pseudonitzschia seriata, Prorocentrum micans, P. compressum, P. mexicanum, Alexandrium affine, A. catenella, Ostreopsis lenticularis* are also reported.

AN ELISA FOR THE DETERMINATION OF DOMOIC ACID IN SHELLFISH

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As part of a programme to develop ELISA for the more important algal toxins contaminating shellfish an ELISA for domoic acid (DA) has been developed. Antigens and plate-coating toxin-protein conjugates have been prepared by two methods (i) Hapten A: domoic acid - NHS activated ester derivatives were prepared in a modification of the method of Newsome *et al.* 1991 giving rise to linkage via any of the 3 carboxyl sidechains and (ii) Hapten B: domoic acid derivatised by a newly developed procedure that attaches protein to the secondary amino group in the domoic acid ring. These haptens have been conjugated to BSA, ovalbumin, thyroglobulin and fetuin by standard methods.

Antibodies raised against Hapten A conjugated to ovalbumin have been used in conjunction with Hapten B - BSA conjugate as the plate coater to develop an indirect competitive ELISA for domoic acid. The ELISA has not yet been fully optimised but currently has a limit of detection of 1 ng DA/ml and a working range of 1 to 1000 ng DA/ml (equivalent to 0.1 - 100 µg DA/g shellfish flesh assuming a 200-fold dilution during extraction cf. maximum permitted level 20 µg DA/g flesh). Improved sensitivity is expected from the optimised assay. Cross reaction with kainic acid is less than 0.01 percent and there was no cross reactivity to glutamic acid or a number of its analogues. Thus these compounds, which would interfere with the membrane binding assay for domoic acid as they bind to the glutamate receptor used in the domoic acid assay, will not interfere with the domoic acid ELISA.

Antibodies raised against Hapten B have been used to establish an ELISA with improved assay sensitivity achieving a detection limit of 0.02ng/ml (4ngDA/g shellfish flesh).

PHYTOPLANKTON DYNAMICS IN BOSTON BAY, SOUTH AUSTRALIA - A PRELIMINARY SURVEY

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Introduction

Boston Bay is situated on the south-eastern side of Eyre Peninsula, South Australia (Fig. 1). It is approximately 17km long and 6km wide with a maximum depth of about 20m. Boston Island, which is 7km long and 3km wide is located in the middle of the bay. Channels at the northern and southern ends of the island allow exchange between Boston Bay and Spencer Gulf. Boston Bay is used extensively for recreation and industry. Seacage tuna farming started in 1990. Each seacage holds about 2000 tuna which are fed daily with pilchards.

Various sources of pollutants contribute between 0.3-0.8 mg/L of total nitrogen daily to the waters of Boston Bay. Dissolved inorganic nitrogen is held to be one of the most important growth-limiting nutrient for phytoplankton in coastal marine waters (Jeffrey and Hallegraeff, 1990). Waste products from fish farms add large amounts of phosphorous and nitrogen to the water column. Previous studies in Boston Bay have revealed cysts of the toxic dinoflagellate *Alexandrium* sp. in the sediments around the main wharf, and cysts from the non toxic phytoplankton *Polikrikos*, *Gonyaulax* cf. *digitale* and *Operculodinium centrocarpum* (Hallegraeff and Andrijanic, 1995).

This preliminary study, assessed the spatial and temporal variation of phytoplankton composition, abundance and chlorophyll a concentrations in Boston Bay.

Materials and Methods

In situ measurements of salinity, water temperature and dissolved oxygen were taken. Initial phytoplankton net hauls were conducted at 18 sites over two days in March and 20 sites June. Additional surveys were undertaken in September and December when water and net samples were taken at three day intervals, on five consecutive occasions.

Water samples were taken with a 2 litre Niskin bottle from 2m below the surface and 1m above the sea floor. A subsample (500ml) was filtered through a 0.45 m Millipore filter, frozen and kept on ice until chlorophyll a measurements could be made. Chlorophyll a was extracted with 90% acetone and measured fluorometrically (Parsons et al., 1984). Phytoplankton species

composition and abundance were determined semi-quantitatively from vertical net hauls. Counting of algal cells was achieved using a Sedgewick rafter cell (McAlice, 1971).

Results

During the sampling period temperature and salinity ranged from 13.5-20.5^{o}C and 35.6-36.7^{o}/oo respectively and there was no obvious stratification in the water column.

Average chlorophyll a levels (both top and bottom) for September were generally higher than December and ranged from 0.287-0.751 g/L and 0.2-0.63 g/L respectively. For both September and December average chlorophyll a levels inside the bay were higher 0.46-0.59 g/L, than on the outside of the bay which showed an average of 0.25 g/L. Large variation in chlorophyll a levels were detected on a daily basis at sites inside the bay. The largest coefficient of variation (CV=34%) was at a site associated with a tuna lease (TC1). A good correlation was found between surface chlorophyll a and chlorophyll a levels near the seabed.

Phytoplankton were identified down to genus or species level with >90 taxa found. Several are potentially toxic (Table 1). Composition of the phytoplankton communities was found to be uniform at all sites but abundance varied temporally and spatially. Diatoms remained an important component of the phytoplankton communities although the proportion of dinoflagellates to diatoms altered temporally. In March and June (Fig. 2a and b) dinoflagellates were a higher proportion of the phytoplankton, whereas in September and December (Fig. 2c and d) diatoms were dominant. Species succession (Fig. 3) was similar to other areas of the world.

Discussion

This survey showed there was no obvious stratification in Boston Bay. The dynamics of the phytoplankton in the bay were affected by altering environmental conditions such as water temperature and tide movement.

The survey of phytoplankton in Boston Bay demonstrated similar dynamics to other regions of Australia. Chlorophyll a levels in Tasmanian waters range from 0.3-2.4 g/L (Jeffrey and Hallegraeff, 1990) and in Boston Bay levels were well within this range. Differences in chlorophyll a levels between sites on the western side of the island and the eastern side of the island are attributed to the various nutrient inputs and differences in water movements inside Boston Bay.

The large variance in daily measurements of chlorophyll a levels around the tuna cages were possibly an artefact of benthic algae and detritus falling into the water from the tuna cage nets. The succession of diatoms in spring and summer to dinoflagellates in autumn and winter in Boston Bay and surrounds is a common phenomenon (Hallegraeff and Reid, 1986).

The potentially harmful algae found, although not in large numbers, will require careful monitoring in future. Particularly as it is not known at what cell abundance, potentially toxic algae of the type found in Boston Bay, are able to kill large fish such as the bluefin tuna *Thunnus thynnus*.

Acknowledgements

The authors would like to thank the many people who assisted in the compilation of this report: The Tuna Boat Owners Association of Australia (TBOAA) for providing the funding, the staff at the Lincoln Marine Science Centre, Brenton Hage (SARDI, Port Lincoln), Dr. G. Hallegraeff (University of Tasmania), Helen Luloffs (Flinders University of South Australia), Dr J Mitchell (Flinders University of South Australia), Stephanie Seddon (SARDI-Aquatic Sciences), Alistair Smart (TBOAA), Mark Stewart (TAFE College-Port Lincoln) and Gavin Wright (SARDI-Aquatic Sciences).

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Table I: Potentially harmful phytoplankton found in Boston Bay. The location and known effects of these taxa are listed below with the appropriate references.

Algae	Organisms affected	Numbers in Boston Bay	Location	Reference
Dinoflagellates				
Dinophysis acuta	toxic to shellfish, causes diarrhetic shellfish poisoning (DSP)	<2cells/ml	rare in Australia, North-west Spain, Sweden, Netherlands	Edler and Hageltorn, 1990, Hallegraeff, 1991, Reguera et al., 1993.
Dinophysis fortii	DSP in shellfish	<2cells/ml	no incidents of human poisoning in Australia	Hallegraeff, 1991
Gymnodinium sanguineum	non-toxic, large numbers correlated with juvenile oyster mortalities (anoxia)	1-4cells/ml	Fisher Island	Bricelj, et al., 1992
Gonyaulax sp.	causes anoxia in large numbers, causes paralytic shellfish poisoning (PSP)	<2cells/ml	Hong Kong	Shumway et al., 1990; Lam & Yip, 1990
Scrippsiela sp.	non-toxic, causes anoxia in large numbers	<2cells/ml	world wide	Hallegraeff, 1991
Ceratium furca	one report of diarrhetic shellfish poisoning (DSP)	10-500cells/ml	South Africa	Shumway et al., 1990
Ceratium fusus and C. tripos	damage to shellfish due to anoxia	10-2000cells/ml	Puget Sound, Korea and New York Bight	Shumway et al., 1990.
Raphidophycae				
Chattonella sp.	damage to finfish due to anoxia and toxins	10-140cells/ml	world wide	Honjo, 1994
Heterosigma sp.	damage to finfish due to anoxia and toxins	10-200cells/ml	world wide	Honjo, 1994
Diatoms				
Chaetoceros sp.	large numbers, may cause mechanical damage to fish gills	100-40000cells/ml	world wide	Hallegraeff, 1991
Pseudonitzschia sp.	amnesic shellfish poisoning (ASP)	5-2500cells/ml	California and Australia	Walz, et al., 1994; Hallegraeff, 1994

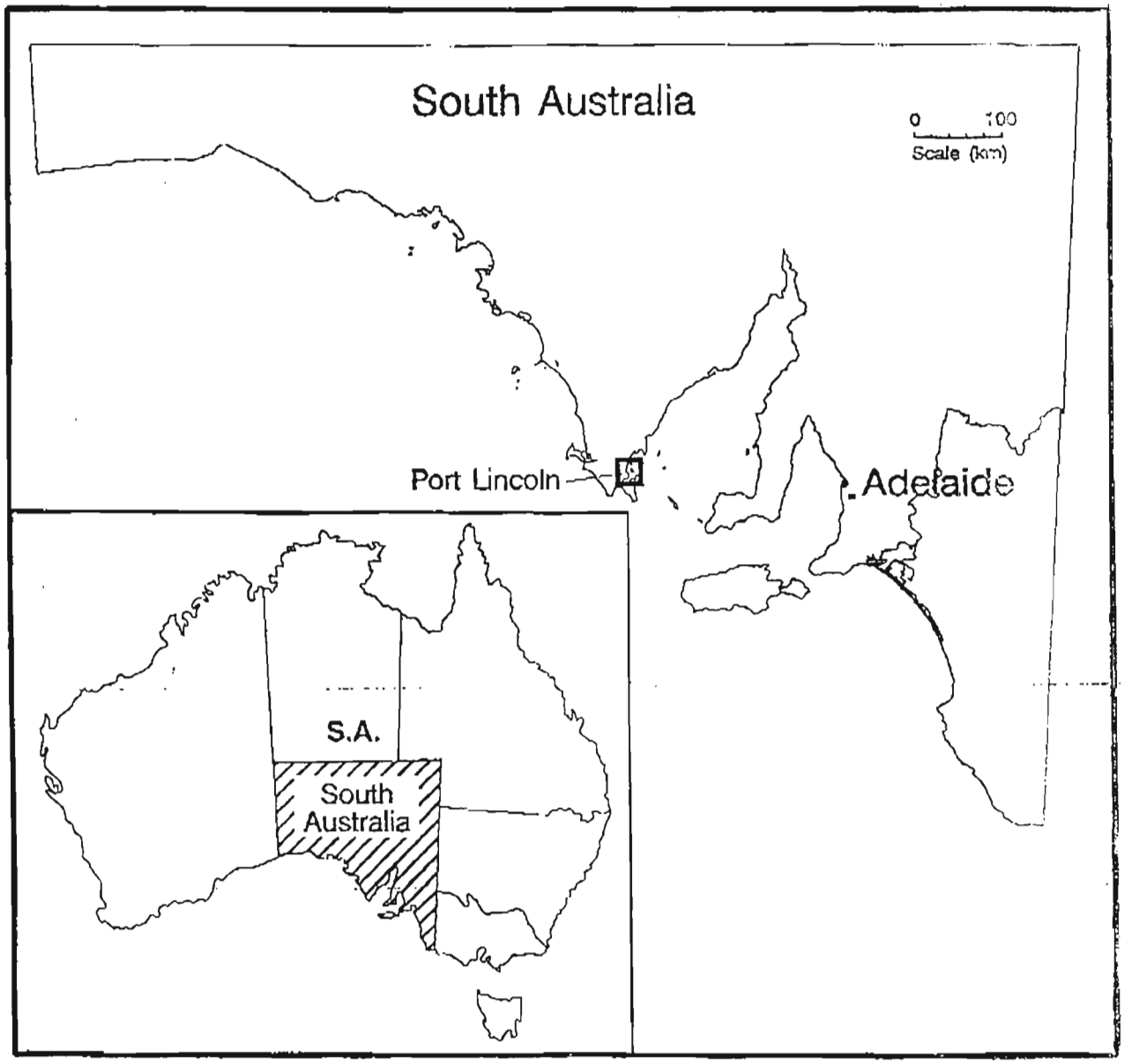
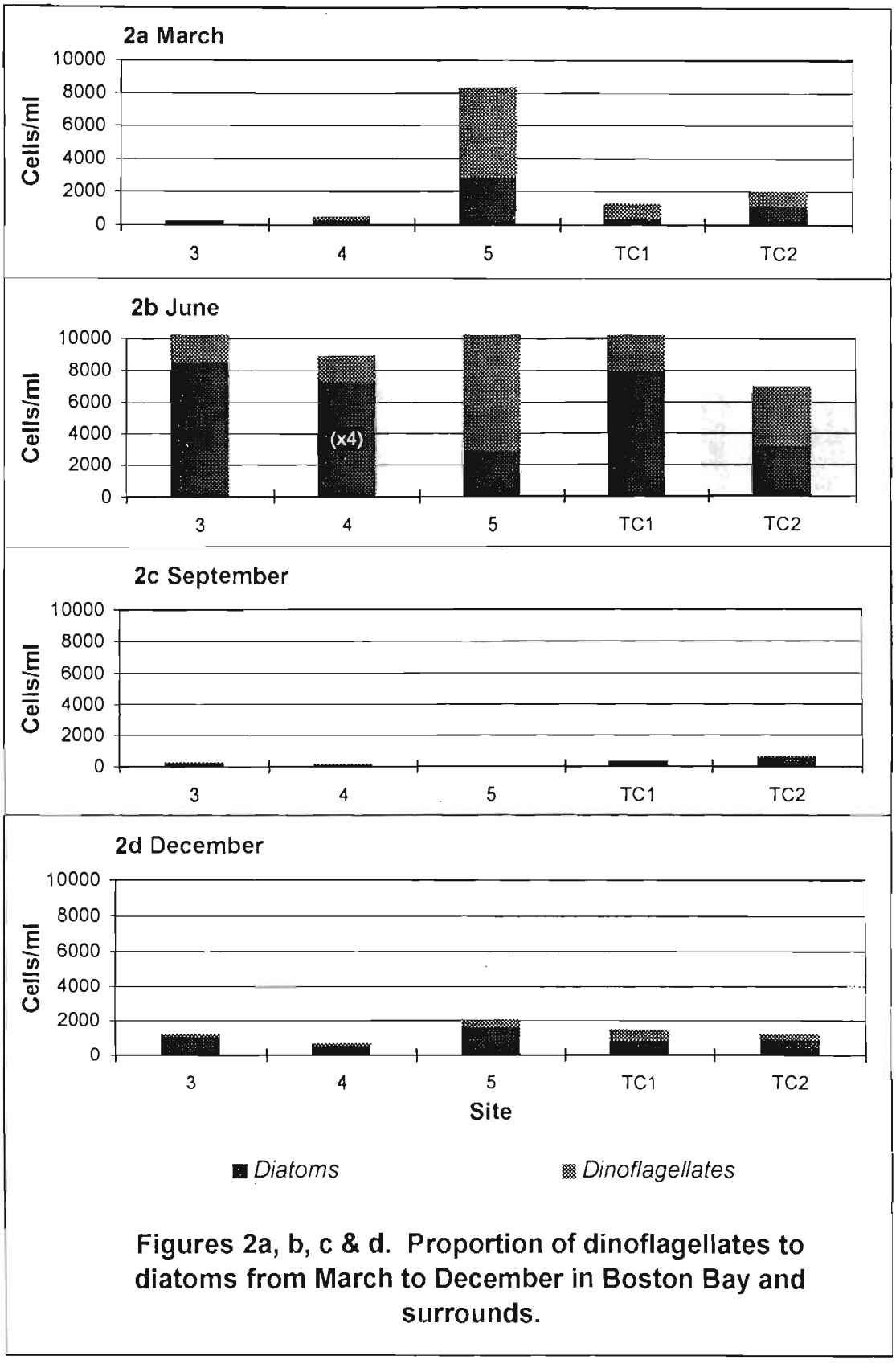


Figure 1. Map of Port Lincoln, South Australia.



Figures 2a, b, c & d. Proportion of dinoflagellates to diatoms from March to December in Boston Bay and surrounds.

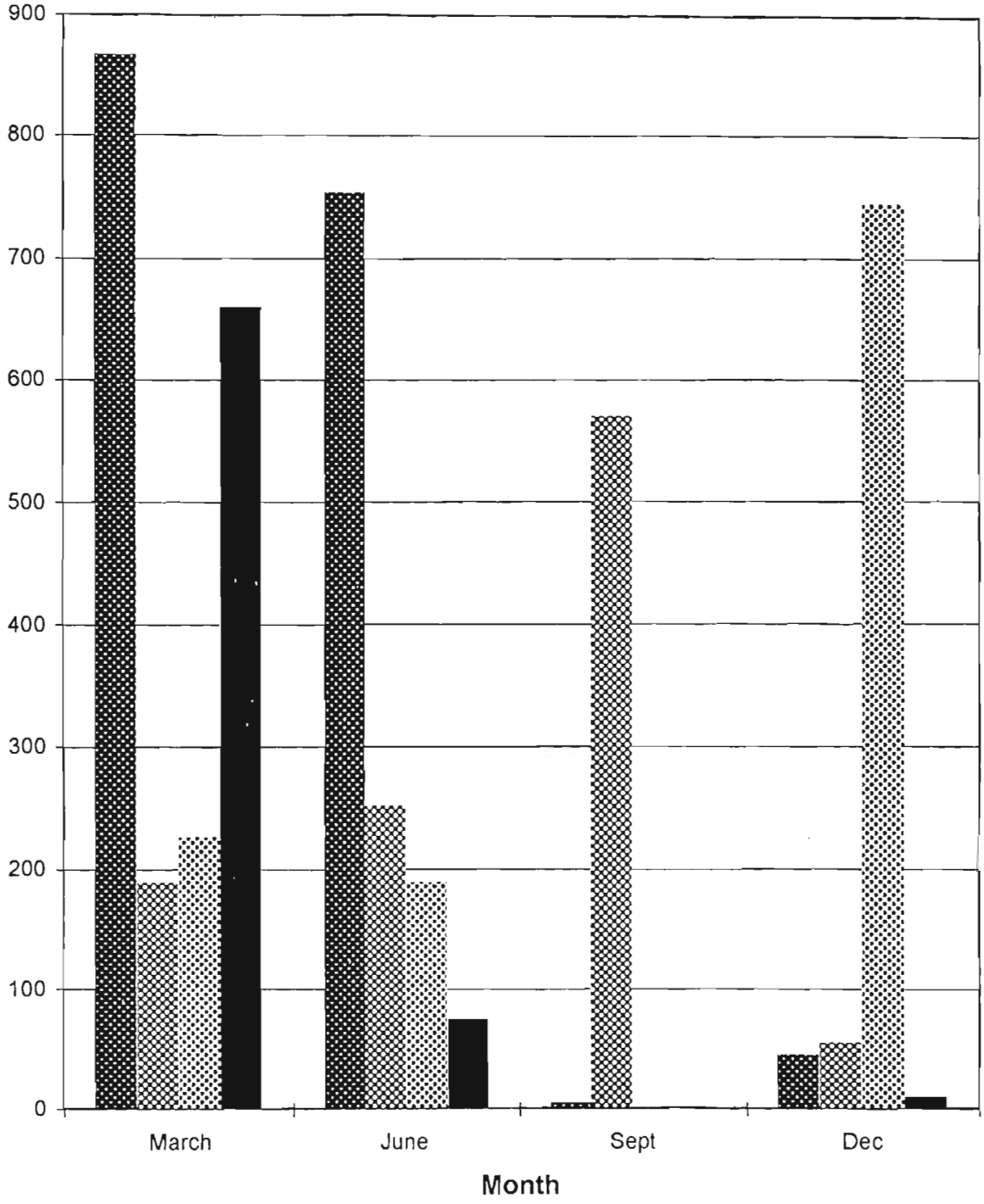


Figure 3. Species succession from March to December 1995 in Boston Bay and surrounds.

PACIFIC COAST HARVESTING CLOSURES ON THE INTERNET - PILOT PROJECT

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We have developed a home page that can provide information on shellfish harvesting closures for algal toxins, sewage contamination and other contaminants by fisheries management area and subarea, the geographical units that are referred to in the harvesting closure notices issued by DFO. The web page address is:

www.ios.bc.ca/plankton/closures/closures.htm

Users access a map of British Columbia, select north or south coast, which each display maps of the management areas. They can then zoom in on a particular area and see a display of its subareas. By clicking on a subarea, they access a file that provides the detailed information on closures. Also included are links to the legal descriptions of the areas and subareas, and to an address and phone list of DFO field offices. As this is still in a pilot project state, users are fully cautioned that the data may not be up to date, and that they should consult with their local DFO office to obtain current information.

This approach has other potential applications for disseminating fisheries management and research information, either internally or to the public. These could include information on catch statistics, fisheries openings, and biological, physical or chemical data.

INCIDENCE OF RED TIDES IN SETO INLAND SEA, JAPAN: OVERVIEW ON LONG TERM MONITORING AND CURRENT HARMFUL SPECIES

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The long-term monitoring on the incidences of red tide has been performed since 1950 in Seto Inland Sea, Japan. Intense monitoring system was established in 1972. Sixteen Prefectural Fisheries Experimental Stations (including its branch) carrying out the regular oceanographic surveys in coastal waters of Seto Inland Sea throughout a year. If a red tide occurs, Prefectural Fisheries Experimental Station immediately informs data of red tide, i. e., species, density, distribution, water temperature, salinity, and fisheries damages to the Fishery Regulation Office of Seto Inland Sea, Nansei National Fisheries Research Institute, and related fisheries cooperative associations and fishermen. In addition, "Airplane Watch" are carrying out every week in summer by Fishery Regulation Office, which is successful to inform the red tide distribution to the fishermen in time.

The incidence of red tide in Seto Inland Sea rapidly increased from 1960 to 1975. During this period, number of factories were build along the coasts and urbanization was highly accelerated, which resulted in eutrophication of Seto Inland Sea. In 1972, large scale red tide by *Chattonella antiqua* occurred and killed caged yellowtail of 7.1 billion yen worth in Seto Inland Sea. Maximum incidence of red tide reached about three hundred cases a year in 1976. "The Temporary Regulation for the Protection of Environment of the Seto Inland Sea" was established in 1973 to prevent the water pollution and coincidental red tide occurrences. Consequently, this action led to the decrease of red tide occurrences due to regulation of the nutrient input. However, about hundred cases of red tide still occurred every year.

Major red tide species in Seto Inland Sea are genus *Chattonella* (*C. antiqua*, *C. marina*, and recently *C. verruculosa*), *Gymnodinium mikimotoi* (=*Gymnodinium nagasakiense*), *Heterosigma akashiwo* (=*Heterosigma carterae*), genus *Prorocentrum* (*P. dentatum*, *P. triestinum*, *P. minimum*), *Noctiluca scintillans*, and diatoms. Toxic algal blooms due to *Dinophysis* spp., *Alexandrium* spp. and *Gymnodinium breve* were also observed in the Seto Inland Sea. *Chattonella* species and *G. mikimotoi* are the most causative species involving massive killing of fish and shellfish in Seto Inland Sea since 1970. The economical loss due to *Heterosigma akashiwo* red tide are minor as compared with those by the genus *Chattonella* and *G. mikimotoi* in Seto Inland Sea. Currently, the incidences of *Chattonella* red tide significantly have declined, but the red tide by *G. mikimotoi* still causes huge damages on local mariculture. In 1995, massive red tide of *G. mikimotoi* occurred in western part of Seto Inland Sea, and caused 639 million yen worth of economical loss on fish and shellfish. Previous studies demonstrated that

Chattonella and *G. mikimotoi* red tides cause the physical clogging of gills by extreme mucus excretion, damage of the epithelial tissues of gill by hemolytic substances (i.g. polyunsaturated fatty acids), neurotoxin production, and the production of oxygen radicals from red tide cell and/or in the fish tissue. However, precise mechanism of fish-killing is still unclear. Escape from the red tide area and/or stop of the feeding are the most common means to prevent the damage of cultivating fish from red tide. Grazing pressure by zooplankton seems to be low in the occurrence of *Chattonella* and *G. mikimotoi* red tide. However, decline of *G. mikimotoi* red tide sometimes caused by heterotrophic flagellate, *Gyrodinium dominans*.

Since 1988, incidence of the new red tide species, *Heterocapsa circularisquama* (small armored dinoflagellate) has increased remarkably in western Japan including Seto Inland Sea. The red tide of *H. circularisquama* involves mass mortality of bivalves. *H. circularisquama* red tide at Hiroshima Bay in 1995, killed 278 million Japanese yen worth of the culturing oyster and short-necked clam. According to the field observation and laboratory experiments, *H. circularisquama* reduces the clearance rate of bivalves at the density of 50-200 cells/ml, and kills them at 5,000-10,000 cells/ml in association with vigorous "clapping", retraction of mantles and gills, valve closure, and alternation of cardiac activities. Although the toxicity of *H. circularisquama* on bivalves are extraordinary, any fish-killing and shellfish poisoning, and human illness has not been observed in this red tides.

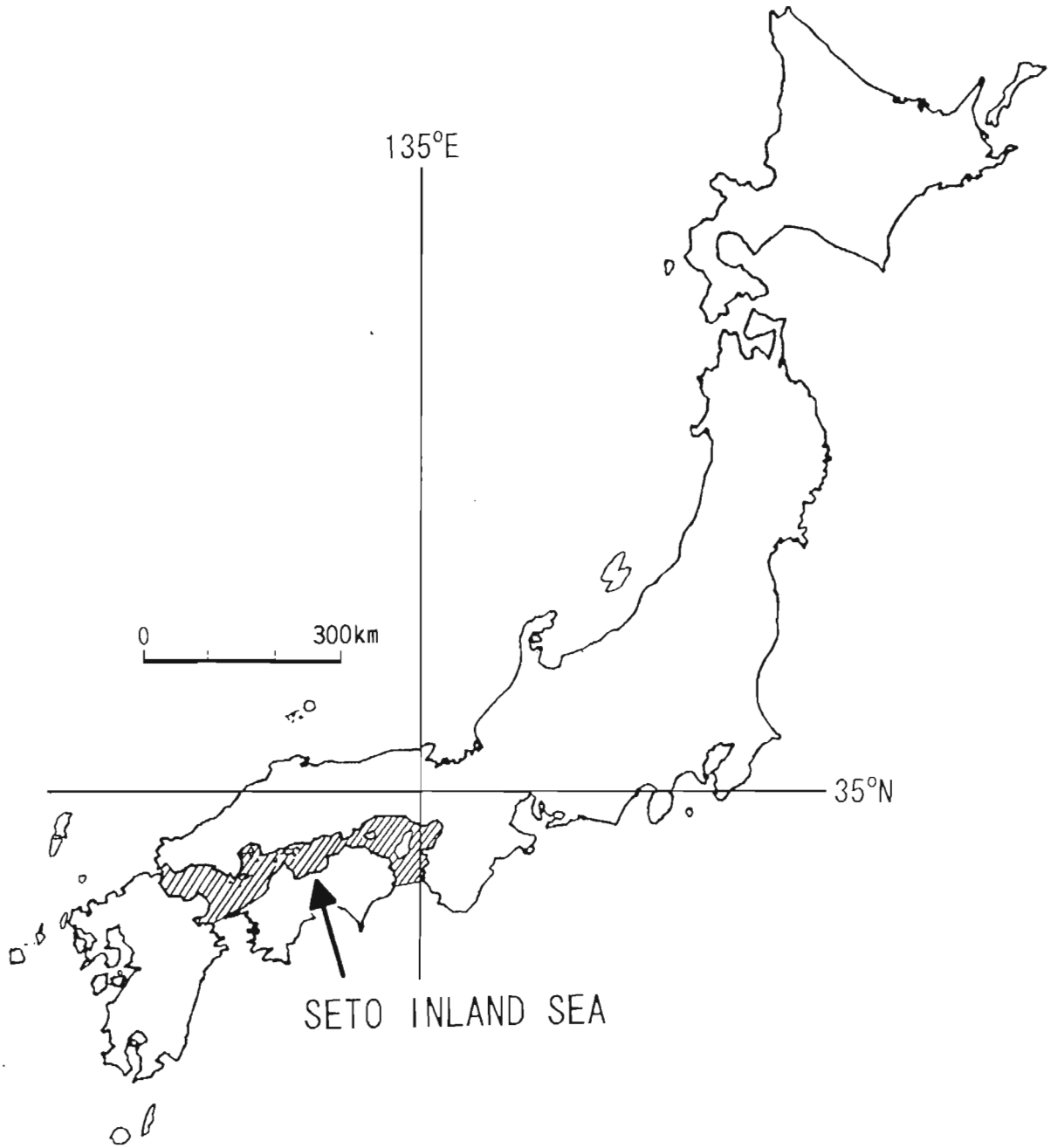

Fig. 1. Location of Seto Inland Sea

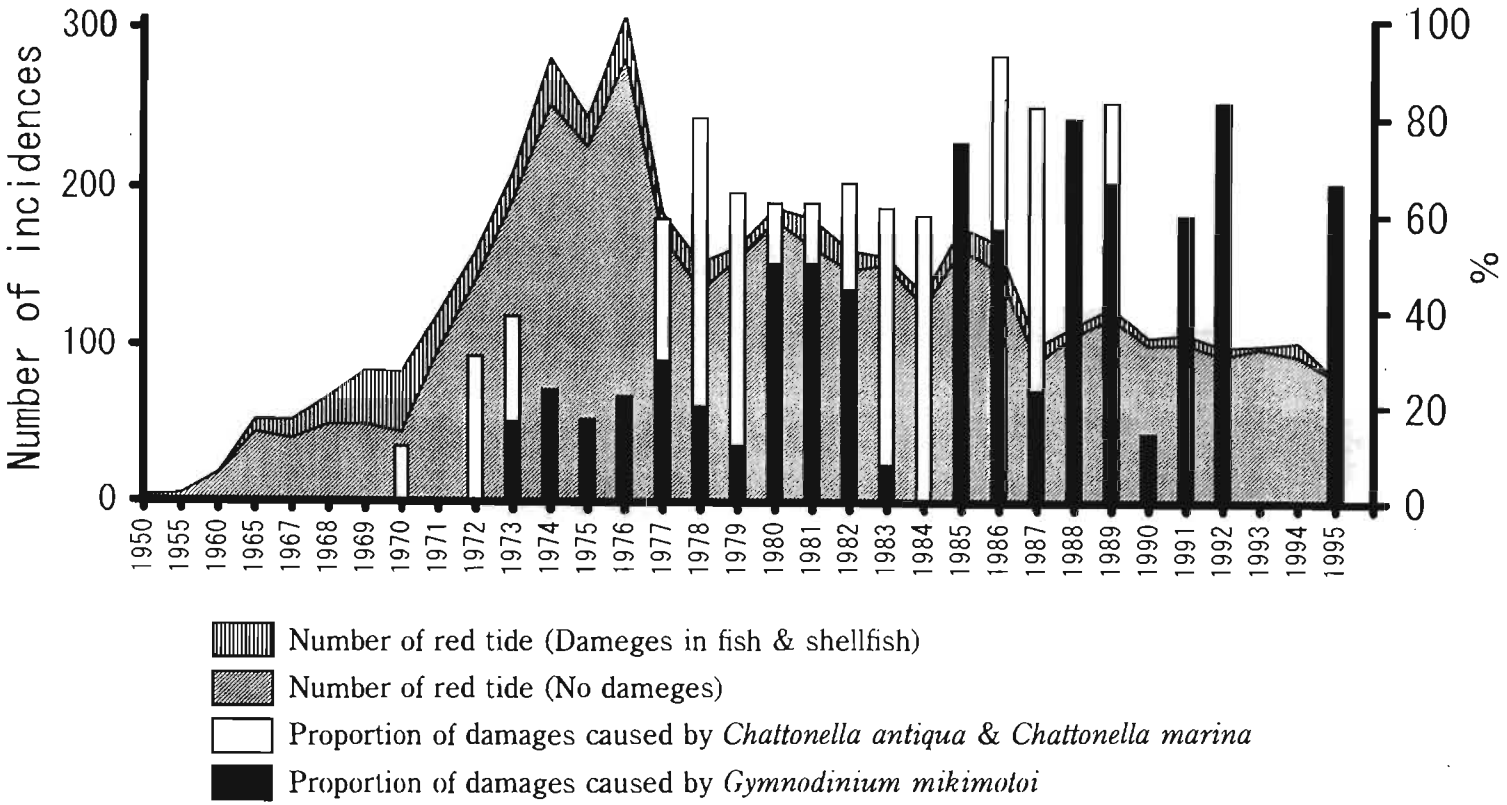

Fig. 2 Changes of the incidences of red tide and its damages on fish and shellfish from 1950 to 1995 in Seto Inland Sea, Japan
The data sets were obtained form "*Red Tide in Seto Inland Sea*" published by Fishery Regulation Office of Seto Inland Sea.

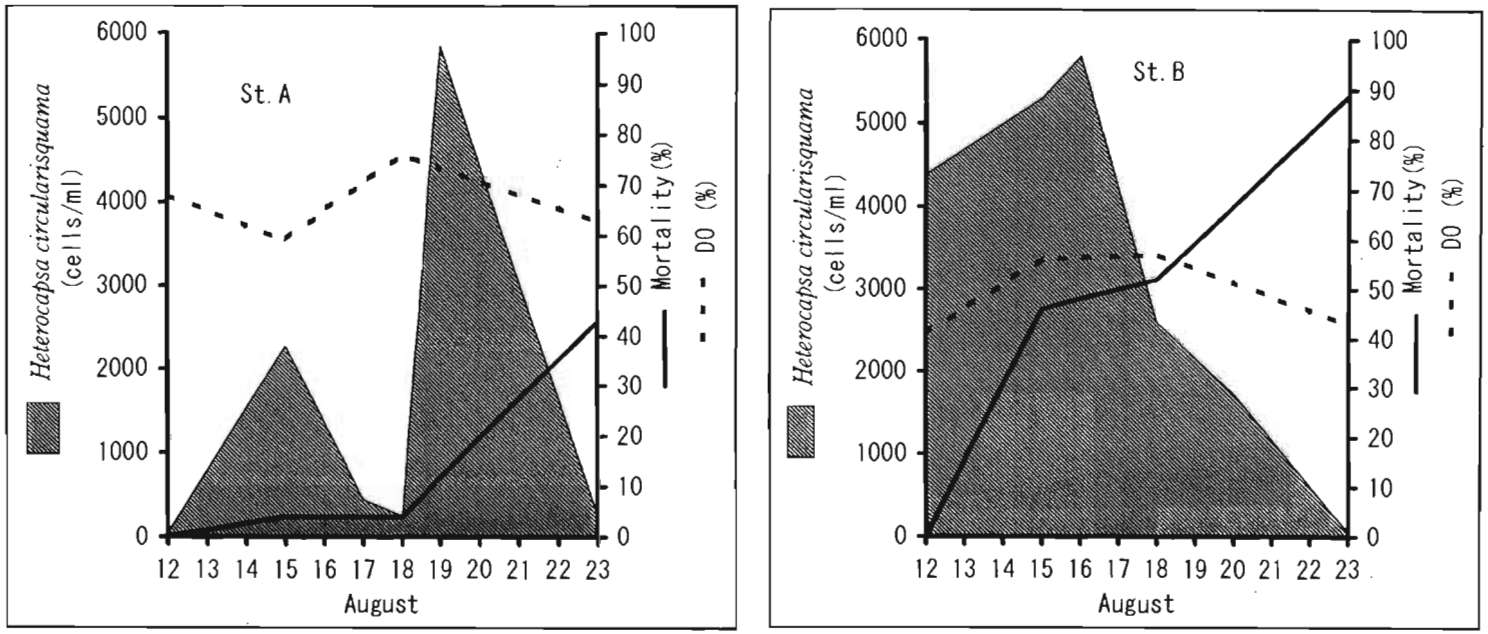

Fig. 3. Changes in cell density of *Heterocapsa circularisquama*, coincidental mortalities of pearl oysters, and saturation of dissolved oxygen at 5m on August 1994, in Ago Bay. (Matsuyama et al., 1996)

WORKING GROUP REPORTS

REPORT OF WORKING GROUP #1

ANALYTICAL METHODS FOR PHYCOTOXINS AND HARMFUL ALGAE

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1. Introduction

The objective of this working group was to review analytical methods for phycotoxin and harmful algal research. The goal is to provide a summary of current, new, and developing methods, and to determine those areas of methodology which show the greatest promise or need. This summary is intended to help direct efforts in developing useful techniques for detecting harmful algae and their toxic products.

2. Requirements for Analytical Techniques

The working group began by separating analytical methodology into two distinct categories: regulatory and research. It was realized that the needs of these two groups were different.

Regulatory:

Regulatory methods ultimately need to be rapid, cost effective, and simple. There is a willingness to compromise both quantitation and the ability to discriminate between all derivatives of a toxin group for a quicker, more cost effective method. It was suggested that a useful regulatory method for toxin detection could be a simple dock-side +/- test. While one would lose the ability to give an absolute number for toxicity, the test would provide the necessary information to harvesters or regulators to make a rapid and informed decision about the toxic status of a product or water sample for a reasonable financial cost. One point noted during this discussion, was that this type of specific rapid method would not have the ability to detect new or unknown toxins which might otherwise be detected using a more general test such as the mouse bioassay.

Approval for the use of such techniques for toxin detection will take some time, and institutions such as Health Canada will have a large impact on their approval and acceptance. Nevertheless, a collective interest in developing such a rapid +/- test was expressed during the session.

Research:

Research methods for toxin analysis have a different directive. These analyses need to be highly accurate, precise, consistent, and robust. Analytical research requires that all derivatives of a toxin be identified and quantified. For this reason there is a concern about reporting in units such as "saxitoxin-equivalents" for paralytic shellfish poisoning toxicities.

3. Detection Methods for Toxins and Harmful Algae

The working group reviewed the current and developing methods, with emphasis on toxins responsible for paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), and amnesic shellfish poisoning (ASP), as these poisonings were recognized as the most important from a Canadian perspective.

Immunoassays:

Two kits for detecting DSP toxins are currently available. An immunoassay dip stick test for detecting domoic acid is being developed. A kit for PSP toxins should be possible though it may require 2 assays; one for neosaxitoxin and related toxins, and another for the saxitoxin group. It was felt that one hurdle impeding the development of immunoassays is the cost and availability of purified toxins to produce antibodies and conjugates, even though only small quantities of toxin are required for the production of antibodies. International collaborative efforts would help this process. The group felt that these assays are valuable because of their specificity and ability to detect the actual toxins, as opposed to detecting toxicity. Also, the cost of setting up equipment for immunoassays is low because the same equipment could be used for tests of all classes of toxins. Nevertheless the point was made that cohesive international support is needed to bring these methods into general use.

Receptor or Enzyme -Based Assays:

The receptor-based assays have the advantage of being integrative. That is, they recognize the toxicity of the toxins, and therefore provide a quick diagnosis for regulatory uses. The approach can be used to detect many of the known toxins already. The assays are cost effective because one set-up could be adapted for tests of different classes of toxins, as is true for the immunoassays. These tests are also rapid. There has been a concern over using radioactively labeled ligands in receptor-based assays, however it was felt that further research and development should be able to overcome this problem through the use of new tags.

Cell-Based Assays (including cytotoxic and non-cytotoxic):

Cell assay kits for PSP toxins and brevetoxins are nearly ready for market distribution and are currently being tested in the field. This PSP kit has the advantage of reporting the specific action of the toxin directly (as a sodium channel blocker), and not just cell death. However, there

was some concern raised over other cell assays which may report non-specific responses. The major disadvantages with the cell assays are the long incubation periods and short shelf-life of the assay kits. Nevertheless it is believed that such an approach may also work for DSP and even CTX toxins.

Enzyme Assays:

Many of the comments made for receptor-based assays also apply here. There was some discussion about the possibility of developing phosphatase assays for detecting both the DSP toxins and microcystins. The assay is already operating in research labs. It was felt that such an assay could be adapted for regulatory use, and may be simpler and more rapid than the ADAM method. However highly specific enzyme-based assays may not detect inactive pro-toxins such as DTX_4 etc.

DNA / RNA Assays:

It was agreed that DNA and RNA based assays (molecular probes) hold a lot of promise for detecting shellfish toxins. These particular assays would apply to the causative phytoplankton species and permit detection of phytoplankton (or any living cell, e.g. bacteria, fungi etc.) with the ability to produce toxins. This type of assay may also lend itself to genetic research into the biosynthetic machinery present in toxic and non-toxic phytoplankton, the molecular features that control it, and resistance factors.

4. Phytoplankton Monitoring

The working group recognized that there remains some debate about the use of phytoplankton monitoring as a predictive tool for shellfish toxin monitoring. However, it was felt that phytoplankton monitoring does provide information which cannot be gathered through toxin analysis alone.

Phytoplankton monitoring is an inexpensive tool which may be useful for prediction of toxic or harmful events in some areas. Regional monitoring also provides information on the introduction of new species to locations, and about the population dynamics of phytoplankton in general. Gaining a better understanding of all phytoplankton may help us to understand the dynamics of toxic populations. Once again molecular probes would be invaluable in this process. Another important consideration is the protection from fish killing species such as *Heterosigma sp.* and *Chaetoceros sp.* that monitoring provides for fish aquaculturists.

In general, it was agreed that phytoplankton monitoring will provide an early warning system until such time as complete toxin testing is fast, reliable, and inexpensive.

REPORT OF WORKING GROUP #2

ECONOMIC IMPACTS OF HARMFUL ALGAL BLOOMS

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1. Introduction

Harmful algal blooms (HABs) adversely affect fisheries in Canada, both through mortalities of fish and shellfish stocks and by the accumulations of toxins in tissues making the fish or shellfish unsafe for the human consumer. The group realized the varying economic impacts resulting from HABs, including considerable losses to the industries on one hand and resulting funding for research and management on the other. In addition, human health may be affected and if there is a failure to protect the human consumer, there can be significant socioeconomic impacts. This was experienced first hand in 1987 when the eastern Prince Edward Island mussel crisis occurred resulting in permanent disabilities and deaths among people who consumed mussels containing high concentrations of phycotoxins.

Wild harvest and aquaculture industries have also suffered serious losses with significant associated socioeconomic losses. At present, the Canadian government allocates substantial resources to research, regulation and management of harmful algal bloom (HAB) problems. Economic analysis would assist in setting some realistic objectives and aid in allocating funding in order to meet current and future Canadian obligations to industries and consumers.

Questions the group addressed were related to:

- the costs of failure of the management system
- the cost of management and research and is it commensurate with the industry's value
- the total economic impact of HABs on industry
- whether economic impacts intersect with policy and political concerns

It was determined that within each of the four concerns there are components that are easily quantified and others where costs are more difficult to measure.

2. Failure of the Management System

If the present monitoring system fails, contaminated products that have been distributed on the market need to be located and recalled. This requires additional manpower, resources, and prompt action. Medical costs for short and long term treatment of affected victims may be relatively easily quantified. Indirect costs of medical problems such as disabilities, loss of productivity, and value of human life are more difficult to quantify, as is the damage to the seafood industry as a whole through loss of consumer confidence and reduced sales. The latter frequently affects seafood products as a whole, in addition to the specific product that was recalled.

3. Management and research

Information on values for government funded programmes such as those for the inspection of products to be marketed, monitoring, and research funding for government, universities and the seafood industry can be easily quantified as can industry monitoring programmes. More difficult to assess are costs associated with whether the resources applied to inspection and research programmes respond to industry and public perception of the scale of the problem and what the value would be of the research that would be done if there was not an economic drive.

4. Impact on Industry

Costs associated with the value of landed stock, delayed harvest due to unsafe levels of toxins in tissues, disposal of stock that is unsafe and obtaining new stock, value of destroyed stock, holding stock for elimination or clearance and the cost of meeting regulatory requirements can be easily quantified. Those costs related to loss of opportunity, value of additional production that would occur in the absence of the problem or with increased monitoring, loss of consumer confidence as noted above (both affected product and other seafood products), and impacts on tourism would be more difficult to determine.

5. Economic implications of policies and politics

Marketing and public education costs can be quantified easily whereas impacts on trade, interprovincial marketing, implementing new monitoring methodologies, meeting changed standards and political influences are more difficult.

6. Concerns

The group discussed issues related to the growth (in many areas) of the aquaculture industry which requires assurance that areas that they are expanding into, or the new species that they are bringing into culture, are of sufficiently low risk from biotoxin contamination losses that they will be viable.

It was recognized that it is difficult to obtain reliable information from both shellfish and finfish aquaculture industries or other government agencies on losses suffered and on resources applied to the problem. Farmers view this as privileged information and are reluctant to divulge losses or their problem source. These concerns are associated both with competitive performance compared to other aquaculture companies and with their dealings with insurers.

Education was stressed by participants as being very important. Although the seafood industry is generally well-attuned to the problem, non-traditional and illegal harvesting pose significant risks to public health and will impact on industry as a whole if people become sick. In addition, consumer awareness needs to be improved, particularly among tourists.

Restoration of consumer confidence after the failure of a monitoring programme usually is difficult, costly and labour intensive because most often the media is quick to alert the public to a problem, frequently overstating the case, but less diligent in reporting on the resolution of the problem. The recent success of the P.E.I. mussel culture industry demonstrates that public and private partnerships can be successful in reversing these effects. It was felt, however, that in general it is difficult to strike the balance in informing the public of public health hazards while maintaining confidence in the product over the long term.

7. Impact of Government Restructuring

The Canadian government is in the midst of a major structural change in seafood product safety management where the Inspection Branch of the Department of Fisheries and Oceans (DFO) is being transferred to the Canadian Food Inspection Agency. We are also at a critical junction in government support for research. Concerns were raised for the implications of these changes on industry. For example, what will be the economic implications to industry, if it is required to fund product safety monitoring? These will include increased costs passed on to the consumers, reduced competitiveness in the export market, and demands for a greater say in management.

Following discussion about downsizing and cutbacks within the government an important question was raised: **"What are the implications if a core research capability to respond to a crisis is not maintained?"** The view was expressed that we would be hard pressed to respond today to a crisis of the scale faced in 1987, when P.E.I. mussels were found to be contaminated by domoic acid in 1987. This was successfully managed through the combined efforts of numerous personnel from several federal agencies, the provincial government, industry and universities.

8. Recommendations

Participants recommended that the Department of Fisheries and Oceans undertake the following reviews. These would establish the value of the industry and the costs to industry of harmful algal blooms and, based on these, establish the level of resources that government should

allocate for research and management of the problem.

- DFO should undertake a basic review of the wild harvest and cultured shellfish industries and the finfish aquaculture industries, combined with the resources applied to research and management of phycotoxins. The review should include an assessment of the costs to industry of harvesting closures on its' profitability. Ultimately, it should include an in-depth breakdown of costs.

- DFO should also undertake, through its economic staff (in collaboration with research staff), a full accounting of selected issues, in order to demonstrate the practicability and value of the review. Examples for inclusion include: an assessment of the economic impact of industry taking on the costs of monitoring for product safety; the value and potential management costs associated with development of mussel aquaculture in the Bay of Fundy; and an assessment of the value of the recreational shellfish harvest in British Columbia, including the impact of harvesting closures, and the economic impact on the seafood industry of one consumer death.

- DFO should also review current communications and public education approaches to industry, media and the medical profession, to assess where changes in approaches will result in better protection of human health and development of more collaborative efforts within industry.

When undertaking these assessments and reviews, DFO should draw on expertise from outside the department such as Health Canada, Statistics Canada, National Research Council and other external sources.

REPORT OF WORKING GROUP #3

BACTERIA AND ALGAL BLOOMS

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1. 0 Introduction

From the discussions held, it was clear that the topic of marine bacteria in relation to algal blooms and marine toxins continues to generate interest and remain controversial. Although considerable progress has been made in the past five to six years, questions remain about the production of marine toxins by bacteria and, in general, about the significance of bacteria within the entire framework of harmful algal bloom dynamics and toxin production. Relative to marine algae, for example, how important are bacteria as a toxin source? The discussion was limited to marine bacteria, as it is now accepted that certain freshwater cyanobacteria are a source of PSP toxins. It was also acknowledged that certain marine bacteria are reported to produce tetrodotoxins. The term "phycotoxins" is often used to refer to those toxins produced by phytoplankton (e.g., PSP and DSP toxins, brevetoxins, domoic acid, etc.). When it is unequivocally demonstrated that bacteria also produce these same toxins, then the term "marine biotoxins" may be a more accurate descriptor.

2.0 Convincing Chemical and Biological Evidence Required

To date, most of the work on bacterial production of "phycotoxins" has been on the PSP toxin group, and it is on this area that this part of the discussion was focused. Two aspects were identified that require solid evidence in order to convince those scientists who remain skeptical about the autonomous production of PSP toxins by bacteria.

2.1 Chemical Evidence

Up to now, all of the chemical evidence for the production of PSP toxins by bacteria has essentially been derived from HPLC retention times. This has been supported, in some cases, by the specificity of both sodium-channel blocking and receptor binding assays (both of which indicate that compounds with the same pharmacological action as PSP toxins are present in the samples). Recent supporting evidence has started to emerge from mass spectrometry, in this case, selected ion monitoring by capillary electrophrophoresis coupled to mass spectrometry (CE-MS). Although progress is being made and the CE-MS results are the first data of this type, selective

ion monitoring is not definitive and a full chemical spectrum by MS-MS or NMR is required to produce unequivocal evidence for the production of PSP toxins by bacteria. The difficulties in this kind of work were discussed, including that relatively large quantities of material may be required, and that complications arise due to the direct injection of liquid samples into the mass spectrometer. Nevertheless, efforts are underway in some laboratories to establish this type of definitive chemical evidence.

2.2 Biological Evidence

Most of the recent work on the autonomous production of PSP toxins by bacteria has focused on bacteria grown in the laboratory in pure culture. However, at this conference a paper was presented (Levasseur et al.) demonstrating that PSP toxins were associated with bacterial-sized particles in seawater during a phytoplankton monitoring season. This work and that of an earlier published study by Professor Kodama made for a lively discussion. Although HPLC results indicat the presence of PSP compounds, evidence has yet to be presented that toxicity in the bacterial size fraction is actually due to bacteria. There was some disagreement about the type of evidence that is required to determine that these particles and subsequent toxicity were indeed due to bacteria. Some felt that, at minimum, the organisms must be characterized using techniques that generate a physiological and morphological profile consistent with that of a bacterium. A minority felt that the organisms must be identified to genus and species level; that providing an identifying "number" was insufficient, a name was required. Given that the vast majority of marine bacteria are recognized as non-cultureable, the difficulties in actually isolating these strains was discussed. However, it was pointed that using bacterial 16S rRNA sequencing would enable the recognition of bacteria and, in some instances, perhaps allow for identification. An additional step would be to develop species-specific molecular probes against the active bacteria. Modern molecular techniques would also allow for the monitoring of the bacterial population in studies of algal bloom dynamics and algal toxin production (see Section 3.0).

Another approach is to prove that the bacteria have the genetic potential, whether expressed or not, to produce a biotoxin autonomously. It was concluded that this can best be accomplished by developing molecular probes against the genes responsible for biotoxin production. In order to do this, it was suggested that the biosynthetic pathway must first be deduced to identify which enzymes are involved. However, it was pointed out that it would be easier to use molecular techniques to determine which genes were involved, and that this would eventually lead to information on the biochemical pathway, as well as aid in developing the probes. It was recognized that the elucidation of metabolic pathways becomes more difficult as the complexity of the pathway increases, e.g., as for PSP toxins. It was noted that this type of work is actually underway using bacteria, which are ideal model systems because they are much easier to work with than dinoflagellates. For example, biotoxin-producing bacteria could be used to produce molecular probes against that portion of the genome which is involved in coding for toxin production. This task may be facilitated by use of bacterial mutants that no longer have the capability to produce a given biotoxin. Once developed, the molecular probes can be used to probe any other bacteria (or other organism) for the potential to produce a given biotoxin.

3.0 Role and Significance of Bacteria in Relation to Marine Biotoxins

Given that the identity of a biotoxin can be confirmed and that marine bacteria can be proven to be involved in biotoxin production, the remaining discussion was devoted to determining the possible significance of these bacteria to the overall scheme of biotoxins in the ocean. Several roles of bacteria, and their relevance, were identified as follows.

3.1 Algal Bloom Dynamics

Although it was recognized that marine bacteria can sometimes play a significant role in controlling algal bloom dynamics, this topic was not discussed in detail in the Workshop Session. It is known, for example, that bacteria produce exudates that are capable of either stimulating or inhibiting phytoplankton growth. This may have an important impact on the development and/or decline of algal blooms, both benign and harmful.

3.2 Toxicity of the Algae

The Working Group discussed how marine bacteria, in addition to being autonomous producers of marine biotoxins, may also play a role in influencing the toxicity of the algae. Examples were given of how the removal (using antibiotics) of bacteria from cultures of *Pseudo-nitzschia multiseries* resulted in a decrease in domoic acid production by this diatom. Re-addition of bacteria resulted in an enhanced production of toxin, although the mechanism for this action has not yet been identified. The bacteria, themselves, were not shown to be capable of domoic acid production.

A long discussion was then devoted to the problem of providing convincing evidence that a culture of phytoplankton is indeed bacteria free (axenic). In the strictness sense, is there even such a thing as an axenic algal culture? In classical "proofs", the use of various organic media may miss the presence of "non-culturable" bacteria, and visual examination to verify the absence of bacteria is likewise unreliable. Essentially, then, these questions revert to the same arguments that were mentioned above (Section 2.2). It was therefore concluded that molecular techniques are necessary to convincingly prove that a culture is axenic. This becomes important, it was pointed out, because the presence of even a few toxigenic bacterial cells may contribute toxicity that may be mistakenly attributed to the algae.

During the discussion of axenic cultures, it was questioned whether the antibiotic treatment used to kill the bacteria can alter the toxin biosynthetic pathway of the algae. It was noted that antibiotics, although supposedly specific to prokaryotes, can also adversely effect eukaryotes. With one exception, the potential effects of antibiotic treatments themselves on algal growth and toxin production are therefore unknown. Indeed, it was pointed out that *Pseudo-nitzschia multiseries* cultures retained their ability to produce domoic acid when bacteria were reintroduced, even after antibiotic treatment to render the original diatom culture axenic.

The relevance of bacterial involvement in biotoxin production by algae was then discussed. Bacteria could be an explanation for several as-yet-unexplained observations about changes in toxicity of certain algae. For example, it is well known that some strains of *Alexandrium tamarense* are more toxic than others. There are even non-toxic strains of this, and other, dinoflagellate species. Recently, at least three cases of toxic *Pseudo-nitzschia pungens* have been reported. The involvement of bacteria in this toxicity cannot be ruled out, although addition of bacteria to non-toxic strains of *P. pungens* has not induced toxicity. Similarly, changes in the bacterial species composition within non-axenic (= xenic) cultures of algae may explain some of the changes in toxicity observed over a period of months to years.

Another controversial area that was mentioned, but not discussed in detail, is the question of the existence of intracellular bacteria and their possible role in controlling algal toxicity. One view was expressed that if intracellular bacteria do exist, then they may simply be considered to be an integral part of the original organism, much as any other organelle. Nevertheless, the role of intracellular bacteria, if any, and if they do exist, must be discerned. This is an important and active area that clearly that requires new approaches, including molecular techniques, to resolve.

3.3 Toxicity of the Shellfish

Bacteria, either directly or indirectly, have the potential to influence the toxicity of wild and aquacultured molluscan and other shellfish. As discussed above, bacteria may produce marine biotoxins autonomously. If so, and given the known ability of molluscan shellfish to feed on bacteria-sized particles and on aggregations of particles, then bacteria must be considered another possible vector to toxify the shellfish. The importance of this source, relative to the larger algal cells, remains to be determined. Experiments are required to determine if biotoxin-producing bacteria, alone or in association with other small-size class particulate material, may toxify the shellfish. Likewise, additional field observations are needed to determine if shellfish become toxified in the absence of known toxigenic algal species.

The ability of bacteria to modify the toxicity of the algae was also deemed important in ultimately affecting the level of toxicity reached by the shellfish. For this reason, it was felt that an understanding is required of factors that influence bacterial abundance and species composition. For example, certain marine bacteria may be selectively inhibited by antibiotics used in fish food. Similarly, other bacteria may be selectively stimulated by nutrients from sewage outflows, agricultural run-off, or storm events. Counter to this, it was argued that it may not even be necessary to know if bacteria or algae are the source of the biotoxins; one needs only to measure the toxicity level directly in the shellfish. However, it was ultimately concluded that a basic knowledge is required of the functioning of an ecosystem, including the role of bacteria, in order to improve our predictive capability.

A final area mentioned, but not discussed in detail, is the role that bacteria may play in altering (increasing or decreasing) the potency of biotoxins directly in the gut of the shellfish. This may be accomplished via the biotransformation of the toxins to render them more, or less, potent.

Likewise, bacteria may increase toxicity by producing toxins directly within the shellfish, or, conversely, they may decrease toxicity by metabolizing the toxins. Little appears to be known about these bacterial roles.

3.4 Other Possible Roles of Bacteria

Aside from their involvement in biotoxin production, it was suggested that marine bacteria may have other relevant roles. Net pen liver disease has become a major problem for farmed salmon in Pacific waters. It is known that microcystin-LR is implicated in the salmonid deaths, and that this toxin has been traced to their food, i.e., crab larvae and copepods. However, the ultimate producer of the toxin has not been identified since cyanobacteria, the usual producers of microcystin-LR, have not been detected in those waters. An alternative, bacterial, source for this toxin may therefore be considered.

In the realm of bacterial-algal interactions, it was pointed out that algae may act as carriers of certain pathogenic bacteria. The example was given of a wide range of marine phytoplankton that are known carriers of the bacterium *Vibro cholerae* implicated in cholera outbreaks in several areas of the world. This suggests an alternative pathway for pathogens to enter the human food chain, and raises additional concerns if the pathogens are shown to be associated with the apparently increasing frequency and intensity of harmful algal blooms.

4.0 Viruses in Relation to Bacteria and Phytoplankton

Even though the Workshop Special Session focused on bacteria, it was considered that viruses should not be ignored, and they were therefore included at the end of the discussion. Indeed, viruses are now recognized as being increasingly important in microbial food web dynamics. Viruses are able to infect both bacteria and phytoplankton. A host-specific virulent phage has the potential to alter the bacterial species composition, and this could have ramifications on biotoxin production and bloom dynamics. By infecting phytoplankton, viruses are capable of triggering the decline of algal blooms. Thus far, the only harmful alga to be studied in this respect is the "brown tide" organism *Aureococcus anophagefferens*, which can be infected and lysed by a virus.

Although viruses, alone, do not have sufficient genetic material to produce biotoxins on their own, they may be capable of transferring this capability into the genome of other organisms, including bacteria and phytoplankton. This lateral gene transfer could account for the observed production of the same marine biotoxins by diverse groups of organisms. Again, research is required in this little studied area.

5.0 Conclusions

The discussions pointed out numerous areas in which bacteria could play an important role in the dynamics of harmful algal blooms and in biotoxin production. This provided ample

justification for carrying out research in this field. In order to make continued progress, however, we must apply mass spectroscopic methods for biotoxin identification, and develop molecular techniques for use in laboratory and field situations.

If this is deemed to be such an important area, the question was raised as to why only six groups world wide are currently carrying out research on bacterial production of biotoxins. We were reminded that the study of bacteria in relation to biotoxin production and bloom dynamics is still at its infancy. It takes time for scientists to justify the importance of this research to managers and granting agencies in order to obtain research funding, and then to gear up laboratories for this type of research.

6.0 Recommendations for Research

- Verify the identity of marine biotoxins produced by bacteria via the use of mass spectrometry techniques.
- Develop molecular probes to characterize, verify the identity of, and determine the frequency of occurrence and location of biotoxin-producing bacteria.
- Develop molecular probes against genes that code for biotoxin production in various organisms, e.g. viruses, bacteria, and phytoplankton.
- Apply molecular probes against toxigenic organisms to study algal bloom dynamics in relation to the presence of viruses and bacteria.
- Investigate the effects of bacteria on algal toxin production.
- Study the uptake of toxigenic bacteria and bacterial aggregates by shellfish to determine the relative importance of the bacterial contribution to shellfish toxicity.
- Study the accumulation of biotoxins by shellfish in the field, and document cases of shellfish toxicity in the absence of known toxigenic algae.
- Investigate the possible role of bacteria in modifying (increasing or decreasing) the toxicity within the shellfish gut.
- Study the role of viruses in controlling the population dynamics of bacteria and harmful algal blooms.
- Investigate the lateral transfer of extrachromosomal genetic material (e.g., viruses, plasmids) that codes for biotoxin production.

REPORT OF WORKING GROUP #4

UPTAKE AND DEPURATION OF ALGAL TOXINS

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Introduction

The occurrence of phycotoxins in marine foodwebs poses a hazard to public health and represents a significant economic threat to commercial harvesters of wild and cultured shellfish. Harvest closures by regulatory authorities responding to phycotoxin incidents can result in severe socioeconomic hardship in coastal regions dependent upon the seafood industry. Although it is generally accepted that bivalve molluscs acquire phycotoxins by filtering toxigenic microalgal cells from the water column, there is still insufficient knowledge regarding the uptake and depuration kinetics of phycotoxins in bivalves, as well as the mode of transfer and fate of toxins in other commercially important marine species including benthic crustaceans and pelagic fish.
The present Working Group convened to discuss research strategies concerning the uptake and depuration of phycotoxins in marine animal species, during which the following issues were addressed:

1. Rationale for research
2 Target species of interest to researchers
3. Research methods
4. Uptake and physiological effects
5. Depuration

1. Rationale for research

The Working Group expressed strong support for continuation and expansion of research in this field, however the suggested rationale for research differed among group members. While some proposed that research should be conducted to provide a basis for resource/risk managers to open and close fisheries, others suggested that the focus should be on developing counter-measures for industry to deal with toxic commercial species (i.e. - depuration methods). Clearly there is a need to accomodate both industry and regulatory concerns, and to increase our knowledge of these issues to alleviate the adverse socio-economic impact of harmful algal blooms.

2. Target species of interest to researchers

Several informative studies concerning paralytic shellfish toxin kinetics in important commercial molluscan species, mainly those of the east coast of North America, have appeared in the recent literature (cf. references). However information on toxin kinetics is still lacking for many other marine species. The regular closure of the crab fishery on the Pacific coast due to high levels of domoic acid underscores the need for studies on phycotoxin transfer to commercially important crustacean species. Also of concern is the paucity of information on phycotoxin kinetics in finfish, particularly associated rates of toxin detoxification. Toxin pathways in species considered to be non-traditional seafood products (e.g. - whelks, sea urchins, sea cucumbers) have received little attention although diversification of ethnicity in North America in recent years has led to their increased utilization as food. Several members of the Working Group stressed that no species should be overlooked given the wide array of animals that are harvested for human consumption.

Following the above outline, it was recommended that phycotoxin accumulation should be examined in all tissue compartments of animals, rather than only those body parts that are consumed traditionally. Scallops, for example, are often considered safe for consumption when phycotoxins are present since only the adductor muscle is normally consumed and it rarely accumulates toxins in significant quantities. However, emerging markets for scallops now encourage the consumption of "roe-on" (gonad attached) and half-shell products, which dictate that toxin assessment of different body tissues are required to ensure the public safety.
It was of general opinion of the Working Group that phycotoxin kinetics must be considered species-specific, necessitating acquisition of toxin uptake and depuration data for each species. Assumptions that phycotoxin dynamics in one species will be copied in another should be made with caution due to the incredibly diverse physiological feeding behaviours exhibited among various species in marine ecosystems.

3. Research methods

The Working Group discussed the merits and problems associated with conducting toxification studies in laboratory conditions as opposed in studies performed *in situ*. Although field conditions offer animals a natural environment which is less likely to induce stress-related complications that are often observed in laboratory studies, determination of specific toxin kinetic parameters (uptake, biotransformation, depuration rates) in the field is difficult. Laboratory feeding studies offer researchers the benefit of controlling environmental variables, including algal concentration and cellular toxin content. Thus, researchers should be able to calculate the quantity of toxin ingested by an animal based upon the number of toxic cells consumed.

Several problems associated with laboratory feeding studies were discussed by the Working Group. One of the most critical problems included variability in the toxicity of algal food stock. Cellular toxin content can vary enormously in unialgal cultures depending upon culture conditions and phase of growth. While experimental studies often attempt to feed algae grown

under uniform conditions to shellfish, batch culture cells will almost always vary in toxin content. Members of the Working Group suggested that an attempt should be made to employ large-scale continuous culture systems to grow toxic algal food stock. However this has proven difficult since cell densities under these conditions are often inadequate for meaningful intoxication and necessitate excessive volumes of algae to feed test animals.

The Working Group discussed the design of laboratory experiments for phycotoxin uptake. Some members noted that simple upwellers, such as those used in aquaculture facilities, were sufficient for exposing bivalve molluscs to toxigenic algae. However, others argued that more elaborate mesocosms such as recirculating aquaria are needed to ensure that animals are always exposed to a homogeneous algal supply. Recirculating systems are essential for experiments involving benthic species such as *Prorocentrum lima* and dinoflagellate cysts which will sink or adhere to surfaces in the absence of vigorous resuspension. Furthermore, the possibility of using recirculating flumes was discussed, since the flow created by these systems most closely resembles natural feeding conditions.

Unique complications were experienced by researchers attempting to toxicify benthic scavengers such as crabs with phycotoxins. Problems were reported in providing animals with a homogeneous toxin supply, due to the variability in toxin accumulation in feed organisms (mussels, scallops, clams). One suggestion was that toxins could be fed in the form of capsules, however questions exist as to the stability of various phycotoxins in this form. Behavioural aspects must also be taken into consideration in the case of crabs and other cannibalistic animals. Group members noted problems with feeding animals in tanks due to the tendency of more dominant and aggressive individuals to eat more than others. Thus, researchers were forced to "hand-feed" individual crabs pre-weighed quantities of toxic food.

4. Toxin uptake and physiological effects

Opinions within the Working Group differed as to the goal of exposing animals to toxigenic algae. Some members argued that the main objective should be to load shellfish with phycotoxins merely as a starting point for depuration studies, whereas others noted that useful information can be obtained during the toxin uptake phase. Important toxin kinetic parameters determined from uptake experiments include the time needed for toxin levels in animals to surpass regulatory limits and the toxin level at which tissues become saturated. This information would provide regulatory authorities and growers with an estimate of the time required for shellfish to attain varying degrees of toxicity. Also of interest during the uptake phase is the rate of toxin transfer from the digestive system to other anatomical compartments. If toxins are transferred very slowly to certain tissues (eg. weeks), then it may be possible for growers to harvest animals for market at the initiation of a toxic bloom event.

Another parameter that should be of concern during toxin uptake studies is the metabolic fate of the toxins. Are toxins completely degraded by enzymatic and digestive processes in shellfish, or are they merely converted to more, or alternatively, less toxic compounds? Evidence

has shown that transformation pathways of phycotoxins within shellfish tissue varies enormously depending on the specific toxins, algal species, shellfish species and anatomical compartments. The benefits of using *in vitro* incubation experiments to study toxin biotransformation were discussed. These studies involve incubating purified toxin solutions in tissue homogenates, thereby eliminating variation due to feeding rates and algal toxicity.

The Working Group noted that assessment of physiological effects exhibited by animals following ingestion of toxigenic algae was of considerable ecological importance. Responses observed in bivalves include mortalities, alterations in pre- and post-ingestive feeding behaviour, and changes in motor activity. Of considerable importance is that studies should distinguish between chronic and acute effects when physiological observations are reported. Receptor binding studies were suggested as an effective analytical technique for examining specific activities of toxins in test animals. Such studies would be very useful since it is possible that observations of physiological incapacitation may be a result of laboratory conditions rather than toxin activity, although in most cases the use of control animals can obviate this concern.

5. Depuration

Terminology regarding the process of elimination of phycotoxins from marine animals was discussed by the Working Group. It was noted that in North America, the term "depuration" is usually associated exclusively with fecal coliform contamination in shellfish. Indeed, the U.S. National Shellfish Sanitation Program (NSSP) defines depuration as "...the process of using a controlled aquatic environment to reduce the level of bacteria and viruses in live shellfish..." (Somerset, 1991). The Working Group concluded that "depuration" remains the most suitable term to describe the active elimination of phycotoxins. Care should be exercised at all times to qualify "depuration" either from fecal contamination or from phycotoxins.

Although strategies such as ozone treatment and chlorination have been attempted to enhance depuration processes, efficient and economically feasible phycotoxin depuration techniques have yet to be developed (Shumway et al., 1995). Several members of the Working Group suggested that assisting industry with depuration methods should be an area of focus for researchers. Concern was raised that regulatory authorities should be made more aware of methods presently used by industry to depurate phycotoxins from seafood products. The high degree of toxin variation often observed between individual animals suggests that following a period of depuration, toxins may be absent from some animals but may still be present at dangerous levels in others. The number of animals sampled for testing, the period of depuration deemed adequate for safe consumption of the animals, and methodologies to expedite depuration are all questions requiring resolution prior to safe, effective and economically efficient depuration of phycotoxins from commercial shellfish.

Another area of interest was the treatment of wastewater flushed from toxic shellfish. Toxic dinoflagellates have been observed to survive and divide following passage through bivalve digestive systems, thus there is a very real potential for "clean" waters to be seeded by live toxic

cells released in the feces of depurating shellfish. If toxic animals are to be moved to a "clean" area for depuration, then wastewater must be treated suitably to kill any live cells that may be released during the initial 2-3 days of depuration. The Working Group was less decisive as to the treatment of wastewater following the release of live cells from the bivalve guts, although UV treatment of initial recirculated water prior to flushing may prove effective. Suggestions were made that soluble toxins in the wastewater should be treated, however, others argued that the concentration of toxin would be sufficiently dilute to render it harmless to other marine animals.

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