Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae

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ABSTRACT

Martin, J. L. and K. Haya (Editors). 1999. Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2261: x + 149 p.

The Sixth Canadian Workshop on Harmful Marine Algae was hosted by Fisheries and Oceans Canada, Maritimes Region, in St. Andrews, New Brunswick, on May 27-29, 1998. There were 78 attendees representing government research and management departments, universities and industry. Fifteen posters and 21 oral presentations featured topics on harmful algal bloom organisms responsible for amnesic shellfish poisoning (ASP), paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), or fish mortalities. Discussion groups were held on aquaculture insurance, AOAC methodology for PSP toxin determination, regulation of marine products for the presence of natural toxins, and eutrophication and the potential introduction of *Pfiesteria* and *Pfiesteria*-like organisms.

see intro

RÉSUMÉ

Martin, J. L. and K. Haya (Editors). 1999. Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2261: x + 149 p.

Le ministère des Pêches et Océans Canada, région des Maritimes, fut l'hôte du sixième atelier de travail canadien sur les algues marines nuisibles, à St. Andrews, Nouveau-Brunswick, le 27-29 mai, 1998. Il y avait 78 partitipants en recherche et en gestion des divers ministères gouvernementaux, d'universités et du secteur privé. Quinze affiches et 21 présentations orales traitaient des algues marines responsables de l'intoxication paralysante par les mollusques (IPM), l'intoxication amnésique par les mollusques (IAM), l'intoxication diarrhéique par les mollusques (IDM) ou des mortalités de poissons. Des groupes de discussion out consideré l'assurance aquacole, les méthodes de l'AOAC pour la détermination des toxines de l'IPM, la réglementation des produits de la mer pour la présence de toxines naturelles ainsi que l'eutrophisation et l'introduction potentielle de *Pfiesteria* et des organismes semblables à *Pfiesteria*.

ACKNOWLEDGMENTS

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INTRODUCTION

Occurrences and problems associated with harmful and toxic marine algae have a long and continuing history on both Canada's Atlantic and Pacific coasts. Historical paralytic shellfish (PSP) toxin data and anecdotal evidence indicate that shellfish harvesting areas are closed to harvesting during a part of the year for the entire Pacific and a large portion of the Atlantic coasts; for example, St. Lawrence Estuary and Bay of Fundy, as well as parts of the Gulf of St. Lawrence, Newfoundland and Nova Scotia. More recently, the amnesic shellfish poisoning (ASP) toxin or domoic acid was first detected in blue mussels in eastern Prince Edward Island in 1987. Subsequent additional research and monitoring showed domoic acid and other implicated phytoplankton species besides *Pseudo-nitzschia multiseries* in areas on both the Atlantic and Pacific coasts. Compounds known as spirolides are being linked to large thecate dinoflagellates on the southeast shore of Nova Scotia. The occurrences of *Dinophysis* toxins are more widespread since 1990. Not only are Canada's shellfish industries affected, but the salmonid aquaculture industry has suffered substantial losses in recent years due to algal species such as *Chaetoceros* sp. and *Heterosigma* sp.

Following the domoic acid occurrence in 1987, the Canadian Department of Fisheries and Oceans established a national advisory committee called the Phycotoxins Working Group representing the DFO regions. The terms of reference for the Working Group include: promoting the exchange of new scientific information on harmful marine algae and their effects in a timely fashion; fostering the development of cooperative and collaborative scientific programs; and encouraging new research initiatives. One method of meeting these aims is through sponsoring national workshops on harmful algal blooms, and exchanging research results and ideas about related topics. Through these forums for knowledge exchange, the Phycotoxin Working Group is provided aid in developing recommendations for research priorities in Canada.

These proceedings are a written record of the Sixth Canadian Workshop on Harmful Marine Algae held at St. Andrews, New Brunswick, Canada, May 27-29, 1998. A different approach was taken at this workshop, where authors gave an overview or introduction to their posters prior to the poster session.

Earlier workshops were held in: Moncton, NB - 1989 (Bates and Worms 1989); Dartmouth, NS - 1990 (Gordon 1991); Mont-Joli, QC - 1992 (Therriault and Levasseur 1992); Sidney, BC - 1994 (Forbes 1994); and St. John's, NF - 1996 (Penney 1996). This report contains abstracts, extended abstracts and short papers from presentations, keynote addresses and discussion groups, and a list of participants from the 6th Canadian Workshop.

PREVIOUS WORKSHOP PROCEEDINGS IN THIS SERIES

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: 58 p.

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: 66 p.

Therriault, J.-C. (Ed.). 1992. Proceedings of the Third Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 1893: 154 p.

Forbes, J. R. (Ed.). 1994. Proceedings of the Fourth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2016: 92 p.

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

*HETEROSIGMA CARTERA*E, A MAJOR KILLER OF PEN-REARED SALMON IN BRITISH COLUMBIA

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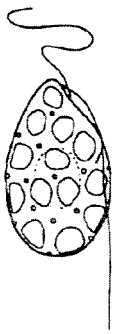
DESCRIPTION OF THE ALGA

H. carterae (10-18 μm in length, formerly *akashiwo*) belongs to the division Chromophyta and the class Raphidophyceae, which also includes species of *Chattonella* (30-50 Tm), *Fibrocapsa* (20-30 μm), and *Oltmannsia* (26-32 μm) (Throndsen 1997). *Olisthodiscus luteus* (12-19 Tm) often misidentified in North America as *Heterosigma* is considered to be the same species (Hara and Chihara, 1987). The morphological shape of *Heterosigma* is round, ovoid and can be flattened depending on its physiological condition (Ajzdajcher 1991). Of the 2 flagella, the anterior pulls the cell while the posterior is less active and trails. There are up to 20 yellow-brown chloroplasts in the cell, and surface mucocysts, mucus-producing vesicles, on the surface that form the glycocalyx film surrounding the cell (Honjo 1992). The alga excysts at 14-15°C to grow vegetatively at low or high irradiance, between 15-30°C, and has strains that can tolerate salinities of 12-40 ppt (Tomas 1978). Recently, a *Heterosigma* bloom was observed at Broughton Island, B.C, where the seawater surface temperature was from 9-10°C. Isolates of this bloom were successfully acclimated to grow at 21°C in the author's laboratory. Growth at diverse ranges of temperature and salinity suggests that *Heterosigma* strains can develop to accommodate the environmental conditions in specific habitats. Vegetative cell division can be as rapid as 2-5 divisions per day, with nutrient harvesting at night in deep water and vertical migration to the surface during daylight for photosynthesis (Honjo and Tabata 1985). Blooms are generally observed in British Columbia in summer when the water column is stratified after the formation of a stable pycnocline. Blooms of *Heterosigma*, which turn the water brown, are responsible for mortalities of aquacultured fish throughout the world.



DISTRIBUTION

Heterosigma, as a ubiquitous alga, causes severe economic losses worldwide to aqua-cultured fisheries such as yellowtail, black sea bream, black sea bass, flounder and salmon. Damage to cultured fisheries in the Seto Inland Sea of Japan, from 1972 to 1987 were estimated at 2 billion yen (Okaichi 1989; Honjo 1992). Blooms, some causing fish mortalities, have occurred in Scotland (Ayres et al. 1982; Gowen, 1987; Johnson, 1988), Spain (Figueiras and Niell, 1987), Eastern Russia (Ajzdajcher, 1991), New Zealand (Chang et al. 1990, 1993; MacKenzie, 1991; Pridemore and Rutherford, 1992), Malaysia (Taylor 1990), Korea (Park, 1989; Yoon et al. 1992), China (Tseng et al. 1991), and the United States (Taylor and Horner 1994).

In B.C., from its first discovery in Nanoose Bay in 1986 to 1996, this alga has caused the loss of about $20 million of pen-reared salmon (Black et al. 1991). However, from July to October of 1997, dense and extensive blooms of *Heterosigma* were responsible for fish mortalities estimated at between $10 - 20 million.

TOXICITY

Species of raphidophycean flagellates are known to possess haemolytic, neurotoxic, haemagglutinating compounds, in addition to producing superoxide and hydroxyl radicals that cause fish mortality (Shimada et al. 1991; Onoue et al. 1990; Ahmed et al. 1995; Khan et al. 1995; Yang et al. 1995). Recent studies by Khan et al. (1997) have indicated that ichthyotoxicity of *Heterosigma* is less than the other raphidophycean flagellates, *Chattonella antiqua* or *Fibrocapsa japonica*, but that the toxicity may reflect the smaller size of the *Heterosigma* cell. Cultures of the toxic strain of *Heterosigma* from Kagoshima Bay produced 4 neurotoxic compounds that corresponded to the brevetoxins PbTx-2, PbTx-3 oxidised PbTx-2, and PbTx-9. These neurotoxins exhibited a violent paralysis leading to the death of juvenile red sea bream at cell densities in excess of 120,000 cells/mL. However, no abnormal behaviour was evident in fish exposed to 30,000 cells/mL of *Heterosigma* containing these toxins. It should be noted that wild blooms of *Heterosigma* are not always toxic, suggesting that formation of toxigenic agents is dependent on its physiological condition.

Other ichthyotoxic compounds produced by both *Chattonella* and *Heterosigma* are superoxide and hydroxyl radicals in addition to hydrogen peroxide (Oda et al. 1992a, 1992b; Yang et al. 1995). The lethality of these reactive oxygen species to juvenile rainbow trout, *Oncorhynchus mykiss,* was reduced by addition of catalase and superoxide dismutase to the medium of *Heterosigma* exposed to the fish (Yang et al. 1995). Apparently these reactive oxygen species only appear when bacterial loads in the medium are high. Their production resembles a phagocytic activity known as the respiratory burst. However, cultures of *Heterosigma* with added *Vibrio* that were grown by the authors showed no lethality to fish. It is known that cultured toxic algae are often less toxic than those in the natural environment (Kodama et al. 1982). The effects of natural blooms of *Heterosigma* on freshwater- and saltwater-acclimated juvenile chinook salmon, *Oncorhynchus tshawytscha*, indicated that the rate of fish mortality was independent of the ambient oxygen level but was dependent on the density of algae and the ambient water temperature (Black et al. 1991). Fish exposed to the blooms showed no signs of respiratory distress, no signs of hyperventilation, no coughing, but did lose equilibrium, assumed a vertical position in the water before sinking, could be handled without difficulty with no reaction to stimuli, and exhibited a typical anaesthetic behavior. No abnormalities or pathology to gills or internal organs were evident in the moribund fish. Cell free ambient water and cells from centrifuged bloom water when exposed to juvenile chinook demonstrated no lethality. The cause of death was therefore considered due to a labile toxigenic agent (Black et al. 1991), most likely reactive oxygen radicals.

As the fish exhibited an anaesthetic behavior to the blooms, studies were subsequently conducted by the authors on the cholinesterase activity of brain tissue of chinook salmon killed by cultured *Heterosigma*.

Many neurotoxic compounds are known to inhibit the cholinergic transmission in fish brain and muscle. However, data presented in Fig. 1 clearly illustrates that no reduction in acetylcholine esterase activity resulted from exposure to lethal cells of *Heterosigma.*

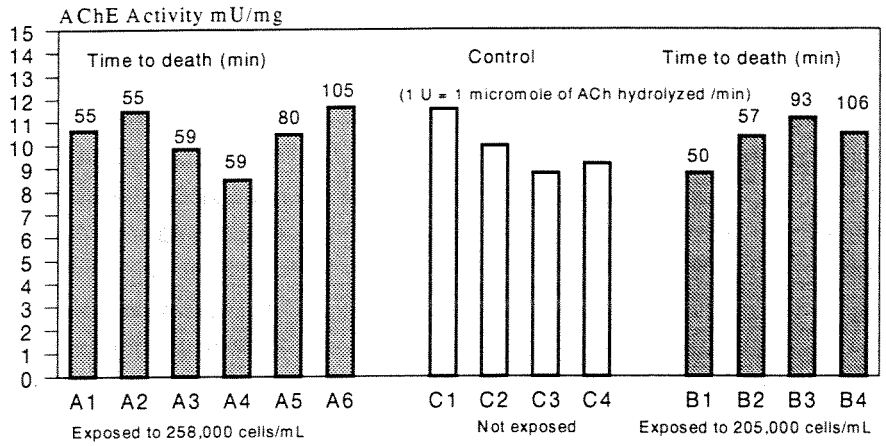


Fig. 1. Brain cholinesterase activity in chinook killed by *Heterosigma carterae* cultures

EFFECTS ON AQUACULTURE AND ECOLOGICAL IMPLICATIONS

Loss of salmon to blooms of *Heterosigma* in 1997, about $20 million worth, was equivalent to the combined loss of fish caused by this alga from the start of pen rearing of salmon a decade earlier. Comments received by the authors from aquaculturists around Vancouver Island on this unusual phenomenon were compiled and are presented in Table 1. The areas are indicated on the map (Fig. 2).

Apart from the severe losses of farmed salmon, the loss of oyster spat in a flupsy (floating upwelling system) is the first record in B.C. of the lethal effects of *Heterosigma* on juvenile oysters. This observation has ecological implications for natural recruitment of molluscan bivalve species that have generally been considered immune to the effects of *Heterosigma.* Other interesting observations were that certain areas on the coast are free from harmful algal blooms, further emphasizing the importance of farm site selection. That mussel fouling of netpens assisted in salmon survival of a *Heterosigma* bloom in the Sooke Harbour Basin suggests the potential for polyculture as a means of mitigating the effects of harmful blooms.

Table 1. Comments by aquaculturists on observed *Heterosigma carterae* blooms that occurred around Vancouver Island in July to October, 1997

Area on Map	Cell Count (cells/mL)	Implications
(A) Holberg Inlet	100,000–750,000	30-40% salmon mortality, alga to 7 m depth
(B) Kyuquot Sound	500,000	25–30% salmon mortality
(C) Clayquot Sound	350,000	80% salmon mortality, alga to 18 m depth
(D) Tofino	5,000–10,000	no salmon mortality
(D) Meares Island	no harmful algae	no salmon mortality
(D) Steamer Inlet	300,000	80+ % salmon mortality
(E) Effingham Inlet	>100,000	major salmon mortality
(F) Kingcome Inlet	< 500	no salmon mortality
(F) Simoom Sound	>50,000	minor salmon mortality
(G) Hardwicke & Thurlow Is.	no harmful algae	no salmon mortality
(H) West Redonda Island	dense blooms	100% oyster spat mortality contained in a "flupsy"
(I) Island Scallop Hatchery	alga dominated culture ponds	water intake 25 m
(J) Departure Bay (PBS)	90,000	78% salmon mortality
(K) Gulf Islands	8,000	2,500 salmon killed
(L) Sooke Harbour Basin	60,000	salmon mortality, but reduced by mussel fouling on nets

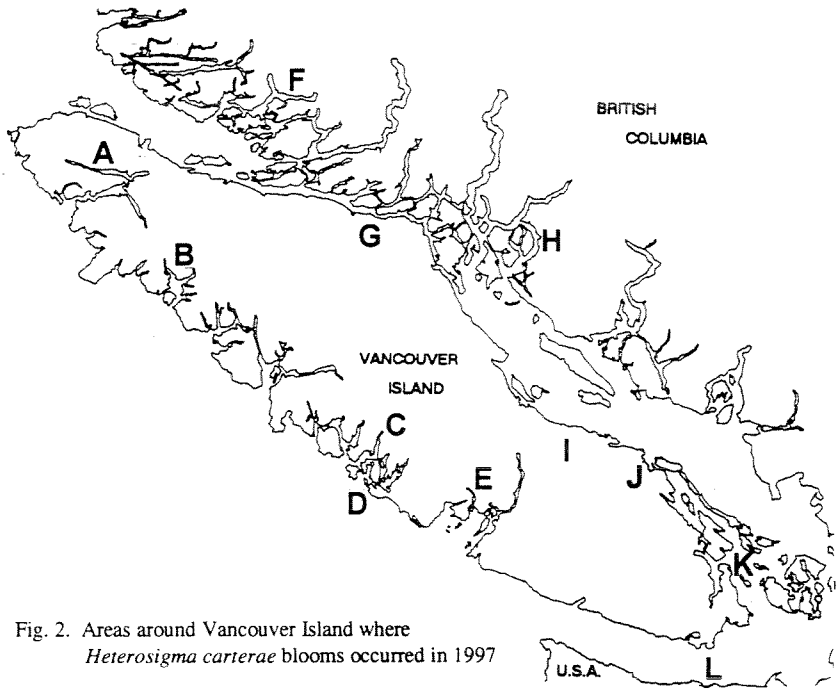


Fig. 2. Areas around Vancouver Island where *Heterosigma carterae* blooms occurred in 1997

During the summer of 1997 the ocean salinity on the east and west coast of Vancouver Island was substantially lower than in the previous year. Hydrographic data from some of the lighthouses in the Strait of Georgia from June through October in 1996 and 1997 are presented in Fig. 3. Mean temperatures during these months at five locations in both years were virtually identical.

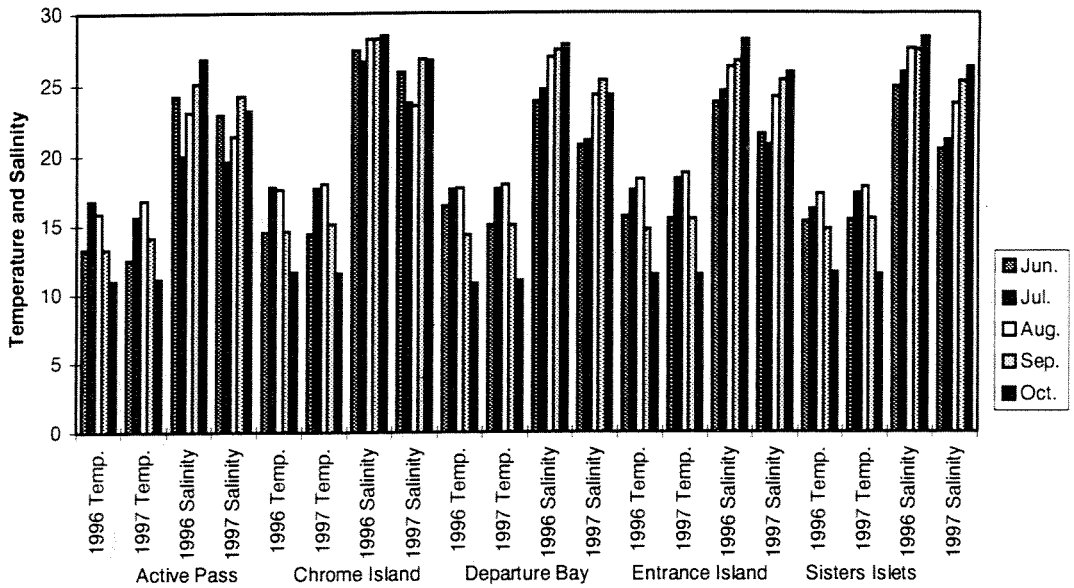


Fig. 3 Strait of Georgia hydrographic conditions 1996/1997

However, the salinity in these locations in all months was substantially lower in 1997 than in the previous year. This reduced salinity was probably caused by a colder than usual spring and early summer in 1997. This prevented a normal snow-melt in the coastal mountains, which with the sudden onset of summer heat produced considerable freshwater runoff to rivers, such as the Fraser river. Additionally, heavy rainfall in the summer of 1997 increased freshwater runoff into inlets along the west coast of Vancouver Island.

Hydrographic conditions at farm sites on the west coast of Vancouver Island at Clayoquot Sound are illustrated in Fig. 4. Temperatures at most site were similar, dissolved oxygen was generally elevated in 1997, and salinity at most sites was lower, some greater than 10 ppt. As mentioned previously, *Heterosigma* is remarkably tolerant to low salinity and probably out-competes most of the other microalgae when salinity declines. Thus, the widespread distribution of *Heterosigma* throughout southern B.C. reflected the reduced ocean salinity from June to October in 1997. One method of bloom avoidance at fish farms is perimeter skirting of pens with polyester tarps, which prevent advection of the surface blooming *Heterosigma* into the pens. This also allows for upwelling of deeper colder water, either by aeration or the use of air-lift or hydraulic pumps, which inhibits growth by lowering the water temperature. De-stratification of the water column by vertical convection also inhibits growth of *Heterosigma*, which during daylight is generally within the top 4 m of the water column (Whyte 1997). In 1997, the alga was dispersed to a depth of 25 m and skirting of pens and upwelling of deep water proved much less effective at reducing fish losses than in previous years.

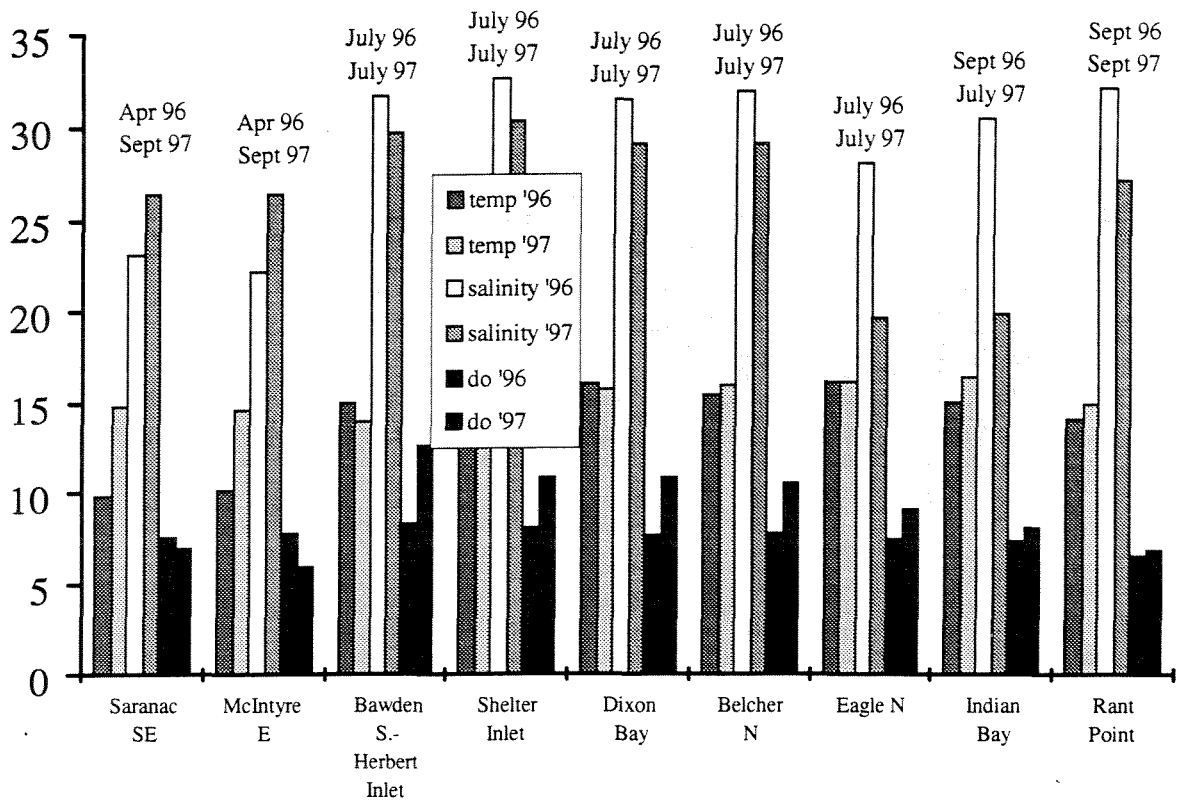


Fig. 4. Clayquot Sound hydrographic conditions

Apart from the direct impact on finfish *Heterosigma* can change significantly the abundance of other phytoplankton in its environment. Natural blooms of the alga can inhibit growth of *Skeletonema costatum* and species of *Chaetoceros* and *Thalassiosira* (Pratt 1966; Honjo et al. 1978). Later studies by Honjo (1993) demonstrated that the glycoprotein constituting the glycocalyx of the cell is a species-specific allelopathic substance. Although the growth of *Skeletonema costatum* was inhibited in the presence of *Heterosigma*, growth enhancement of *Prorocentrum triesterum* occurred (Honjo 1993). Several species of the genus *Prorocentrum* are known to produce Diarrhetic Shellfish Poisoning (DSP) toxins (Bauder et al. 1996).

Growth development and survival of copepods (Uye and Takamatso, 1990), rotifers (Egloff, 1986), planktonic tintinnids (Taniguchi and Takeda 1988), juvenile oysters, and clams (Walne 1970), are severely impacted on exposure to *Heterosigma*. The low salinity in the Strait of Georgia during 1997 would favour the growth of *Heterosigma* and the demise of associated phytoplankton and zooplankton. Reduction in the primary and secondary producers would affect fish populations and could account in part for the observed lack of coho salmon in the Strait. *Heterosigma* blooms may therefore play a more significant role in fish recruitment and population dynamics in coastal waters of B.C. than has been recognised to date. The economic impact of *Heterosigma* blooms on the aquacultured fisheries is well known, but the impact on the traditional wild fisheries has yet to be fully determined.

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HARMFUL ALGAL BLOOMS IN THE USA: AN UPDATE

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Harmful algal blooms continue to plague many U.S. coastal areas. *Pfiesteria piscicida* and *Pfiesteria*-like organisms currently are receiving the most publicity and funding. *Pfiesteria* has been documented in North Carolina, Delaware, and a fish farm in Maryland; about 10 species of *Pfiesteria*-like organisms have been found in North Carolina, Maryland (Chesapeake Bay), and Florida (St. John's River, St. Lucie River, and the Indian River Lagoon). Not only have massive fish kills been reported from these areas, but human illnesses also have occurred and the Center for Disease Control and Prevention (CDC) has been directed by Congress to develop and implement a disease-surveillance system to identify and monitor possible health effects caused by exposure to *Pfiesteria* and *Pfiesteria*-like organisms.

Other U.S. coastal areas also report HAB events. While the domoic acid scare on the west coast in the early 1990s appears to have dissipated for now, blooms of *Pseudo-nitzschia* species continue to occur and health agencies and researchers remain alert in case the toxin returns. In Washington, PSP caused several millions of dollars in losses during the winter, striking in November just at the beginning of the holiday season. Extensive areas were affected and toxin levels were high. Another HAB event occurred in mid-July when *Heterosigma* struck in central Puget Sound causing more than $3 million in losses to one company.

Elsewhere, the brown tide in Texas is apparently in remission after 7 yr, but *Gymnodinium breve* blooms in 1996 and 1997 killed fish along the coast and in bays prompting state agencies to devise a monitoring plan. *G. breve* also has been a problem along the Florida east and west coasts. Only the Northeast seems to have had little HAB activity during the last 2 yr.

IRON AND NITROGEN NUTRITION IN THE BROWN TIDE ORGANISM *AUREOCOCCUS ANOPHAGEFFERENS*

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INTRODUCTION

Blooms of the Pelagophyte, *Aureococcus anophagefferens*, are responsible for the brown tides that occur in the Peconic estuary on Long Island. The occurrence of these blooms does not appear to be correlated with the level of macronutrients (N,P) present in the water column. Laboratory studies suggest that the iron requirement for *A. anophagefferens* may be as high as 9 μM (Cosper et al. 1993) and this trace metal has been implicated in controlling bloom formation (Gobler and Cosper 1996). These laboratory studies used an under-chelated modification of the synthetic medium Aquil and iron may have precipitated out during the course of these experiments. In this abstract, we re-examined the iron requirement for growth of *A. anophagefferens* in artificial seawater under carefully controlled conditions of trace metal availability. The effects of nitrogen nutrition on the growth and nitrate reductase activity in *A. anophagefferens* will be reported separately (Szmyr et al., J. Phycol, *in preparation*).

MATERIALS AND METHODS

Aureococcus anophagefferens (CCMP1708), isolated from the Peconic Estuary on Long Island, NY, was grown in ASP artificial seawater enriched with modified f/2 nutrients (Guillard and Ryther, 1962) supplemented with 5.0 μM citric acid and 1.0 μM selenium. The cultures were grown at 20°C using a 14:10 L:D cycle at 50 μE m^{-2} s^{-1} in acid washed polycarbonate flasks. To reduce iron contamination present in the salts used to prepare the seawater, stock solutions were passed through Chelex-100 resin prior to use. The total iron concentration in the different treatments was measured by graphite furnace atomic absorption spectroscopy by Chris Gobler at State University of New York in Stony Brook. Two different conditions of iron availability were utilized; (1) iron was complexed with twice its concentration using EDTA or NTA, and (2) cultures were grown in trace metal buffered media using 100 micromolar excess EDTA. Triplicate cultures were grown through 4-5 transfers to dilute internal iron pools and growth was measured by *in vivo* fluorescence or visual cell counts.

RESULTS AND DISCUSSION

Aureococcus anophagefferens cultures grown on 1 μM added iron (total Fe concentration = 1.3 μM) gave maximum cell numbers (9 x 10^9 cells•L^{-1}), maximum fluorescence (~850), and growth rate (~1 div per day) similar to that obtained in iron-replete (11 μM) cultures. Thus the iron-quota for our *A. anophagefferens* was lower than 1 μM. Efforts to limit the growth of *A.*

anophagefferens by iron in culture included the use of small (20 mL), medium (800 mL) and large (12,000 mL) flasks in an effort to change the container surface to volume ratio. Special polycarbonate tubes were used that can be inserted directly into the fluorometer unopened. Direct measurements indicated that the major source of iron contamination in our experiments was due to the salts used to prepare the media. Chelex treatment of the ASP base salt solution reduced the contamination level to 90 nM iron. Chelex treatment of the ASP base salts and the f/2 macronutrients further reduced our iron contamination to < 25 nM. All three sources of iron tested gave similar results (Table 1). Even at 0 μM added iron, the cell maximum yield of *Aureococcus* was not significantly different from the 11 μM iron-replete controls. A representative experiment using trace-metal buffered media where high concentrations of EDTA were used to control the free iron concentration in solution is shown in Fig. 1.

Table 1. A summary of the different experiments used to calculate the maximum iron quota for the growth of *A. anophagefferens* in defined artificial seawater.

Experimental chelator and concentration	Iron concen-trations added in the different experiments	Actual concentration of iron as measured by GFAA	Average cell yield in cells $\cdot L^{-1}$	Calculated Q_{Fe} based on the "no added iron" culture
2x EDTA	0-11 μM	90 nM – 11 μM	4.2×10^9	< 25 amol per cell
2x NTA	0-11 μM	25 nM – 11 μM	5.4×10^9	< 6 amol per cell
100 μM EDTA	0-11 μM	25 nM – 11 μM	4.2×10^9	< 5 amol per cell

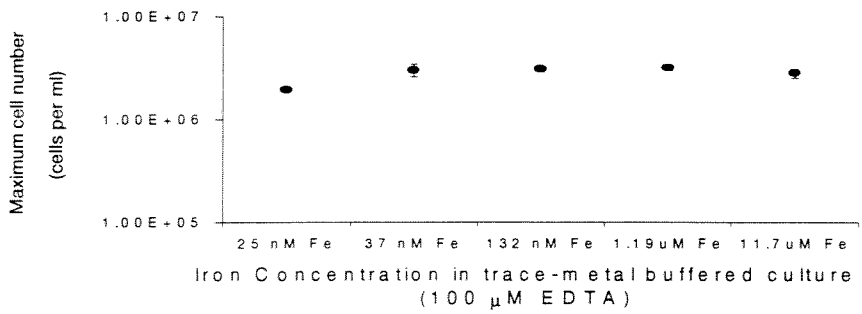

Fig. 1. The effect of iron on the maximal cell number of *A. anophagefferens* (± s.d.) in 100 μM EDTA trace-metal buffered cultures. The iron concentration is the added iron plus the 25 nM contaminate iron.

Measurement of the actual iron contamination in the three different synthetic seawater media by atomic absorption spectroscopy provides an upper estimate of the minimum iron quota (Q_{Fe}) needed for growth of *A. anophagefferens* in culture. The calculated value of 5 amol $cell^{-1}$ is similar to that reported for the oceanic diatom *Thalassiosira oceanica* (Sunda et al., 1991), and lower than the 6-100 amol $cell^{-1}$ required by the coastal species, *T. pseudonana* and *T. weissflogii*

(Anderson and Morel, 1982). *Aureococcus anophagefferens* CCMP1708 has a very low iron requirement, with the minimal iron necessary to support growth below 25 nM.

ACKNOWLEDGEMENTS

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EFFECTS OF SI/P LOADING RATIO AND SUPPLY MODES ON THE POPULATION DYNAMICS OF *ALEXANDRIUM TAMARENSE*

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Alexandrium tamarense is one of the dinoflagellates which produces paralytic shellfish poisoning (PSP). Although PSP in Japan has originally been a problem in the northern region, it has been expanding to the southern region in recent years. In Hiroshima Bay which is located in the southern region, the PSP by *A. tamarense* was observed in 1992 (Yamamoto and Yamasaki 1996). Since then, it has become an annual event during spring in Hiroshima Bay. The occurrence of PSP is a serious problem in Hiroshima Bay, because the bay has the highest production of cultured oyster in Japan.

Tarutani (1997) observed that silicate and phosphate were the two major nutrients limiting the growth of phytoplankton assemblages in Hiroshima Bay. In the present study, the population dynamics of *A. tamarense* in the estuarine region of Hiroshima Bay were analyzed using a numerical model which simulates species competition for Si and P supplied in continuous (everyday) and intermittent (every 7 d) modes.

The species considered in the model are *Skeletonema costatum* and *Heterocapsa triquetra* which are dominant species during the bloom period of *A. tamarense* (Tarutani 1997). The numerical model basically consists of equations expressing nutrient uptake (Micaelis-Menten equation) and cell growth (Droop equation). After uptake, the time required for processing phosphorus in the cell was considered in the model to express the time lag between uptake of phosphate and cell division. All parameters required for the equations were obtained by experimental studies (Tarutani and Yamamoto 1994; Tarutani 1997).

The Si/P loading ratio affected the competition between diatoms and dinoflagellates. Since *S. costatum* had the highest uptake rates of Si and P and also the highest growth rate, only a Si/P loading ratio less than 1.5 allowed coexistence with the other two dinoflagellates (Fig. 1). These results indicate that a low Si/P loading ratio is necessary for dinoflagellates to become dominant.

The nutrient supply mode affected the species composition of the phytoplankton assemblage. Under the continuous nutrient supply mode, *H. triquetra* always dominated over *A. tamarense* (Fig. 1). On the other hand, the intermittent nutrient supply increased the probability of *A. tamarense* coexistence even when the Si/P loading ratio was as high as 5 (Fig. 2). This was thought to be largely dependent on the P cell quota (phosphorus storage ability of the cell) of *A. tamarense*. These results obtained in the present study suggest that the proliferation of the toxic dinoflagellate *A. tamarense* in estuaries can be prevented by controlling the riverine nutrient loading ratio and the supply intervals. This is a sort of bioremediation.

ACKNOWLEDGMENTS

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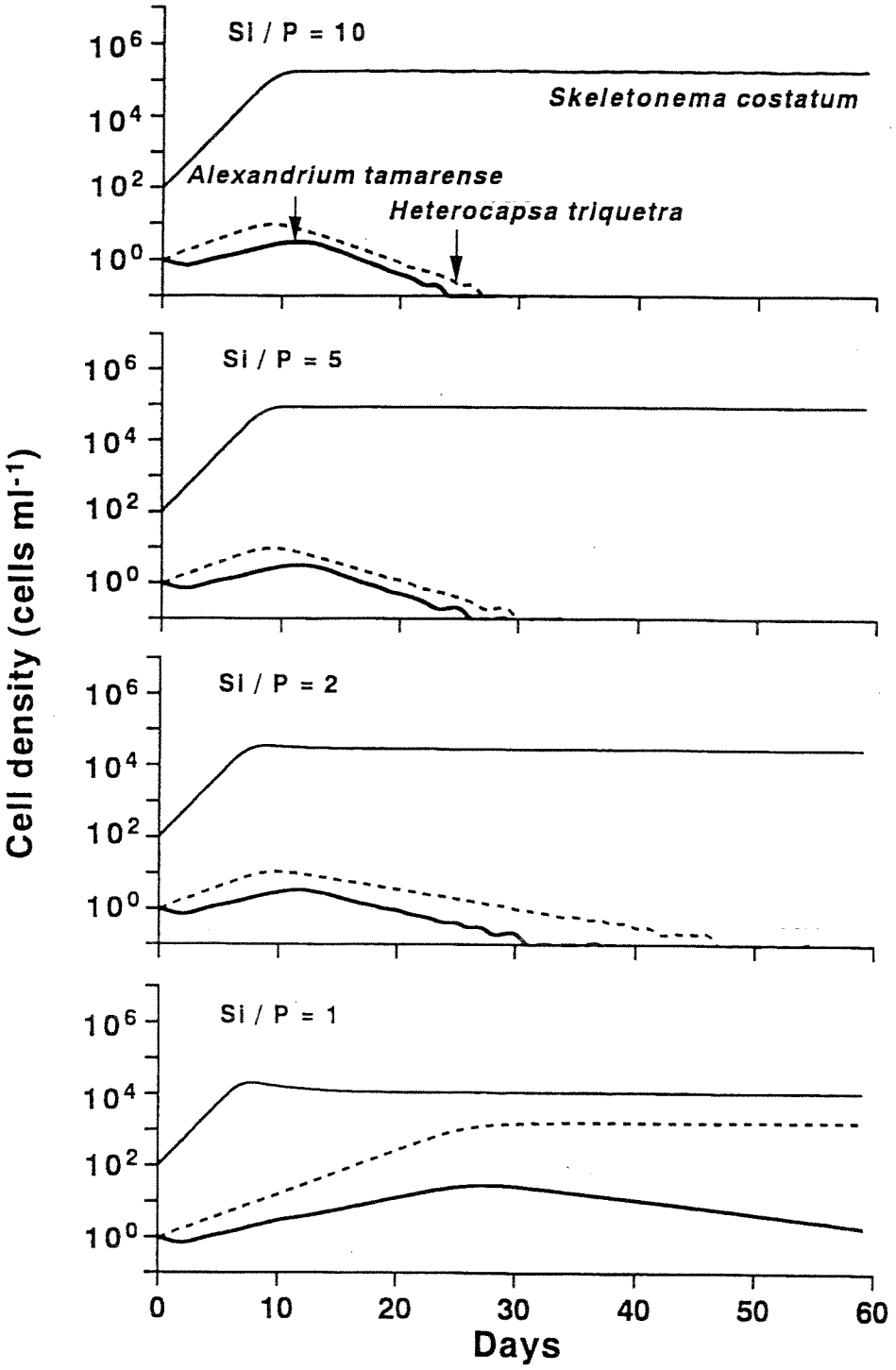

Fig. 1. Simulated population dynamics of three marine phytoplankton species (*Alexandrium tamarense*, *Heterocapsa triquetra* and *Skeletonema costatum*) under continuous (everyday) nutrient supply mode. Dilution rate: 0.25 d^{-1}, phosphate load: 0.2 μM d^{-1}.

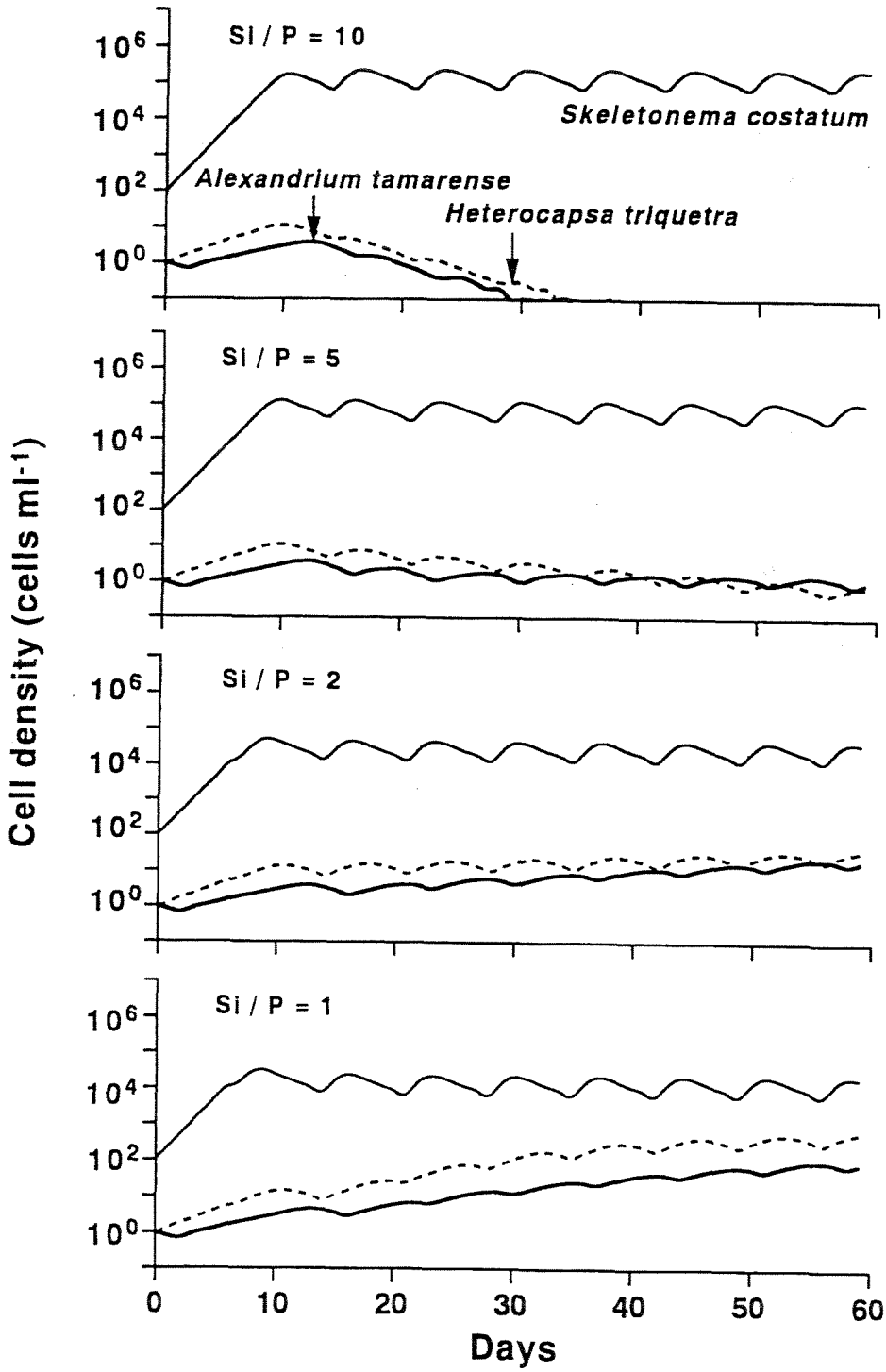

Fig. 2. Simulated population dynamics of three marine phytoplankton species (*Alexandrium tamarense, Heterocapsa triquetra* and *Skeletonema costatum*) under intermittent (every 7 d) nutrient supply mode. Dilution rate: 0.25 d^{-1}, phosphate load: 0.2 $\mu M\ d^{-1}$.

ENVIRONMENTAL CONDITIONS RELATED TO DSP TOXIN DYNAMICS AT A NOVA SCOTIAN AQUACULTURE SITE

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Diarrhetic shellfish poisoning (DSP) toxins have been detected periodically in mussels from an aquaculture site in Nova Scotia. Planktonic and epiphytic microalgal populations were studied at a mussel farm in Mahone Bay, Nova Scotia to establish the source of the toxins at the site. The only organism identified as a producer of DSP toxins at the site was *Prorocentrum lima*, an epiphytic dinoflagellate. This species is frequently found as an epiphyte upon the phaeophycean macroalga, *Pilayella littoralis*, which commonly fouls aquaculture lines in the region. *Prorocentrum lima* is the most likely candidate species for the low level DSP shellfish toxicity in Nova Scotian coastal waters.

A field experiment was conducted during the summer of 1997 to determine the role of mussel aquaculture on the settlement and growth of both the toxic dinoflagellate and associated fouling macroalgae. Six miniature live-mussel socks (20 cm long, mean number of mussels = 177) and six dummy socks (created with empty mussel valves) (20 cm long, mean number of mussels = 114) were hung along a horizontal long-line at an aquaculture site. After four and nine weeks, three socks of each treatment were harvested and the biomass of fouling macroalgae and concentration of *P. lima* cells were determined. Macroalgal fouling, which was almost entirely by *Pilayella littoralis*, was greater on the live-mussel socks than on the dummy socks (ANOVA, $P<0.05$). Densities of *P. lima* cells per gram dry weight of fouling biomass were higher on the dummy socks than the live-mussel socks (ANOVA, $P<0.05$). The data suggest that mussel culture provides a microenvironment that favours the colonization and growth of macroalgae, but does not enhance the growth of the dinoflagellate. The data also imply that growth and colonization densities of the dinoflagellate are not directly dependent on fouling biomass.

PRODUCTION OF DSP TOXINS THROUGH THE CELL CYCLE OF THE BENTHIC DINOFLAGELLATE *PROROCENTRUM LIMA*

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The benthic dinoflagellate, *Prorocentrum lima,* has been circumstantially implicated as a potential source of diarrhetic shellfish poisoning (DSP) toxins at commercial shellfish aquaculture sites in Nova Scotia. Cell cycle events and DSP toxin production were studied in triplicate unialgal batch cultures grown at 18±1°C, under a photon flux density (PFD) of 90±5 μmol m^{-2} s^{-1} on a 14h/10h light/dark (L/D) photoperiod. Cultures in exponential growth phase were synchronized in darkness for 17 d. The synchronization was verified by staining double-stranded DNA with the green fluorochrome SYTOX, after which the cellular DNA content histogram was determined using a flow cytometer equipped with an argon laser (excitation at 488 nm).

The cellular DSP toxin content, RNA:DNA ratio, chlorophyll *a*, and cell concentrations were quantified through successive cycles of cell division. The results indicated that the cell populations became asynchronous about 3 d after transition from darkness to the 14:10 L/D photoperiod. This may be due to the prolonged division cycle (5-7 d) that is not phased by the photoperiod. Thus the cell cycle events do not seem to be tightly regulated by L/D cycles, although the cell populations were synchronized during a period of extended darkness. For an epibenthic dinoflagellate, such as *P. lima*, this may be attributable to shade-adaptation typical of its natural habitat, where cells can grow optimally at a very low PFD, and to its low growth rate (μ = ~ 0.11-0.15 d^{-1}). Unlike planktonic *Prorocentrum* spp. (Pan and Cembella 1998), cytokinesis in *P. lima* occurred early in the dark and ceased by "midnight" (Fig. 1). For *P. lima*, chlorophyll *a* levels increased early in the light; this is consistent with the pattern for planktonic *Prorocentrum* spp.

Four components of the DSP toxin complex were determined in *P. lima* cells during successive cell division cycles: okadaic acid (OA), OA C8-diol-ester (OA-D8), dinophysistoxin-1 (DTX1) and dinophysistoxin-4 (DTX4). Cellular levels of these toxins ranged widely throughout the cell cycles: 0.37-6.6 (OA), 0.02-1.5 (OA-D8), 0.04-2.6 (DTX1), and 1.8-7.8 (DTX4) fmol $cell^{-1}$, respectively. Initially, cellular levels of OA, OA-D8 and DTX1 decreased after *P. lima* cells were inoculated into fresh nutrient-replete growth medium, but the DTX4 content remained fairly constant. No DSP toxins were produced during the prolonged period of darkness. Soon after the cultures were returned to the 14h/10h light/dark regime, cellular levels of all four toxins increased gradually. In the light, the increase of DTX4 in dark-synchronized cells occurred early in the photoperiod, whereas the induction of OA and DTX1 production was delayed by 3-6 h. This indicates that DTX4 synthesis is initiated in G1 and persists into S phase ("morning" of the photoperiod), whereas OA and DTX1 production occurs later during S and G2 phases ("afternoon") (Fig. 1). No toxin production was measured during cytokinesis, which happened early in the dark.

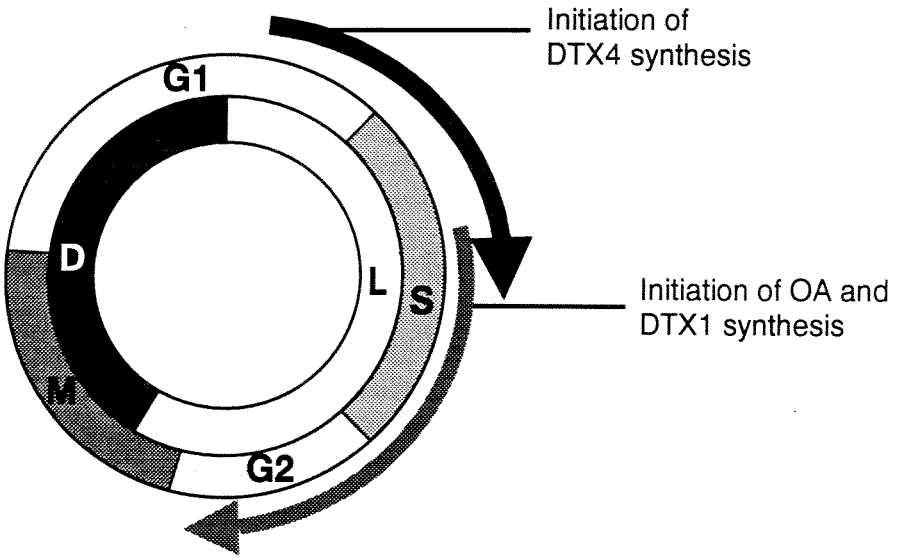

Fig. 1. Schematic description of the progression of cell division cycle events and production of DSP toxins in *Prorocentrum lima*. Inner ring: L/D cycle, outer ring: cell division cycle, arrows: production of toxins

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DOMOIC ACID TOXICITY OF LARGE NEW CELLS OF *PSEUDO-NITZSCHIA MULTISERIES* RESULTING FROM SEXUAL REPRODUCTION

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INTRODUCTION

During normal vegetative growth in culture, the pennate diatom *Pseudo-nitzschia multiseries* decreases in cell length and also in cellular content of the neurotoxin domoic acid (DA), causative agent of Amnesic Shellfish Poisoning (Bates et al. 1998). Diatoms commonly undergo sexual reproduction (auxosporulation) in order to regenerate their large cell size (Drebes 1977). We have recently documented sexual reproduction in species of *Pseudo-nitzschia* (Davidovich and Bates 1998). After mixing exponentially growing cells of the opposite mating type and of the appropriate minimal cell size, we observed pairing of parent cells, gamete production, fusion of gametes to form zygotes, and enlargement of auxospores within which formed the long initial cells. We call the latter "large new cells" after they exit the auxospores and undergo the first vegetative division. Here we document the DA production by large new cells, relative to parent cells. We also report on the range of cell sizes that favour auxosporulation. Such information may provide insights into the timing and toxicity of *Pseudo-nitzschia* spp. blooms in the field.

MATERIALS AND METHODS

Apical cell length measurements. Apical cell length of *Pseudo-nitzschia* spp. was measured at 40x magnification with an ocular micrometer mounted on a Dialux-20 (Leitz) microscope.

Field samples. Vertically integrated water samples containing *Pseudo-nitzschia* spp. cells were collected from New London Bay, Prince Edward Island (PEI) from November 23, 1990 to December 1, 1996. The majority of samples were collected during September to November, with a limited number collected in January and February.

Laboratory experiments with *Pseudo-nitzschia pungens*. Changes in apical cell length of the nontoxic *P. pungens* (10 different clones) growing in 75 mL of f/2 medium at 20°C and ca. 100 $\mu mol\ m^{-2}\ s^{-1}$ (10:14 h L:D) were followed from November 22, 1996 to April 17, 1998.

Laboratory experiments with *Pseudo-nitzschia multiseries*. Aliquots (0.3 mL) of exponentially growing cultures of *P. multiseries* (clones KP-104 and KP-105) isolated by K. Pauley on December 2, 1993 from Cardigan Bay, PEI, were mixed together in 15 mL of f/2 under the above growth conditions in order to induce sexual reproduction (Davidovich and Bates 1998). The resulting offspring of the KP-104 x KP-105 cross were:

Clone Name	Date isolated into culture
CLN-1	January 17, 1997
CLN-2	February 13, 1997
CLN-3	February 13, 1997
CLN-4	February 13, 1997
CLN-5	February 17, 1997

Two batch culture experiments were carried out in order to measure the growth curves and production of DA by the offspring clones (CLN-1 to CLN-5) of KP-104 x KP-105:

Experiment 1 (July 30, 1997): Clones CLN-1 and CLN-2
Experiment 2 (February 23, 1998): Clones CLN-3, CLN-4, and CLN-5.

Under the above conditions, 350 mL of f/2 medium were grown in flasks (triplicate in Exp. 1, and duplicate in Exp. 2) for 58 d. Samples were periodically removed to measure cell concentration (all cells counted in 5 µL aliquots) and DA concentration in the "whole culture" (=cells plus medium), using the FMOC HPLC technique (see Bates et al. 1991).

RESULTS AND DISCUSSION

Field samples. *Pseudo-nitzschia multiseries* and *P. pungens* species cannot be distinguished with the light microscope, so they were grouped as *Pseudo-nitzschia* spp. Cells in samples from New London Bay ranged in length from 35-150 µm (median = 105 µm; n = 1,627) from 1990-1996 (September to February) (Fig. 1). This size range exceeds that reported by Hasle and Syvertsen

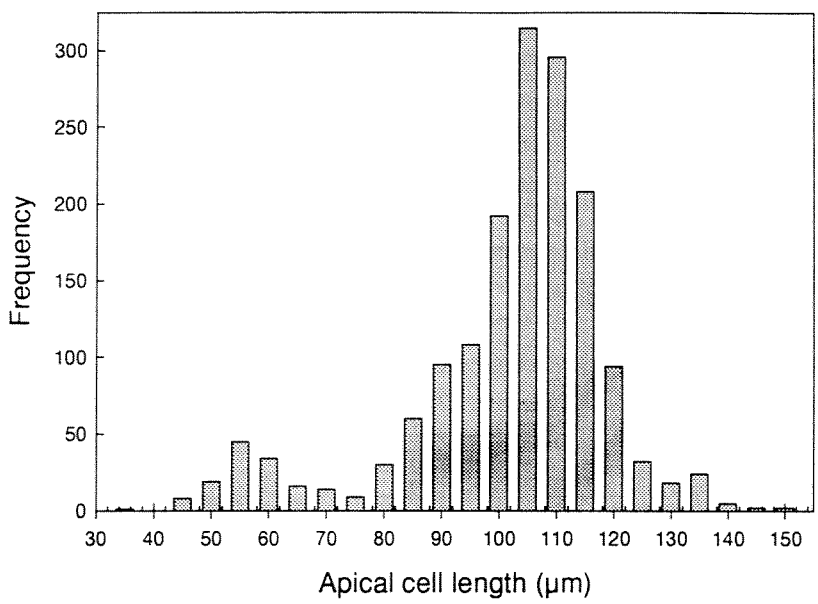

Fig. 1. Size-frequency distribution of *Pseudo-nitzschia* spp. cells from New London Bay, PEI.

(1996) for *P. multiseries* (68-140 µm) and *P. pungens* (74-142 µm). In comparison, large initial cells of *P. multiseries* from laboratory cultures reached a maximum apical length of 202 µm, but are more typically in the range of 120-173 µm (Davidovich and Bates 1998).

A low number of cells fell into a small size category of 35-75 µm (mean = 56 µm). These were all collected in January and February, but represent only a few samples, each of which contained few *Pseudo-nitzschia* cells. The apical lengths of sexually reproducing *P. multiseries* cells in our laboratory experiments were in this same size range (39 and 59 µm for clones KP-105 and KP-104, respectively, but the range can extend from 33-104 µm). In January-February, *Pseudo-nitzschia* cells in the field are, thus, in the size range and also exposed to the photoperiod (ca. 10 h of light) most conducive for sexual reproduction (see Hiltz et al. 1999). Additional samplings are required to determine when auxosporulation occurs in the field, especially since no sexually reproducing cells have thus far been seen in any field samples.

Laboratory experiments with *Pseudo-nitzschia pungens*. Ten clones of *P. pungens* decreased in apical cell length by about the same amount during the course of the measurements (Fig. 2); i.e., by ca. 2.5 µm per month, or ca. 30 µm per year, consistent with clones of *P. multiseries* in a previous study (Davidovich and Bates 1998). There appears to be a slowing in the rate of size decrease after October-November, 1997. Experience with other *Pseudo-nitzschia* cultures shows that the cells eventually stop dividing and die. Mixtures of the *P. pungens* CL-clones (shown in Fig. 2) produced some gametes and zygotes, but no initial cells.

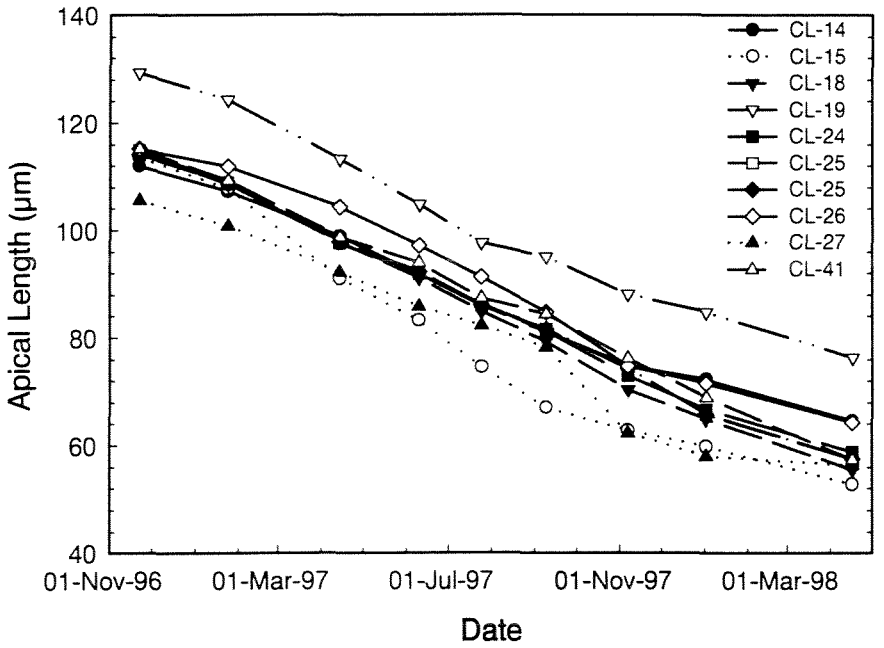

Fig. 2 Decrease in apical cell length of *Pseudo-nitzschia pungens* clones in batch culture.

Laboratory experiments with *Pseudo-nitzschia multiseries*. By the time the parent cells were sexually reproducing in culture, their cellular DA level had decreased to near the detection limit (< 0.1 pg DA $cell^{-1}$). The toxicity of the large new cells was similarly low during the first 2 mo after

isolation. Cellular toxin content then increased dramatically over the next several months, after which it was deemed worthwhile to carry out time-course experiments.

All five clones showed similar growth curves in batch culture (Fig. 3A). Production of DA by the large new cells in the "whole culture", expressed per mL (Fig. 3B), was substantially

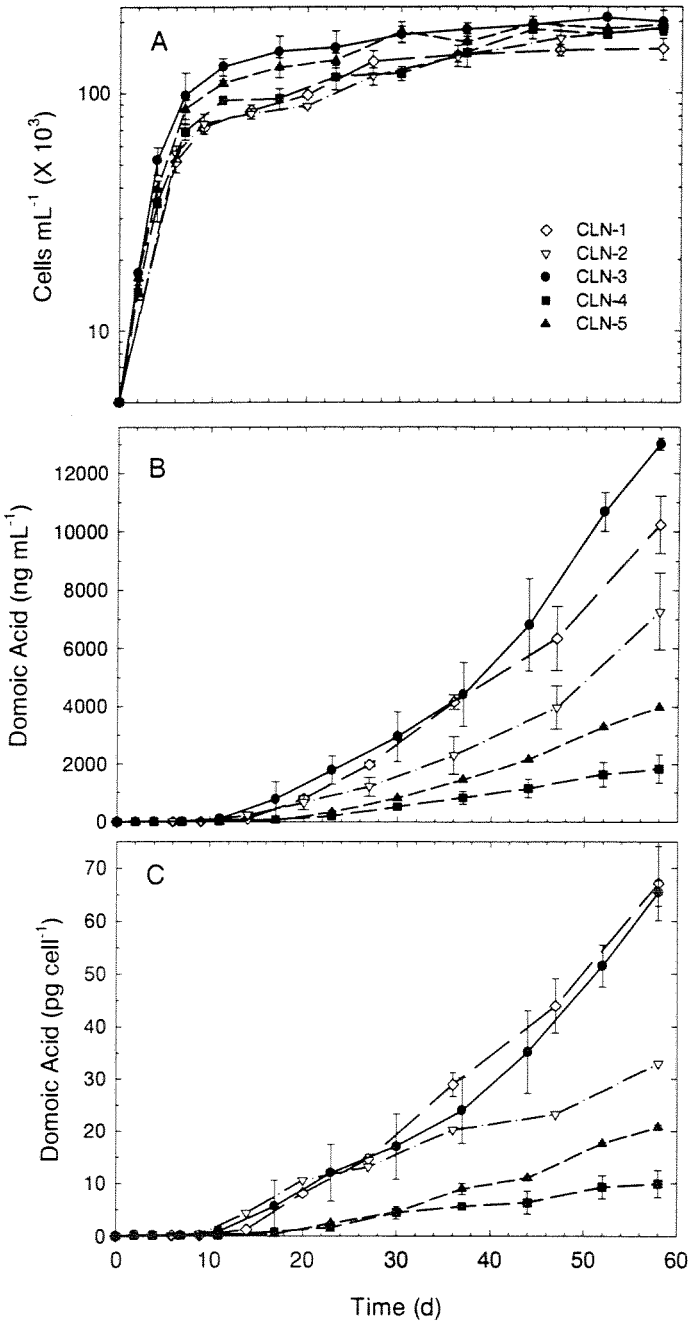

Fig. 3. (A) Growth; (B) domoic acid production per mL; and (C) domoic acid production per cell of five clones of *Pseudo-nitzschia multiseries* large new cells in batch culture. Experiments were carried out 5 mo (for CLN-1 and CLN-2) and 12 mo (for CLN-3, CLN-4 and CLN-5) after isolation.

greater than that ever previously documented (usually < 2,000 ng mL^{-1}). For example, after 58 d of growth, clone CLN-3 produced 13,000 ng DA mL^{-1}. The other clones, however, produced less DA, ranging from 1,850 ng mL^{-1} for CLN-4 to 10,250 ng mL^{-1} for CLN-1.

Likewise, the concentration of DA, expressed per cell (Fig. 3C), was exceedingly elevated. Clones CLN-1, CLN-2, and CLN-3 had the highest levels (67, 33, and 65 ng DA $cell^{-1}$, respectively). This is about 10 times more cellular DA than has previously been measured in the field or in other laboratory experiments (Bates et al. 1990; Bates 1998). The lower DA concentrations of clones CLN-4 and CLN-5 may be attributed to their older ontogenetic age when tested (12 mo since the initial cells were first isolated). However, clone CLN-3 was also 12 mo old, yet it produced high levels of DA. Therefore, the lower DA levels may be due to natural variability, even though these are sibling clones.

CONCLUSIONS

It is well known that most diatoms decrease in size during normal vegetative growth and that most regenerate their large cell size via sexual reproduction; *Pseudo-nitzschia multiseries* is no exception. It is also becoming clear that the production of domoic acid by *P. multiseries* tends to decrease over a period of several years in culture. By the time of these experiments, the parental clones had almost lost their capacity to produce DA. However their offspring, the large new cells, were able to produce up to an order of magnitude more DA than had ever previously been documented. It is presently not known if the ability to produce DA is causally related to cell size; this may just reflect a general change in physiology as the cells become smaller and then rejuvenate their large size. The variability in DA production by sibling offspring clones also poses some interesting questions that are currently being investigated.

We are beginning to document the apical length range within which *P. multiseries* cells are able to sexually reproduce (thus far 33-104 µm). Information about cell length and cellular DA content in laboratory cultures may eventually aid in understanding the timing and toxicity of *Pseudo-nitzschia* spp. blooms in the field. For example, our measurements of the apical length of archived *Pseudo-nitzschia* spp. cells in field samples led to the conclusion that short cells collected in January - February are in the appropriate size range to sexually reproduce. Additional samples must be collected in order to be able to document when populations of larger cells first appear. Field measurements of DA per cell and per cell volume are also required to corroborate laboratory studies of the same. Nevertheless, we can presently conclude that the higher DA content of large new cells has implications for the toxicity of natural blooms.

Acknowledgments

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MACRONUTRIENTS-BASED PREDICTION OF DOMOIC ACID LEVELS IN THE PENNATE DIATOM *PSEUDO-NITZSCHIA MULTISERIES*

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The pennate diatom *Pseudo-nitzschia multiseries* has been implicated in the amnesic shellfish poisoning in Cardigan Bay, Prince Edward Island. The same diatom has also been found elsewhere worldwide. Studies in the last decade show that this diatom significantly produces the neurotoxin, domoic acid, under physiological stress caused by limitation of nutrients, such as phosphate and silicate. Similar conditions were found in the field when the toxigenic blooms of the diatom were prevalent. Based on our data from the laboratory, we have developed a model, which expresses the predicted amount of domoic acid per cell as a function of the dissolved nitrogen, phosphate, and silicate present in the medium. The optimal model selected may be expressed as a linear combination of four terms:

$$DA = -2.82 - 8.31\times10^{-3}\,P + \; + 6.58\times10^{-2}\,Si^{1/2} + F1(P{:}N) + F2(Si{:}N),$$

where $F1(P{:}N) = 41.21\,P{:}N + 3.24\times10^{-2}\,(P{:}N)^{-1/2} - 1.98\times10^{-4}\,(P{:}N)^{-1}$

and $F2(Si{:}N) = -18.30\,Si{:}N + 8.42\,(Si{:}N)^{1/2} + 1.37\times10^{-1}\,(Si{:}N)^{-1/2} + 1.21\times10^{-3}\,(Si{:}N)^{-1}$

The appropriateness and goodness-of-fit were validated using established statistical tests. A nomogram is provided to facilitate the calculations of domoic acid levels using this model. The 95% confidence intervals of expected DA levels computed from this model range in width from 0.12 to 0.81 pg cell^{-1} over the range of the experimental data. The model works best for DA levels under 3 pg cell^{-1}.

With identification of the diatom and measurement of nutrients, this model can provide early warning of impending amnesic shellfish poisoning episodes. This may be especially useful in areas where direct and timely analysis of toxin may not be possible because of lack of analytical facilities or may pose an excessive financial burden for private shellfish growers.

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IDENTIFICATION GUIDE FOR THE MARINE PHYTOPLANKTON OF THE ST. LAWRENCE.

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Phytoplankton plays an important role in marine ecosystems. Precise identification of phytoplankton species is thus essential for many studies, as for example, for toxic algae monitoring programs and for more fundamental projects on the distribution and structure of phytoplankton communities. Unfortunately, there is currently no reference documentation available covering most of the phytoplankton species of the St. Lawrence system. This guide should help in the identification of the species present in these waters. Such a document should also be an important tool in the evaluation of biodiversity of the St. Lawrence providing a synthesis of accumulated knowledge for a variety of users. It should also help in confirming the presence of potentially harmful species.

This guide contains more than 400 marine phytoplankton species which were identified, measured and photographed. The phytoplankton groups described include diatoms (110 spp.), dinoflagellates (120 spp.), flagellates, including cryptophytes (5 spp.), chrysophytes (18 spp.), euglenids (3 spp.), prasinophytes (18 spp.) and prymnesiophytes (30 spp.), as well as some of the most common species of protozoa (100 spp.) including heterotrophic flagellates, choano-flagellates and ciliates. It also includes a brief description of all the species identified along with 160 photographic plates made from 1200 light and electron microscope photographs. Measurements, taxonomic references and distribution of the species are also included.

New records from this area include: the dinoflagellates - *Alexandrium ostenfeldii*, *Dicroerisma psilonereiella*, *Ensiculifera carinata*, *Fragilidium subglobosum*, *Gonyaulax alaskensis*, *Gyrodinium corsicum*, *Polykrikos schwartzii*, *Protherythropsis vigilans*; the diatoms - *Cerataulina pelagica* and *Lennoxia falveolata* which were very common and abundant; and several species of flagellates - *Chrysochromulina brevifilum*, *C. parkeae*, *C. polylepis*, *C. vexillifera*, *Plagioselmis prolonga var. nordica*, *Teleaulax amphioxeia*.

This project received the financial support of SLV 2000 Research program of the DFO, Laurentian Region.

UTILIZING DATA FROM VOLUNTEER PLANKTON INVESTIGATORS IN A BIOTOXIN PROGRAM

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In 1997, the state of Maine successfully implemented a volunteer-based, state-wide phytoplankton observer network. This program incorporated the use of 22 volunteer groups/organizations and collected samples on a weekly basis from approximately 60 locations, which were assigned by the Department of Marine Resources (DMR), based on historical data from over 20 yr of monitoring for Paralytic Shellfish Poisoning (PSP). Volunteers were outfitted with the necessary equipment (field microscopes, nets, data sheets, and miscellaneous supplies) and trained in sampling technique, phytoplankton identification, and data reporting by officials from the United States Food and Drug Administration (USFDA), DMR, the University of Maine Cooperative Extension Service (UMCE), and Bigelow Laboratory for Ocean Sciences. Data collected, designed to target *Alexandrium*, *Dinophysis*, *Pseudo-nitzschia*, and *Prorocentrum*, were transmitted via FAX to the DMR. Over 500 samples were recorded for the 1997 sampling season, these observations were used to augment DMR's present shellfish monitoring program and will hopefully provide an early-warning system in the event of episodes of toxin. Although 1997 was a low toxin year, the two incidents of PSP documented by DMR's monitoring program were corroborated by volunteer observations. 1997 *Dinophysis* spp. abundance and distribution information is new to researchers in Maine and has lead to DMR's purchase of equipment to perform Diarrhetic Shellfish Poisoning bioassays. Funding for this program was received from the USFDA and the NOAA/NESDIS Penobscot Bay Project.

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VOLUNTEER PHYTOPLANKTON PROGRAM

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A group of volunteer field plankton observers can be equipped with nets and small field microscopes to be a significant asset to a marine biotoxin management program. Although plankton observations are not likely to replace toxicity testing, they can make a program more cost effective by focusing toxicity testing on times, locations and toxins of greatest concern. Following collection of plankton with a net, the samples are examined with a small field microscope that can have a portable video camera attached to aid in training observers and in documenting observations on videotape. An informal demonstration was given on the use of the equipment.

SHELLFISH TOXIN MONITORING IN THE MARITIMES REGION - 10 YEARS SINCE THE ESTABLISHMENT OF THE ENHANCED MOLLUSCAN MONITORING PROGRAM

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INTRODUCTION

The 1987 domoic acid shellfish poisoning incident in Prince Edward Island caught the regulatory agencies by surprise as the historical evidence indicated that shellfish from the southern Gulf of St. Lawrence had never been implicated in seafood poisoning. Routine monitoring over the previous years did not indicate the presence of paralytic shellfish poisoning (PSP) toxins and investigations of shellfish poisonings could not establish a link to known toxins. Such was the confidence in having a toxin-free area that the Department of Fisheries and Oceans Inspection Branch rarely sampled for toxins. The number of samples averaged less than 20 per year. On November 27, 1987 evidence of a new toxin was established by mouse bioassay analysis at the DFO Inspection Black's Harbour, New Brunswick (NB) laboratory and the Health Protection Branch (HPB) laboratory in Ottawa, Ontario. Mouse bioassays of leftover mussels from illnesses in both Charlottetown, Prince Edward Island (PEI) and Montreal, Quebec (PQ) revealed uncharacteristic symptoms. The symptoms, which included barrel rotation, bilateral scratching and aggressiveness, suggested that brain functions were affected by this "new" toxin. The resulting public recall of all bivalve shellfish products from PEI no doubt helped to keep the number of illnesses from rising, although by this time greater than 100 people were ill and 4 deaths were ultimately blamed on this toxin. Once all products affected by the recall were detained and/or destroyed the focus of the investigation turned to identifying the responsible agent. A large task force consisting of the DFO Inspection and Science Branches, Environment Canada, Health Protection Branch, National Research Council (NRC) and others was organized for the purpose of identifying this toxic factor. A team consisting largely of NRC and some DFO Inspection staff and led by Dr. Jeffrey Wright of the NRC in Halifax succeeded in identifying domoic acid (DA) as the toxin responsible for the illnesses (Wright et al. 1989). A High Performance Liquid Chromatographic (HPLC) method for DA was developed shortly afterwards and permitted the routine and rapid detection of DA in shellfish tissues (Quilliam et al. 1988) The availability of this new method now permitted the Inspection Branch to monitor shellfish for DA since the spring of 1988.

The Moncton, NB laboratory became responsible for all the DA analyses for the former Gulf Region (PEI, Eastern NB and Northern NS) and the Halifax, Nova Scotia (NS) Chemistry Laboratory was responsible for all the DA analyses for the former Scotia-Fundy Region (Black's Harbour, Yarmouth, Halifax and Sydney districts). The establishment of a mouse bioassay unit in

Moncton at the same time enabled the former Gulf Region to monitor for PS toxins. The existing laboratory in Black's Harbour performed all bioassay analyses for the former Scotia-Fundy Region. This function continued until the establishment of a bioassay unit in Halifax in early 1989. The Halifax laboratory then assumed responsibility for all of Nova Scotia with the exception of the Gulf area.

The Gulf Region had never developed a comprehensive shellfish monitoring program and therefore had little historical data on which to base an effective and efficient sampling plan. The Halifax and Sydney, NS areas, although having a more comprehensive sampling program, had similar problems with historical data. Southwest NB and Yarmouth, NS had extensive monitoring programs and a long history of PSP problems.

The widespread attention given the DA episode resulted in an immediate drastic decrease in shellfish and, to a lesser extent, finfish consumption. A recurrence may have seriously harmed the credibility of the agencies and the industry. It was within this environment that the Inspection Branch decided an all-encompassing shellfish monitoring program was needed as it was better to err on the side of caution. In some cases, shellfish areas were to be sampled twice per week for PSP and DA, with some areas meriting up to three extra additional sub-samples. Clearly, a strong partnership between the industry and government was required. This cooperation still exists today in that some shellfish farmers and producers provide samples from their aquaculture sites on a regular basis. These programs were named the Gulf Region Phycotoxin Monitoring Program and the Scotia-Fundy Enhanced Molluscan Monitoring Program.

The responsibility for sample collection in all districts was, for the most part, the responsibility of the Inspection Branch. The human resources of the Branch were soon overwhelmed, with the result that the collection of samples was contracted out in most of the districts. This proved extremely expensive and consequently all districts opted out of this scheme. A concomitant reduction in the number of samples permitted the Inspection Branch to employ their own inspectors for all sampling. We report on the first 10 yr following the domoic acid outbreak.

DISCUSSION

The data used in this paper are a compilation from the archives of past and existing laboratories. Every attempt was made to ensure the accuracy of the data, but due to different software formats and conventions employed over the years the numbers may not match previously reported data. These differences are minor and should not exceed 5 % of previously reported totals.

As can be seen in the charts, the sampling levels increased dramatically in 1988, by about 1000%. Such an increase in workload changed the focus of all district laboratories. Microbiological samples had, until this time, constituted the overwhelming majority of the workload. Sample preparation and/or mouse bioassays now accounted for approximately half of the analysis time. Some laboratories hired additional staff, sometimes on a seasonal basis. Since the environmental conditions leading to the domoic acid episode were largely unknown, a sampling plan based on environmental parameters was impossible. Therefore, only a 100% coverage of all shellfish harvesting areas was acceptable. Even sampling grids had to be

developed because little was known of the spatial distribution of organisms and toxins. As more and more toxin events were detected and monitored, the kinetics of toxin uptake and spatial distribution became better understood. Managers became more confident and could reduce sampling efforts temporally and spatially with no reduction in safety to the consumers.

The introduction of a regulatory phytoplankton monitoring program also removed some pressure from the shellfish sampling regime. With the knowledge of which toxic phytoplankton were present and at what levels, managers could selectively adjust the number of shellfish samples analyzed by either mouse bioassay or HPLC. Official action related to phycotoxin content is based on the actual edible content of shellfish and shellfish products and not to the presence of phycotoxins in the water column. As a result, a policy decision has terminated this CFIA phycotoxin monitoring effort and all resources are now centered on toxin analysis only.

CONCLUSIONS

To our knowledge, every toxin event in the Maritimes Region during the last 10 yr was detected by the shellfish monitoring program. No confirmed illnesses can be traced to any known or novel toxin from open shellfish areas. All of the DA events in PEI and southwest New Brunswick were detected long before the quarantine limits were reached. The two PSP events in the Baie de Chaleur and the annual PSP events in the Bay of Fundy were detected prior to unsafe levels being reached. Managers may feel that a further reduction in sampling could be justified. Given the spatial and temporal variations normally expected, our conclusion is that the number of sites should **not** be reduced unless these areas are closed to the taking of shellfish as a safety measure, i.e. no monitoring - no harvesting.

ACKNOWLEDGMENTS

In closing, we would like to acknowledge the tremendous support and cooperation of our colleagues in Fisheries and Oceans Canada, Science Branch. This group includes, but is not limited to the following: Jennifer Martin of the St. Andrews Biological Station who has consistently provided phytoplankton data from the Bay of Fundy and other information on a very timely basis, and Stephen Bates of the Gulf Fisheries Centre for help in phytoplankton events and the kinetics of DA production. John Hurst of the Maine Department of Marine Resources has long fostered close cooperation with the adjoining New Brunswick area. PSP and DA events do not respect territorial waters and the early warning of toxic events is appreciated and reciprocated on every occasion. Most recently, with the advent of PSP episodes in the Baie de Chaleur, the Laurentian Region is also sharing information as it becomes available. **All** of this cooperation is extremely valuable to our monitoring program and will hopefully continue well into the future.

We also thank Stephen S. Bates for critical review of this manuscript.

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PARALYTIC SHELLFISH TOXIN DOSE-RESPONSE CURVE FOR BAY OF FUNDY NATURALLY CONTAMINATED SOFT SHELL CLAM, *MYA ARENARIA*

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INTRODUCTION

The mouse bioassay has been used to quantify paralytic shellfish poisoning (PSP) toxins since the 1930's (Sommer and Meyer 1937; Medcof et al. 1947). The method was developed in response to shellfish poisonings on the California coast and has become the international standard for PSP. Despite many attempts, via differing strategies, progress at replacing this assay with a non-animal model has been slow. Although some methods can claim success, they have not been incorporated into shellfish toxin monitoring programs due to costs and/or special requirements. Virtually all of these alternate methods also suffer from specificity, in that only the PSP, or in some cases the sodium-channel blocking toxins, can be measured. The mouse bioassay has the added advantage of detecting any toxin or biologically active agent with the only variable being sample preparation and chemical solubility. Regulatory agencies are therefore hesitant to adopt methods which may compromise consumer protection.

One of the problems with the mouse bioassay is that of interfering substances in the biological material being tested (Schantz 1960). Although the mineral acid extract gives very good recoveries of the PSP, it also solubilizes naturally occurring sodium salts. The effects of excessive sodium on the bioassay have been well known for years and are present to some degree in all marine shellfish tissues. The sodium ion has the effect of reducing the intraperitoneal toxicity, as measured by the mouse bioassay, by 20-50% and perhaps greater for some salted products. While this may appear to be an unacceptable procedure, all the epidemiological investigations of shellfish poisonings have been based on mouse bioassay results. Virtually all of the illnesses reported, from mild to severe, have been from a total dosage of 1500 µg and most at values greater than 3000 µg. A death from a reported dose of only 500 µg must be regarded as suspect. At these levels, the influence of sodium has been eliminated through dilution and the values reflect an accurate picture of the toxicity. All tissue extracts causing mouse deaths below 5 min must be diluted in order to give death times of 5-7 min (AOAC 1984). For the inexperienced assayist, as well as the experienced assayist when diluting at highly toxic extracts, judging the dilution factor can be a matter of trial and error. Sometimes 2-4 dilutions are required before the correct dosage is found. This is wasteful of mice and time.

MATERIALS AND METHODS

Data from routine shellfish toxin monitoring samples in southwestern New Brunswick were used to establish the dose response relationship. All samples from 1988-95 requiring dilution were collated. All mice with death times of less than 5 min were used (n = 2035). Mouse weights were corrected using the AOAC table. Over that period of time three analysts were involved with bioassays, eliminating an analyst-based bias in response. Linear regression and other statistics were generated with SAS/STAT® (1989) and Microsoft® Excel spreadsheet programming.

Female CD-1 mice (Charles River strain) weighing 16-23 grams were used for all bioassays. The AOAC (1984) method for paralytic shellfish poison was used for analysis with minor modification; more precise pH control was achieved by using species-specific acidulant. Final pH was adjusted to 3.2-3.8, but seldom required manipulation.

RESULTS

The dose-response curve for *Mya arenaria* is shown in Fig. 1, and the Sommer's Table curve for saxitoxin is in Fig. 2. For comparison purposes only the death times between 1 and 5 min were used; above 5 min dilution is not required, and Sommer's Table ends at 1 min. The regression line for naturally contaminated *M. arenaria* has a slope of 136.54 and an intercept of 1.5349. The line can be expressed as $y=136.54x+1.5349$, where y represents the log toxicity in micrograms and x is the reciprocal of time to death in seconds. The regression line for Sommer's Table using the same time values results in a slope of 127.04 and an intercept of 1.4593. Analysis of the data sets demonstrated a statistical difference ($p<0.05$) between the two slopes and the two intercepts. This difference can be chiefly accounted for by the effects of NaCl but the effect of the matrix can not be totally excluded. Additional analysis using other species and applying toxin profiles from HPLC data may help to further explain this difference. As can be seen in Fig. 3 and 4, the difference in the expected dilution factor varies between 20 and 40%.

A new table, based on the linear regression for naturally contaminated *M. arenaria,* should greatly reduce the number of incorrect dilutions and hence the number of mice used to determine shellfish toxins in the Bay of Fundy. Past experience with *Mytilus edulis* and *Modiolus modiolus* would support a similar dilution pattern although more data are needed for a full analysis.

ACKNOWLEDGMENTS

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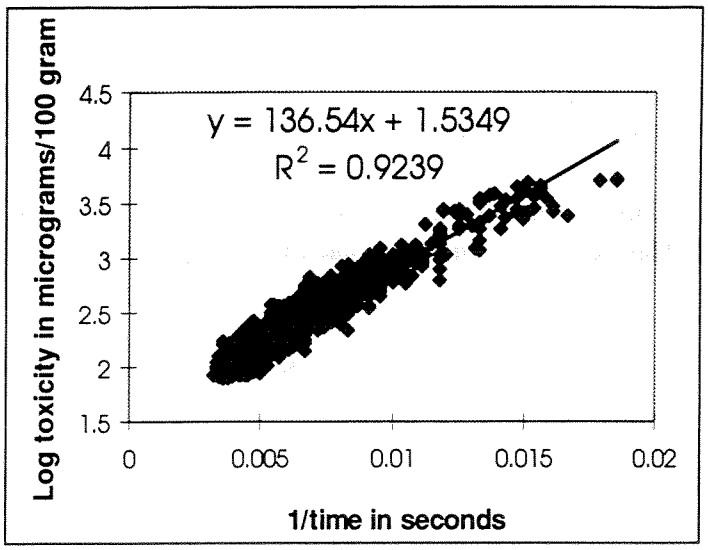

Fig. 1. Linear regression of naturally contaminated *Mya arenaria* from the Bay of Fundy.

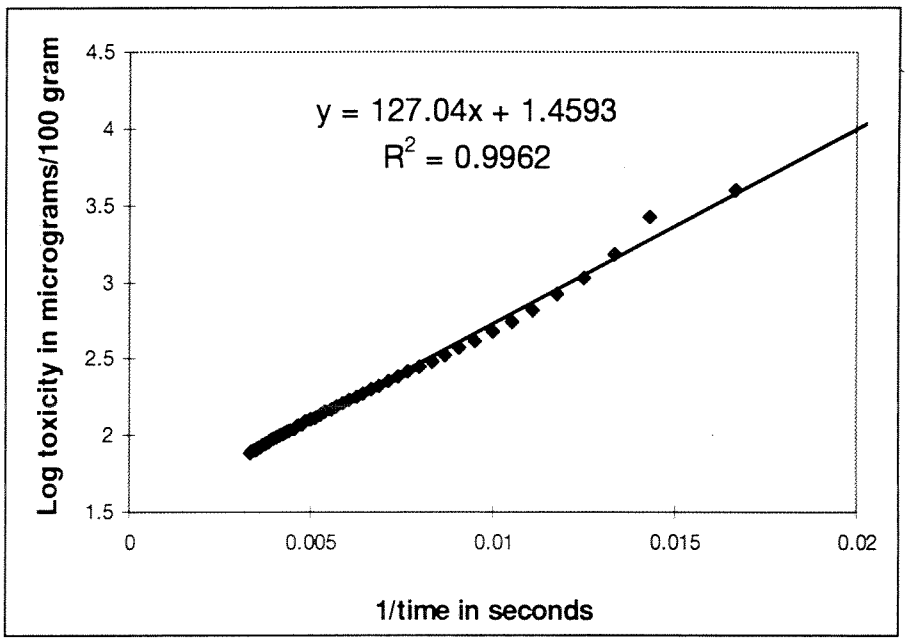

Fig. 2. Linear regression of Sommer's Table using purified saxitoxin.

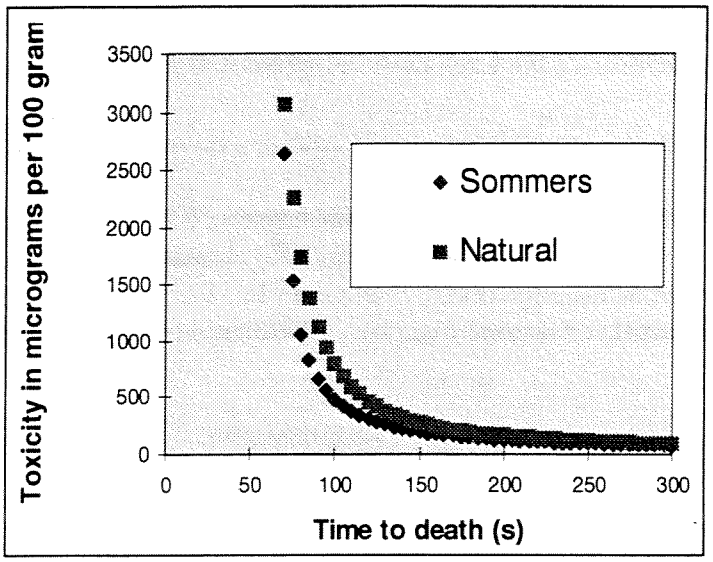

Fig. 3. Comparison of Sommer's Table saxitoxin curve with Bay of Fundy naturally contaminated *Mya arenaria.*

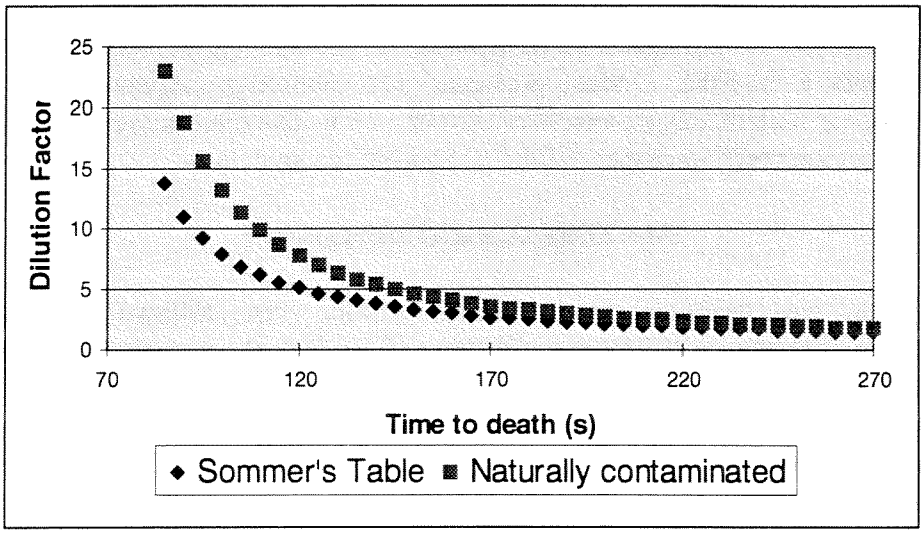

Fig. 4. Comparative dilution factors using Sommer's Table and naturally contaminated *Mya arenaria.*

OPTIMIZATION OF THE MOUSE BIOASSAY FOR MARINE TOXINS - RESULTS OF AN INTERLABORATORY CHECK SAMPLE FOR INDIVIDUAL PRECISION AND POSSIBLE REDUCTION IN THE NUMBER OF ANIMALS EMPLOYED

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INTRODUCTION

The Association of Official Analytical Chemists (AOAC) granted the mouse bioassay procedure for paralytic shellfish toxins with official first action status in 1959 (McFarren 1959). Since then the procedure has been used throughout the world with little, if any, modification. The Canadian regulatory requirement is to use at least three mice per sample regardless of toxicity level. While this provides a high level of confidence in the results, it should be possible to reduce the number of animals for samples with non-detectable levels of toxins. The primary concern is still to keep the number of false negatives to a low level. Over the years, a few collaborative studies have identified various levels of compliance to the existing AOAC method (Adams and Furfari 1984; Park et al.1986). Most problems were caused by deviations from established procedures either in determining conversion factors or in the dilution process. Few of these studies focused on the precision aspect of the test and of the analysts.

MATERIALS AND METHODS

The procedures described in the AOAC method were followed with the following changes. Each analyst was asked to inject 20 mice. CD-1 female mice from Charles River were used at all laboratories. All injections were completed within 1 h. Mice were weighed to 0.1 g and injection and death times recorded to the second. All results are confidential to the individual analyst. Analysts were required to fill in a questionnaire detailing factors such as experience, injection location and speed, syringe and needle size.

RESULTS AND DISCUSSION

The results, summarized in Table 1, are ranked by %relative standard deviation (%RSD) and range from 9.74 to 27.3, with a median of 13.4. The confidence level was calculated at 0.05 as demonstrated in Fig. 1. Analyst number 3 was significantly different from the others. Because all the analysts were using the same mice under similar, and in some cases identical, conditions the difference can only be attributed to injection technique. Some auditors believe that a laboratory conversion factor (CF) should be used, as opposed to individual CF. We disagree with this opinion. Each and every analyst must establish their individual CF values and must continue using the same technique. Any change in technique, such as injection speed or location, necessitates a full standardization procedure.

Experience was not necessarily a positive factor in the precision of the analyst. Analysts with very little experience (less than 200 animals) performed well. Although it is not always the case, the speed of injection appears to increase precision substantially, perhaps because the sample is more widely distributed in the peritoneal cavity and has less chance of leaking out. The depth of injection did not influence the precision as much as the speed of injection, but past experience (Richard, unpublished) indicates a greatly reduced number of false-positives due to injury when the injections are not deep. This was not readily apparent with the limited number of mice in this study. Our current data analyses do not yet allow us to assess the suitability of using a two-mouse screening, as opposed to the three currently in use. Statistical analysis of these and additional data will be completed in the near future.

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Table 1. Results from individual analysts in corrected mouse units. Survivors were assessed as 1 mouse unit. Ranked by precision.

Analyst	1	2	3	4	5	6	7	8	9	10	11
	1.88	1.79	2.15	1.80	1.55	1.71	1.54	1.57	2.06	1.63	1.91
	2.07	1.71	2.26	1.96	1.94	1.58	2.10	1.67	2.08	1.59	2.13
	1.71	2.25	1.80	1.77	2.19	1.79	2.23	1.71	1.76	1.62	2.83
	1.56	1.68	2.21	1.63	1.80	1.77	2.24	1.52	1.34	2.20	1.74
	1.95	1.84	2.40	1.66	1.91	1.92	1.81	2.01	1.78	2.62	1.70
	1.82	1.91	2.52	1.72	2.49	1.88	1.84	1.94	1.87	1.92	2.05
	1.66	2.18	1.83	1.76	1.95	1.96	1.87	2.14	1.60	1.66	1.86
	1.87	1.95	2.30	1.71	1.89	1.59	1.82	2.23	1.63	1.88	1.00
	1.99	1.95	2.54	1.84	1.81	1.92	2.42	2.08	1.65	1.00	1.84
	2.08	1.67	1.95	1.66	1.88	1.81	1.95	1.55	1.52	1.97	1.83
	1.94	1.83	1.98	1.00	1.82	1.62	1.43	1.76	1.72	2.23	1.78
	1.79	1.69	1.99	1.56	1.73	1.49	1.92	1.67	1.86	1.71	1.00
	1.65	2.07	2.21	1.83	1.98	1.65	1.76	1.61	1.88	1.93	1.00
	1.59	1.89	1.92	1.95	1.72	1.81	1.71	1.85	1.89	1.89	1.78
	2.21	1.79	2.02	1.68	1.67	1.83	1.94	1.83	2.51	1.80	2.31
	1.69	1.78	2.31	1.81	1.67	1.00	1.65	1.90	1.44	1.98	1.00
	2.11	1.74	2.02	1.85	1.86	1.71	1.45	1.74	1.18	1.99	1.42
	1.86	2.08	2.19	1.81	1.63	1.92	1.83	1.16	1.83	2.13	2.12
	1.82	1.66	2.38	1.65	1.60	2.13	1.95	2.00	1.51	2.06	1.79
	1.80	1.59	2.53	1.66	1.67	1.86	1.80	1.83	1.66	2.02	1.72
Survivors	0	0	0	1	0	1	0	0	0	1	4
Median	1.84	1.81	2.20	1.74	1.82	1.80	1.84	1.80	1.74	1.93	1.79
Mean	1.85	1.85	2.18	1.72	1.84	1.75	1.86	1.79	1.74	1.89	1.74
S.D.	0.180	0.182	0.231	0.199	0.217	0.233	0.253	0.249	0.291	0.324	0.474
R.S.D.	0.097	0.098	0.106	0.116	0.118	0.134	0.136	0.139	0.167	0.171	0.272
R.S.D.%	9.74	9.83	10.60	11.58	11.83	13.35	13.60	13.94	16.72	17.11	27.23

INFLUENCE OF SEDIMENT TRANSPORT ON REMOBILISATION OF SEDIMENTARY CYSTS OF *ALEXANDRIUM TAMARENSE* FROM THE LOWER ST. LAWRENCE ESTUARY

ab

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This study examines the influence of sediment remobilisation on the concentration of benthic cysts of the toxic dinoflagellate *Alexandrium tamarense* from the lower St. Lawrence estuary. Data were collected once a month in the summer of 1996, near Baie-Comeau. Remobilisation events observed include the passage of small ripple currents (0-3 cm), of dunes (>3 cm) and of a major storm event (major flooding in the Saguenay area) that eroded away >15 cm of sediment in late July. No clear relationships were detected between the concentration of cysts in the top 10 cm of sediment (measured every cm) and these events, nor between cyst concentration and surface blooms of this alga. The high degree of sediment remobilisation at this site probably contributes to mixing the top sediment layer enough to remove the benthic signature of surface bloom events. There is thus a great potential for cyst resuspension in this region - this may contribute to its success as a local "seed bed" for this toxic alga.

INVERSE MODELLING OF WATER COLUMN TOXICITY BY BACK-CALCULATION FROM SHELLFISH TOXICITY

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INTRODUCTION

The usual approach to modelling shellfish toxicity is to formulate a system of one or more differential equations relating the toxin distribution in the shellfish to the concentration and cellular toxicity of toxic algae in the water column, and to solve this system of equations for the distribution of toxins in the shellfish as a function of water column toxicity. In cases where the ambient water column is systematically monitored for toxic algae, this is a valuable tool for predicting toxicity and avoiding the harvesting and sale of shellfish that may be hazardous to human health.

Sometimes an incident of toxic shellfish arises with little or no warning, either because there was inadequate prior monitoring for toxic algae, or because no toxicity was detected in the algae during the period preceding the event. In such cases it is useful to be able to work backwards from the shellfish data to infer a time series of water column toxicity that could have given rise to the observed shellfish toxicity. This approach makes it possible to identify and look for other potential consequences (e.g., toxins that might show up in other components of the food web), and it can be used as a diagnostic tool to test the efficacy of toxic algal monitoring.

Working forward in time from the cause (toxic algae) to the effect (toxic shellfish) generally involves solving a system of differential equations and is referred to by mathematicians as a "direct problem". Working backwards from the effect to the cause is usually far more difficult, in some cases even impossible, and this is called an "inverse problem". Here we address the inverse problem of back-casting from shellfish toxicity data to identify the algal concentrations that could have caused them.

MODELS OF SHELLFISH TOXICITY

Although there are different models that can be used to relate the toxicity of shellfish to the toxicity of the algae on which they are feeding, all are basically variants of the standard uptake and clearance model for a single compartment,

(Rate of change) = (What comes in) – (What goes out)

which differ in the number of compartments and classification of variables. For example, the main models used in previous work on toxin kinetics (Silvert and Subba Rao 1992; Silvert and Cembella 1995; Douglas et al. 1997) were essentially one compartment – they described the flux

of a single toxin (or total toxicity) in terms of the total body burden in the shellfish. However, incorporation of data on biochemical transformations of paralytic shellfish toxins in surfclams *(Spisula solidissima)* required descriptions of the dynamics of six toxin analogues in six anatomical compartments, for a total of 36 (6×6) differential equations (Silvert et al. 1997).

Although such models appear very complicated when written out in their entirety with lots of subscripts and mathematical symbols (see for example Eq. 2 of Silvert and Cembella 1992), conceptually and mathematically they are quite simple; the net flux of toxin from one compartment to another is simply proportional to the concentration in the source compartment. The factors of proportionality can be difficult to estimate (e.g., in Silvert et al. 1997 there are over 1000 such parameters). Fortunately, most are small enough to be neglected, but solving such a system of equations is not difficult from a mathematical point of view. This sort of problem, where one starts from a set of source terms (i.e., the concentration of various toxins, or measured or calculated net toxicity, in the water column) and solves a system of differential equations to calculate where the toxins accumulate, is called a "direct problem" and is mathematically routine. However, that does not mean that such problems are always easy when actual data are involved.

Aliasing and Integrated Sampling

One of the greatest difficulties in modelling shellfish toxicity (and in validating such models) is the lack of continuous integrated measures of toxin concentration or inferred toxicity in the water column. Most plankton sampling provides only "snapshots" of the toxic algal biomass or cell concentration – temporally discrete samples are typically collected from fixed depths or as vertically integrated samples from near the surface. From these samples, the concentration of putatively toxic cells and toxin concentrations of bulk samples must be determined to yield toxin concentrations (cells/L × toxin/cell = toxin/L) or toxicity (toxin/L × specific toxicity of toxin components = net toxicity/L). For model validation, we generally ignore errors associated with these terms, but in fact these errors are not inconsequential – although analytical error associated with the determination of toxin concentrations is on the order of ±3% (A. Cembella, unpublished data), replicate counting errors for phytoplankton enumeration are expected to be on the order of ±10% (e.g., for n=400 cells, Venrick 1978). There is also a substantial error which may be associated with the specific toxicity factors used to convert PSP toxin concentration to toxicity (e.g., Oshima 1995); this error term is difficult to quantify, but it may exceed 30% for toxins of poorly characterised toxicity.

For simplicity, it is usually assumed that the cellular toxin concentration is constant in time, and spatial variability can be ignored. The validity of these assumptions may depend on the particular type or suite of toxins which are considered. For example, the profile of paralytic shellfish poisoning (PSP) toxins in dinoflagellates is relatively stable, although the cellular toxicity may vary greatly in cultures under extreme environmental conditions (cf. Cembella 1998). Such large variations may not occur in nature, but Chebib et al. (1993) found substantial intra-seasonal variation in cell toxicity of the *Alexandrium* populations that were analysed by Silvert and Cembella (1995). Variability of cellular toxicity may be a more acute problem for modelling toxin kinetics during blooms of diatoms associated with amnesic shellfish poisoning (ASP) – significant production of domoic acid by the causative organisms (*Pseudo-nitzschia* spp.) may often be

induced only under environmental stress (reviewed by Bates 1998). Estimates of "average toxicity" for natural blooms of these species must therefore be viewed as highly suspect.

Even more serious potential errors are associated with the implicit assumption that samples from the water column are representative of ambient cell concentrations. This is often not a reasonable assumption. Toxic cell concentrations can shift dramatically on relatively short time-scales (hours to days) due to advective transport, tidal cycles, wind-driven mixing and other hydrodynamic processes, whereas sampling intervals for toxic phytoplankton monitoring are usually on longer time-scales (weeks). In addition, many toxic phytoplankton are capable of vertical migration and/or form highly stratified sub-surface layers associated with density discontinuities in the water column (e.g., the pycnocline) (reviewed by Cullen and McIntyre 1998). Farmed shellfish are usually distributed throughout the water column at varying depths, which can lead to significant differences in exposure to toxic algae (Desbiens and Cembella 1990). Thus, in areas where the toxic algal distribution is "patchy" – either in the horizontal or vertical dimension, or where advective transport can expose shellfish to different water masses over relatively short periods of time, there is a substantial likelihood that the sample will not be representative.

The temporal aliasing problem is illustrated by data from the St. Lawrence estuary in which a single water sample with a high concentration of toxic *Alexandrium* cells did not give rise to any noticeable increase in *Mytilus* toxicity, presumably because the cells represented a transient patch, and the mussels were not exposed to them long enough to accumulate a significant amount of toxin (see Fig. 1 of Silvert and Cembella 1995). The same data set also demonstrated that the mussels became toxic several days before any substantial toxicity was detected in the water samples, and unless we accept that there can be alternate sources of PSP toxins, the only plausible explanation is that toxic algae were present, but that the sampling missed them.

BACK-CALCULATION: THE INVERSE PROBLEM

Solving direct problems is relatively easy from a mathematical point of view, although aliasing and related problems may introduce serious errors, but going the other way is another matter; if the solution to a system of differential equations is known, it can be extremely difficult and even impossible to work backwards to find the source terms that could have led to that solution. Working backwards in this way is referred to as an "inverse problem" and has received relatively little attention from mathematicians. There are several good reasons for this.

Impossible Cases

Inverse problems pose many mathematical difficulties, the worst of which is that they may have multiple solutions, or even none at all. It is easy to see that the inverse problem of toxin uptake may not have any solution in certain cases, and this can be difficult to deal with.

Consider a simple one-compartment model of uptake and detoxification,

$$dC/dt = uX - eC \qquad (1)$$

where C is the concentration of toxin in the shellfish, X the concentration in the water column, and u and e are constant parameters characterising the uptake and detoxification rates (Silvert and Subba Rao 1992). This equation is easy to solve, by numerical means if nothing else works, but the inverse problem of determining X given the time series for C is much more difficult and, as stated above, may even be impossible. This can easily be seen from the following example: since X cannot be negative, C cannot decrease faster than $-eC$. If C decreases more rapidly than this, i.e. if $[\ln C(t_1) - \ln C(t_2)] / [t_2 - t_1] > e$, then there is no biologically meaningful solution, and a purely mathematical analysis would require that $X < 0$.

So what does a mathematically impossible solution mean? It is not really surprising that this can happen, since data are prone to error. The difficulty arises because when we approach a direct problem like solving the above equation for C, given X, any errors in X generate quite reasonable errors in C, but when we go the other way an error in C may create an impossible problem. For example, if there are two adjacent points such that $[\ln C(t_1)-\ln C(t_2)]/[t_2-t_1] > e$, there are several possible explanations; $C(t_1)$ may be too high, $C(t_2)$ may be too low, or the parameter e may be too small.

In Silvert and Cembella (1995), inverse modelling of PSP toxicity of mussels from Cap Chat produced small negative values of *Alexandrium* toxicity for exactly this reason, and we suggested that this could be avoided by smoothing the data. Unfortunately, smoothing these kinds of data is not an easy task. Using any of the standard curve-smoothing methods, such as cubic splines, always leads to negative values, which are exactly what one wants to avoid. It is not possible to use logarithmic transformations to avoid negative values, since the data often contain zeros. We have therefore concluded that some negative concentration values are likely to occur in the inverse solution, and that although these may appear very strange, it is better to learn how to interpret them than to find some complicated mathematical way to get rid of them.

Multi-compartmental Models

Multi-compartmental models are much more difficult to invert than single-compartment models. A simple but commonly used two-compartment model is described by the equations

$$dC_1/dt = uX - (e_1 + x_{21})C_1 + x_{12}C_2 \qquad (2a)$$

$$dC_2/dt = \qquad - (e_2 + x_{12})C_2 + x_{21}C_1 \qquad (2b)$$

where C_1 and C_2 are two toxin concentrations, e_1 and e_2 are the detoxification rates, and x_{21} and x_{12} are exchange parameters giving the rates at which toxin passes into compartment 2 from compartment 1 and *vice versa* (cf. Eqs. 3 and 4 of Silvert and Cembella 1995). The chief simplification in this model is the assumption that toxin is initially ingested into compartment 1, which typically includes the viscera, and that there is no direct uptake into compartment 2, which consists of other tissues. Generalisation to more compartments is complicated but straightforward (Vandermeulen et al. 1983, Silvert et al. 1997).

The direct solution of this system of equations, namely solving for C_1 and C_2 given X, is not much more difficult than the direct solution of a one-compartment model, but the inverse

solution is far more difficult, and even more difficult in practice than it is in theory. To see this, suppose that we have a complete set of data including all parameter values and time series for both C_1 and C_2, and we want to determine X. By comparing Eq. 2b with Eq. 1 we see that just as one can solve the one-compartment model for X given C, in the two-compartment model one can solve for C_1 given C_2, so the system is "over-determined" (or equivalently, given the time series for C_1, it is possible to solve Eq. 2b for C_2 as a direct problem). If the relationship between C_1 and C_2 is not exactly as described by Eq. 2b, then the discrepancy has to be resolved before attempting to determine X. However, once C_1 and C_2 are known, solution of Eq. 2a for X is very similar to the solution of Eq. 1 for X.

More serious difficulties arise because we have to deal with imperfect data. It is rarely possible to measure C_1 and C_2 independently, and in most cases the data we have to work with are total toxin concentrations, namely $C_1 + C_2$, and the existence of two compartments has to be inferred from model fitting rather than direct observation (Silvert and Subba Rao 1992; Silvert and Cembella 1995). Even in cases where detailed toxin measurements from separate organs are available, different anatomical compartments may not correspond to compartments with different toxin dynamics (Silvert et al. 1997). This means that the system of equations 2a and 2b is not simply to be solved for X as a function of C_1 and C_2, but rather must be solved for all three variables X, C_1, and C_2 from knowledge only of $C_1 + C_2$. Although this is not a completely hopeless task, it is not one for which there appears to exist any rigorous mathematical approach, and it is probably better to rely on trial-and-error methods rather than attempt to develop a computational "black box" to solve this type of problem. The number of parameters is low enough so that by simply building the model in a spreadsheet and adjusting the parameter values semi-randomly, one can get a reasonable fit (Silvert 1979).

DISCUSSION AND SUMMARY

It is clear that back-calculation of water column toxicity from shellfish toxin data can provide a useful check on direct sampling for toxic algae, although the methodology is not always straightforward. The major question is perhaps whether this is useful, given that the main practical reason for water column monitoring is to warn in advance of potential toxin episodes before the shellfish are affected!

Even so, there are good reasons to carry out this sort of analysis. One reason is simply to test the efficacy of water sampling to see how reliable it is as a means of warning of increases in toxicity, and to help develop optimal sampling protocols. It seems likely that a higher sampling frequency may be needed in estuaries and in zones affected by coastal currents than in more hydrodynamically static systems, for example, and back-calculation from observed events can be used to test this idea. Although the mathematical problems may seem forbidding, they are not actually a major issue. There are two chief ways in which shellfish toxicity data can be used to determine toxin concentrations or toxicity in the water column. One, which does not actually involve back-casting, is to solve the direct problem and compare the theoretical simulation results with actual measurements of shellfish toxicity. If they agree, then the overall consistency of the data set is clearly satisfactory. If they do not agree, then visual inspection of the results is probably more informative than any exact mathematical calculation would be; if the shellfish are more toxic

than indicated by the simulation results, the obvious conclusion is that the monitoring missed some toxic algae that the shellfish grazed upon, whereas if they are less toxic, it suggests that the sampling hit a "hot spot" where the algae were more concentrated or toxic than average. Examples of this sort of analysis can be found in Silvert and Cembella (1995).

The second way of using shellfish toxicity data to evaluate water column measurements is to back-cast to establish general trends that may affect the overall reliability of monitoring. For example, selective feeding may lead to ingestion rates higher or lower than expected, or highly localised spatial distributions of algae may lead to error. Silvert and Cembella (1995) found that levels of PSP toxicity in mussels at the beginning of the spring bloom were much higher than could be explained by water column toxicity and used back-casting to estimate what levels of algal toxicity would have been needed to explain the observed mussel toxicity.

Of course all of these analyses are only as reliable as the underlying models, and a discrepancy between simulation results and data might simply reflect errors in the models. By using very simple models based on a limited set of assumptions, we can be reasonably confident that any discrepancies can be tied directly to weaknesses in the assumptions, and not to mathematical errors in a complex computational model. For example, Silvert and Subba Rao (1992) were not able to simulate the maximum levels of domoic acid found in mussels during a serious toxicity episode, even when the detoxification rate was set to zero – since the basic assumption in the model is that mussels accumulate domoic acid by ingesting toxic algae, the only possibilities for the discrepancy are that the parameter values (feeding rate, etc.) are not correct, or that the domoic acid comes from some other source. These models are basically tools for carrying out bookkeeping, and if the books do not balance, it is necessary to look for a plausible reason.

For shellfish species with relatively slow toxin uptake and detoxification rates, it can prove valuable and cost-effective to monitor other species like mussels (e.g., *Mytilus edulis*) which take up toxins very rapidly. Inverse modelling is a way to obtain quantitative estimates of water column toxicity by monitoring shellfish, and it could potentially be used to infer concentrations of other pollutants. By using inverse modelling, one can not only identify the concentration of material that was in the water column, whether naturally occurring toxins or anthropogenic pollutants, but also infer the times when it was present; this can be used both to validate direct measurements and to identify possible sources of pollution.

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A COMPARISON OF SAXITOXIN CALIBRATION STANDARDS BY MOUSE BIOASSAY AND CHEMICAL ANALYSIS METHODS

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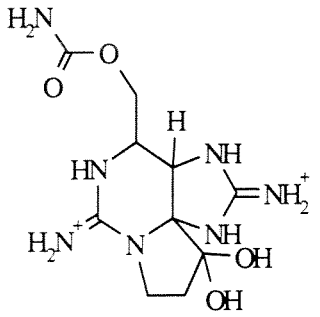
INTRODUCTION

Paralytic shellfish poisoning (PSP) is caused by a group of highly potent neurotoxins which include saxitoxin (STX) and several STX analogs (Burdaspal 1996). This toxin syndrome poses a very serious threat to public health because of the high fatality rate and global occurrence. The regulatory limit for PSP toxins in shellfish is usually set at 800 mg STX equivalents per kg edible tissue.



Saxitoxin

The AOAC mouse bioassay (AOAC International 1995) is used in most countries to screen shellfish for the presence of PSP toxins, although in some countries this assay has been banned due to animal rights concerns. This has led to the development of several new assays such as the neuroblastoma cell assay. The most commonly used chemical analysis method is the combination of liquid chromatography (LC) with on-line post-column oxidation and fluorescence detection (Oshima 1995).

Calibration of the mouse bioassay has always been performed with a standard solution provided free of charge by the US-FDA. Although this solution has been very valuable as the primary reference solution for the mouse bioassay, it was never intended to be used for LC system calibration. The NRC Certified Reference Material Program now markets (at cost recovery) a package of certified PSP toxin calibration solutions (STX, neo-STX, GTX2/3 and GTX1/4) for calibration of analytical instruments.

An additional issue is that new assay kits for PSP toxins are being developed (e.g., the Jellett MIST™ kit). It is important that accurate calibration solutions be provided with such kits, to ensure that there be no discontinuity in results between different determination methods. Therefore we have compared several STX solutions using mouse bioassay and chemical analysis methods such as LC and mass spectrometry.

MATERIALS AND METHODS

Standard Solutions

STX solutions were acquired from: (a) the NRC Certified Reference Materials Program, Halifax, NS (certified concentration = 480 ± 40 mM (140 mg/mL STX-free base in 0.1M acetic acid/water); (b) US Food and Drug administration, Washington DC (stated value = 100 mg/mL STX-dichloride in aqueous 20% ethanol); and (c) CALBIOCHEM, San Diego, CA (stated value = 1000 mM STX-diacetate in 0.1M acetic acid). Accurate dilutions of these solutions were performed using volumetric dispensers with determination of actual dilution factors by weighing dispensed solutions on a digital balance.

Mass Spectrometry

Flow injection analyses were performed on a PE-Sciex API-100 prototype MS using ion-spray ionization (Quilliam et al. 1989).

Liquid Chromatography

LC analyses were performed on a Waters system using the Oshima method with post-column oxidation and fluorescence detection (Oshima 1995).

Mouse Bioassay

Mouse bioassays were performed according to AOAC Official Method 959.08 (AOAC International 1995) using the CD1 strain of mouse with a 1.0 mL IP injection. STX solutions were diluted in 0.003 M HCl.

RESULTS AND DISCUSSION

Ion-spray Mass Spectrometry

Flow injection mass spectrometry with ion-spray ionization (Quilliam et al. 1989) is a useful technique for detecting STX, as well as any impurities that might be present. Analyses using this method revealed impurities in all three standards. The mass spectra are shown in Fig. 1. The peaks at mass-to-charge ratio (m/z) 300 and m/z 282 in each spectrum correspond to the $[M+H]^+$ ion and a fragment ion, $[M+H-H_2O]^+$, respectively, of STX. The m/z 282 ion could also be partly due to the presence of the ketone form of STX which is known to be in equilibrium with the hydrated form of STX.

The NRC standard has some neosaxitoxin (m/z 316) and saxitoxinol (m/z 284) as minor impurities. The US-FDA standard shows what at first appeared to be a major impurity at m/z 328. This was a mystery until it was realized that this standard is dissolved in aqueous 20% ethanol. The m/z 328 ion is most likely due to 12-ethoxy- STX, due to incorporation of ethanol into STX as a hemiketal via equilibrium with the ketone form of STX (Quilliam et al. 1989). It is likely that

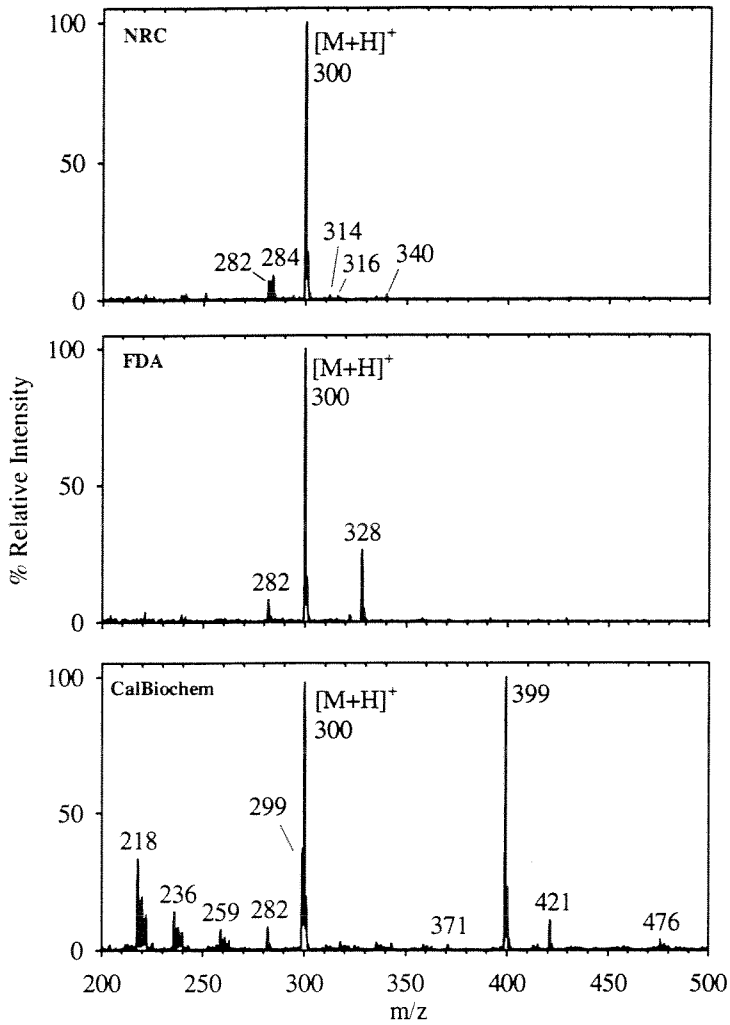


Fig. 1. Flow injection ion-spray mass spectrometry analysis of the three STX standards.

this compound will be greatly reduced when the solution is diluted, but this needs to be tested. The Calbiochem standard showed significant impurity peaks. One of the impurities (giving ions at m/z 421, 399 and 299) has been previously identified as tris(2-butoxy-ethyl)-phosphate, a plasticizer (Wils and Hulst 1993).

LC Results

To determine the concentration of STX in each standard, we prepared diluted solutions in triplicate for each, using very accurate gravimetric techniques, and analyzed them by post-column oxidation LC-fluorescence. The LC system was calibrated with the NRC standard first, as this is our primary standard for LC calibration. The results are presented in Table 1 and Fig. 2. The uncertainties shown are the propagated errors, taking into consideration the relative errors of the LC measurements in both calibration and measurement runs, as well as the uncertainty of the NRC standard. The final uncertainties represent a 95% confidence interval.

Table 1. Results of the LC measurements.

Standard Solution	*Measured Conc'n (mmoles/L)*	*% of Stated Value*
NRC Standard (calibrant)	480 ± 40	(100)
FDA Solution ("100 mg/mL STX-diHCl", 269	230 ± 20	86 ± 7
Calbiochem solution ("1000 mM STX-diAcetate")	790 ± 70	79 ± 7

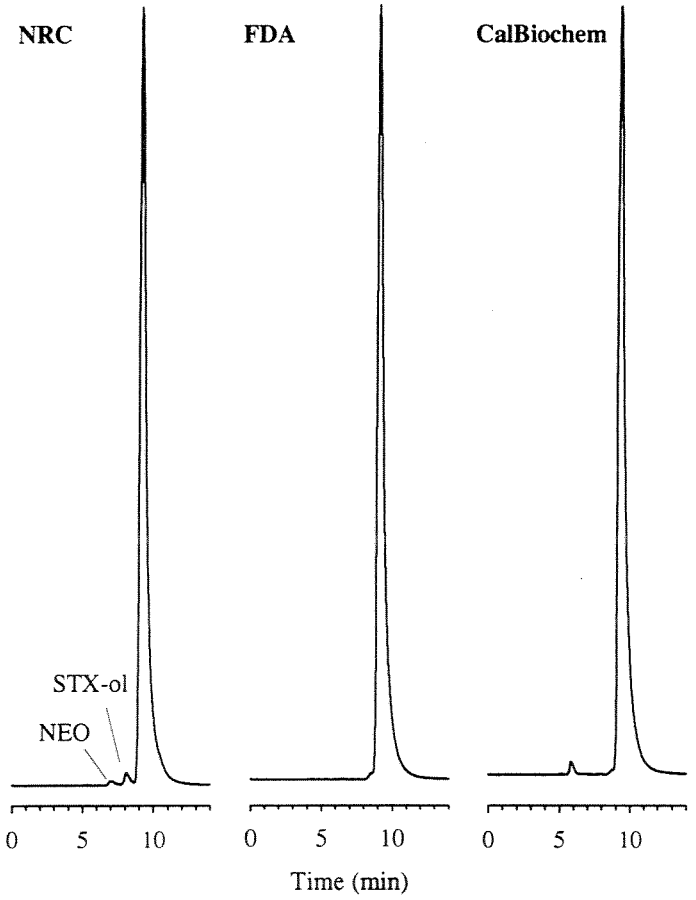


Fig. 2. Normalized chromatograms from the LC analyses of diluted solutions of the three different STX standards, using periodate post-column oxidation and fluorescence detection.

The results are supported in part by the mass spectrometry results which show up impurities, especially in the CalBiochem standard. Aside from such impurities, it may be possible to explain the lower values by difficulties in using gravimetric methods with STX. First, it is very difficult to prepare STX completely free of associated salts. Second, it is very difficult to weigh STX due to its hygroscopic nature. The NRC standard was certified using quantitative NMR techniques rather than gravimetric methods.

Mouse Bioassay Results

After the LC measurements were completed, aliquots of the NRC and FDA standards were diluted in 0.003 M HCl using appropriate dilution factors to prepare solutions at approximately 0.39 mg STX-diHCl per mL. Twenty mice were used for each solution to determine the toxicity as "mouse units (MU) per micromole". The NRC standard gave a value of 1990 ± 170 MU/mmole.

The results are shown in Table 2. Due to the imprecision inherent in the mouse bioassay, it is difficult to distinguish solutions or to determine if a correction of concentrations according to LC values brings the data closer together. However, some guidance may be provided by Oshima's (1995) value of 2483 MU/mmole (based on his standard and the DDY strain of mouse). Correction for the relative sensitivity of DDY vs. CD1 strains (0.87) gives a value of 2160, which is closer to the 2000-2100 range, than is the 1810 value.

Table 2. Results of mouse bioassay measurements

Standard solution	*mouse units/mmole* ± *sd (% rsd)*
NRC Solution	1990 ± 170 (8)
FDA Solution (according to stated conc'n)	1810 ± 200 (11)
FDA Solution Adjusted (according to conc'n by LC against the NRC sol'n)	2100 ± 230 (11)

CONCLUSIONS

There were considerable differences among the measured STX concentrations and the suppliers' stated values. There was also some ambiguity as to whether the STX concentration was being given as that of the free base, the dichloride salt or the diacetate salt. To avoid possible confusion, molarity is a preferred expression of concentration.

The stability of STX solutions was also examined to determine if decomposition might account for low values in some standards. STX solutions (even at 20 mM) are remarkably stable if pH is maintained at approximately 3. No detectable decomposition could be measured over a period of 3 mo with solutions kept at room temperature.

Researchers should be careful when changing from one standard to another, as a discontinuity of data may result. With more precise techniques such as LC, the differences will be noticeable. With an uncertainty of ±10-20% in the mouse bioassay, the differences between the NRC and FDA standards are probably not a major concern. However, we advise that a value of "200 mg FDA-STX(diHCl) equivalents per mL" be adopted by those using the NRC standard for mouse bioassay calibration, rather than the certified concentration value (equivalent to "180 mg STX-diHCl per mL"). In this way, no discontinuity should be observed.

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ELECTROCHEMICAL OXIDATION SYSTEM FOR THE ANALYSIS OF PARALYTIC SHELLFISH POISONING (PSP) TOXINS IN NATURAL SHELLFISH SAMPLES

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INTRODUCTION

High performance liquid chromatography (HPLC) is a powerful tool for the analysis of PSP toxins found in shellfish. PSP toxins lack a significant natural chromophore and must be modified prior to detection. In the traditional "Sullivan" approach, the toxins are oxidized using a post-column reaction system (PCRS) to produce a fluorescent derivative (Sullivan and Iwaoka, 1983). Two additional pumps are required for the PCRS, one to deliver the chemical oxidant which is mixed with the column effluent, the second to add acid to neutralize the oxidant and form the fluorescent product. Disadvantages of this method include the need for this additional equipment, the unstable nature of the oxidant used to form the derivatives, and the sensitivity of the post-column chemistry to changes in temperature and pH. Janiszewski and Boyer (1993) reported fluorescent products could also be obtained using electrochemical techniques (ECOS), thus eliminating the need for cumbersome post-column pumps and unstable reagents. The electrochemical oxidation of saxitoxin and derivatives has been quite accurate in comparisons with the PCRS method using either purified PSP standards or clean algal extracts (Boyer et al, 1997). The task of effective analysis of PSP toxins in a shellfish extract proves to be more of a challenge. In this paper, we discuss the quantitative measurements of saxitoxin and derivatives within the shellfish matrix using the PCRS and ECOS HPLC methods.

MATERIALS AND METHODS

The toxins were separated using a 5μm Alltec C8 (150 mm x 4.6 mm) HPLC column and the isocratic mobile phases of Oshima et al (1995) [*STX and NeoSTX*; 2 mM sodium heptane sulfonate in 30 mM ammonium phosphate, pH 7.1, with 5.7% acetonitrile, *gonyautoxins*; 2 mM sodium heptane sulfonate in 10 mM ammonium phosphate, pH 7.1] at a flow rate of 0.8 mL min^{-1}. In the PCRS, a 1-mL reaction coil in a 50°C heating block was installed between the column and detector. Periodic acid (7 mM in 50 mM pH 9.0 sodium phosphate; 0.4 mL min^{-1}) was added before the reaction coil and acetic acid (0.5 M; 0.4 mL min^{-1}) was added after the coil. For the ECOS, the reaction coil was replaced with an ESA Inc. (Bedford MA, USA) potentiostat equipped with an electrochemical guard cell. Fluorescence was detected for both methods at 330 nm excitation and 390 nm emission wavelengths. Saxitoxin, neosaxitoxin and GTX_{1-4} standards were purchased from the NRC-Canada, Marine Analytical Standards Program. The dinoflagellate extract was obtained from a stationary phase culture of *Alexandrium tamarense* grown in f/2 enriched natural seawater. The cyanobacterium extract was obtained from stationary phase cultures of *Aphanizomenon flos-aquae* (NH5) grown in a minimal nitrogen-limited medium. Algal

samples were sonicated in 0.05N acetic acid and filtered through an 0.45 μm}m syringe filter prior to HPLC analysis. Shellfish extracts of the giant scallop, *Placopecten magellanicus*, were collected by Fisheries and Oceans Canada, and prepared using the standard A.O.A.C. extraction protocol. Prior to HPLC analysis, the extracts were centrifuged at 15,000 rpm and filtered to remove particulates. All samples were run in duplicate on two different days and the toxin concentrations converted to mouse units using the conversion factors in MU μmol^{-1} from Boyer et al (1986): NeoSTX (2100), STX (2050), GTX_1 (1700), GTX_2 (1000), GTX_3 (1800), GTX_4 (1700).

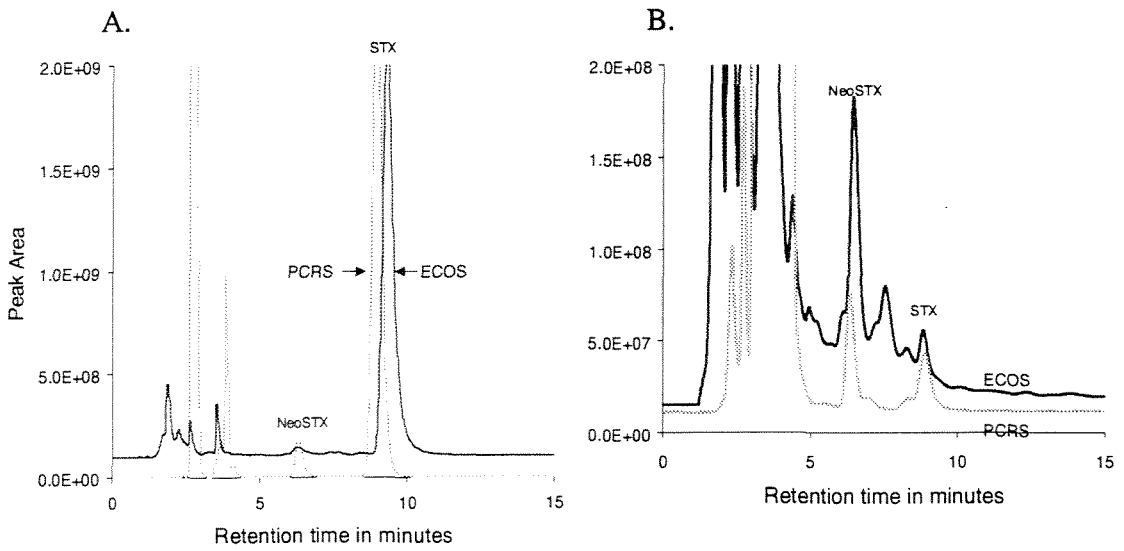

Fig. 1. The HPLC chromatogram of the STX fraction oxidized by either ECOS or PCRS from (a) an extract from *Alexandrium tamarense*, and (b) a scallop digestive gland extract.

RESULTS AND DISCUSSION

Representative HPLC traces from the saxitoxin and neosaxitoxin analysis of the dinoflagellate *A. tamarense*, and the scallop *P. magellanicus*, using either ECOS or PCRS oxidation, is shown in Fig. 1. As expected, the acid extracts from the dinoflagellate were cleaner than those obtained from the shellfish samples. However both shellfish and the dinoflagellate were readily analyzed by HPLC using either PCRS or ECOS oxidation. Agreement between the two different oxidation methods was good, with an R^2 of 0.92 (n=17) for the individual toxin concentrations. Both oxidation methods showed a 10-20% variation in replicate analyses. No significant difference in toxin composition was observed between the different scallop tissues. The composite average for all tissues was 17% STX, 26% NeoSTX, 10% GTX-1, 29% GTX-2, and 18% GTX-3. GTX-4 concentrations were below detection using either oxidation technique. In agreement with the mouse bioassay, most of the PSP toxins were concentrated in digestive gland and mantle tissues (Table 1). Both the ECOS and PCRS techniques gave a total toxin concentration that was less than that measured using the mouse bioassay. The reason for this was uncertain. The conversion factors used to covert μmol toxin to mouse units may be inaccurate, especially for the readily exchangeable gonyautoxins. However, a more likely explanation was

precipitation or degradation of the toxins during shipping from Canada (site of the mouse bioassay) to Syracuse, NY (site of the HPLC assay). Any toxin that had precipitated or absorbed to protein would likely have been removed during the 0.45 µm filtration step immediately prior to HPLC analysis. A detailed study on the effect of sample matrix on the extraction and storage of PSP toxins prior to HPLC analysis is currently in progress.

Table 1. A comparison of the PCRS and ECOS HPLC techniques with the mouse bioassay for the analysis of PSP toxins in different scallop tissues.

Sample (DW3)	*PCRS*	*ECOS*	*Mouse Bioassay*
Scallop digestive gland:			
Total Toxin (MU L^{-1}):	98,800	90,400	
µg STX eq. / 100 g FW:	3,550	3,250	4,770
Scallop mantle:			
Total Toxin (MU L^{-1}):	35,200	19,600	
µg STX eq. / 100 g FW:	1,270	705	1,310
Scallop gonad:			
Total Toxin (MU L^{-1}):	2,120	2,520	
µg STX eq. / 100 g FW:	76	91	49
Scallop remainder:			
Total Toxin (MU L^{-1}):	1,890	1,560	
µg STX eq. / 100 g FW:	68	56	33

Cell on – cell off experiments

One difficulty in the analysis of PSP toxins by HPLC is the presence of "imposter peaks". These are peaks in the HPLC chromatograph that have a similar retention time and fluorescence to the PSP toxins, but are not due to the saxitoxin ring system (Sato and Shimizu, 1997). While the analysis of saxitoxin, itself, is generally free from the imposters, there are several compounds in shellfish and algae that can interfere with the analysis of neosaxitoxin and the gonyautoxins. The ECOS oxidation system offers an easy approach for the identification of these peaks. A sample injection with the electrochemical cell "off" is compared to a standard injection with the electrochemical cell "on". PSP toxins require the cell be "on" to be oxidized and form the fluorescent derivative. In contrast, fluorescence peaks due to imposter compounds show up under both conditions. Similar experiments are possible using the PCRS; however they are complicated by changes in flow rate or pH associated with stopping the post column pumps. An example of an ECOS cell "on", cell "off" experiment using a toxin extract from the cyanobacterium *A. flos-aquae* is shown in Fig. 2.

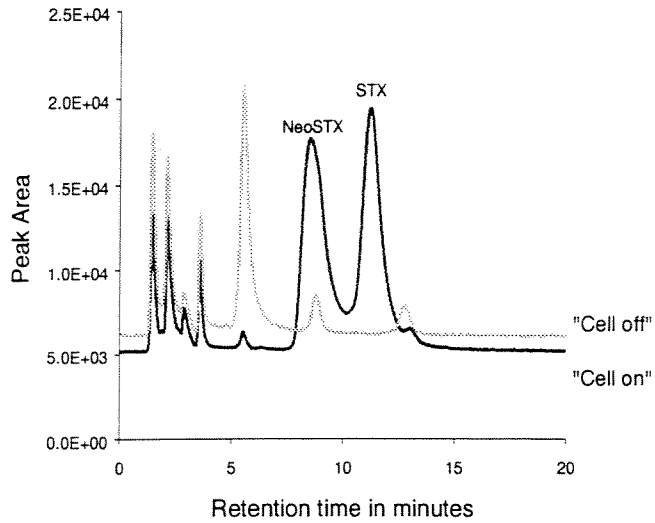

Fig. 2. A cell "on", cell "off" experiment with a toxic extract from *Aphanizomenon flos-aquae*. The peaks associated with STX and NeoSTX disappear when the electrochemical is turned off, exposing a small imposter peak at the same retention time as NeoSTX.. Fluorescent compounds which do not contain the pyrimidopurine ring system found in saxitoxin may either increase in area (peaks at 5.5 and 12.9 min) or remain the same (peak at 3.6 min).

DISCUSSION

The post column reaction system (PCRS) and the electrochemical oxidation system (ECOS) were both equally effective for analysis of PSP toxins from scallop extracts. In contrast to earlier studies (Lawrence and Wong, 1995; Boyer et al. 1997) which used an ESA Coulochem amperometric detector for the oxidation of PSP toxins, these results were obtained using a simple potentiostat and the Coulochem guard cell. No significant differences were observed between samples oxidized using the full amperometric detector or the potentiostat, when fluorescence was detected. The full amperometric detector did allow the oxidation of the toxins to be measured directly (Boyer et al. 1997), but this approach was complicated by the high potentials required and number of other compounds that were oxidized at those potentials. Detection of the fluorescent derivative has the advantage in that the narrow excitation and emission wavelengths (330 nm and 390 nm) provide some selectivity for the saxitoxin ring system. Naturally fluorescent compounds, that are detected within this narrow window, can be excluded by comparing otherwise duplicate analyses with the electrochemical cell turned on and off.

The margin of error for the HPLC analysis using either PCRS or ECOS was within 10-20% for both methods. While this is comparable to error associated with the mouse bioassay, it is below that expected for an instrumental method. The ECOS system eliminates the problem of unstable oxidation reagents, however stability of the standards themselves, remains a problem. A major component in our error was due to running duplicate analysis on different days. The analysis of samples run within the same day usually agreed within 5%. In addition, scallop extracts were not cleaned up prior to HPLC using either solid phase extraction or an equivalent

technique. Disposable *Sep-Pak* cartridges, for example, have been successful in eliminating many of the interfering peaks associated with the analysis of PSP toxins in cyanobacteria (Boyer et al. unpublished) and may have been of benefit here. Further studies applying similar techniques to different shellfish matrices are currently in progress.

ACKNOWLEDGEMENTS

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APPLICATION OF RIBOSOMAL RNA-TARGETED PROBES TO DETECT *PSEUDO-NITZSCHIA MULTISERIES* AND *P. PUNGENS* IN ATLANTIC CANADIAN WATERS

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INTRODUCTION

Around the world, at least seven species of the pennate diatom *Pseudo-nitzschia* are producers or suspected producers of the neurotoxin domoic acid, causative agent of Amnesic Shellfish Poisoning (Bates et al. 1998). Many other *Pseudo-nitzschia* species are non-toxic, but are difficult to distinguish from among themselves or from the toxic species. Electron microscopy is most often used to identify *Pseudo-nitzschia* species. However, this is a laborious and time-consuming procedure that requires expensive instrumentation.

Oligonucleotide (ribosomal RNA)-targeted probes have been developed (Miller and Scholin 1998) to distinguish among 7 *Pseudo-nitzschia* species found in coastal waters of California. They take advantage of unique large unit ribosomal RNA gene sequences, acting as "signatures", for each *Pseudo-nitzschia* species. Here we document that these nucleotide probes are also capable of detecting eastern Canadian species of *Pseudo-nitzschia*. We also examine if differences in cell physiology in cultures affect the amount of labelling by these probes.

MATERIALS AND METHODS

Pseudo-nitzschia multiseries clones

Clones KP-104 and KP-105 were isolated from Cardigan Bay, Prince Edward Island (PEI), in Dec. 1993. Clone CLN-1 was obtained by the sexual reproduction of KP-104 x KP-105, in Jan. 1997 (Davidovich and Bates 1998). Clones CL-44 to CL-48 were isolated from New London Bay, PEI, in Nov. 1996. These Canadian clones were compared to MU-3, isolated from Monterey Bay, California.

Pseudo-nitzschia pungens clones

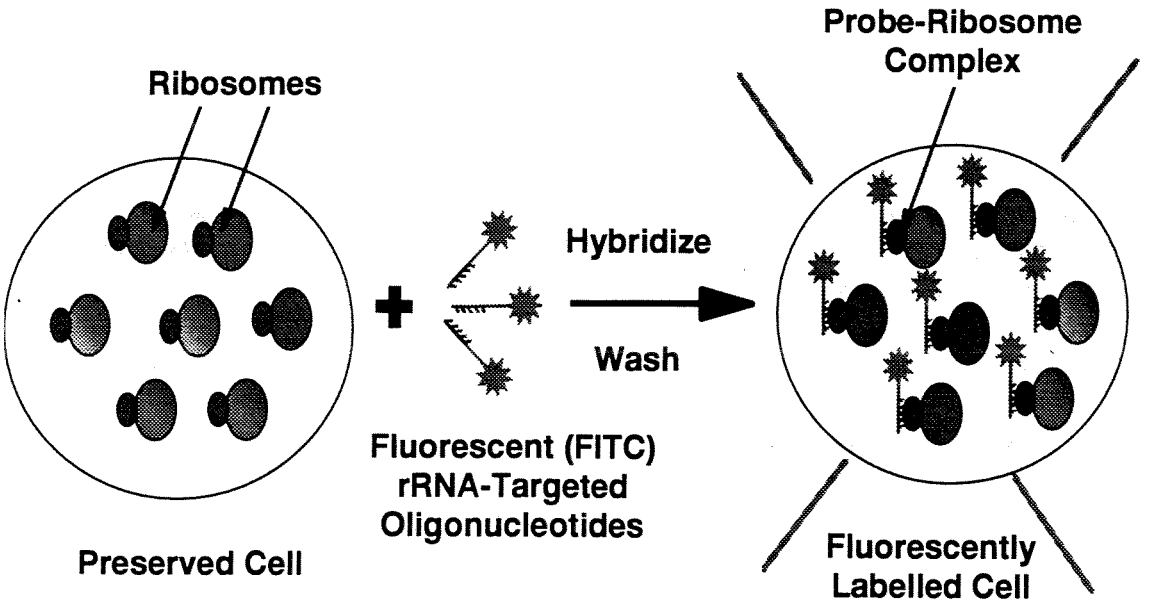
Clones CL-19, CL-24, and CL-71 were isolated from New London Bay, PEI, in Nov. 1996. PU-18 to PU-23 were isolated from Monterey Bay, California.

Physiological experiments

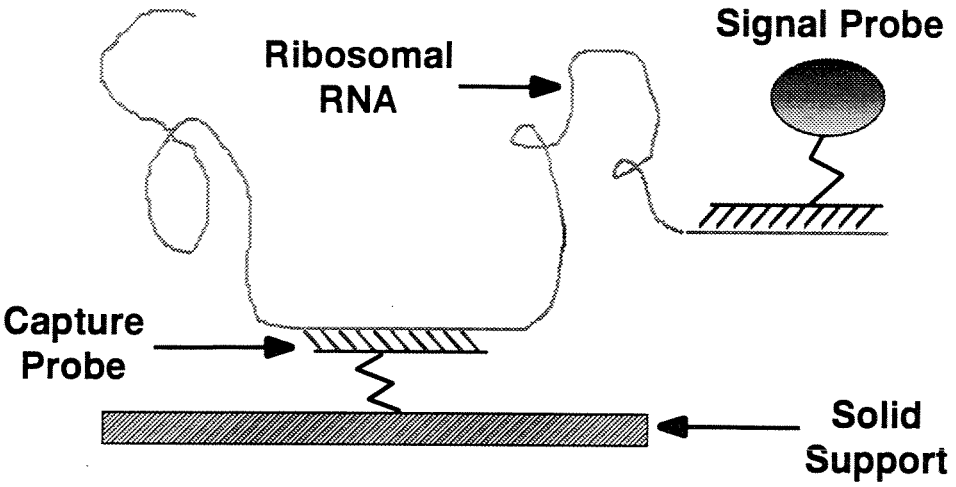
Clones KP-104 and CLN-1 were grown in batch culture (medium f/2) and sampled on days 4, 11, 18, 25 and 32. Clone CLN-1 was grown in Si-limited chemostat cultures (medium f/2) at μ=0.2 d^{-1} and 0.8 d^{-1} (equivalent to 17 and 74%, respectively, of the maximum growth rate).

Two probe assays were tested (Scholin et al. 1997):

1) **Whole-cell hybridization.** Filtered cells were labelled with rRNA-targeted probes and visualized with fluorescence microscopy:



2) **Sandwich hybridization.** Homogenized cells underwent two separate hybridization reactions: capture of target RNA sequences and binding of an enzyme-tagged signal probe to a sequence near the capture site. The RNA is thus sandwiched between two probes.



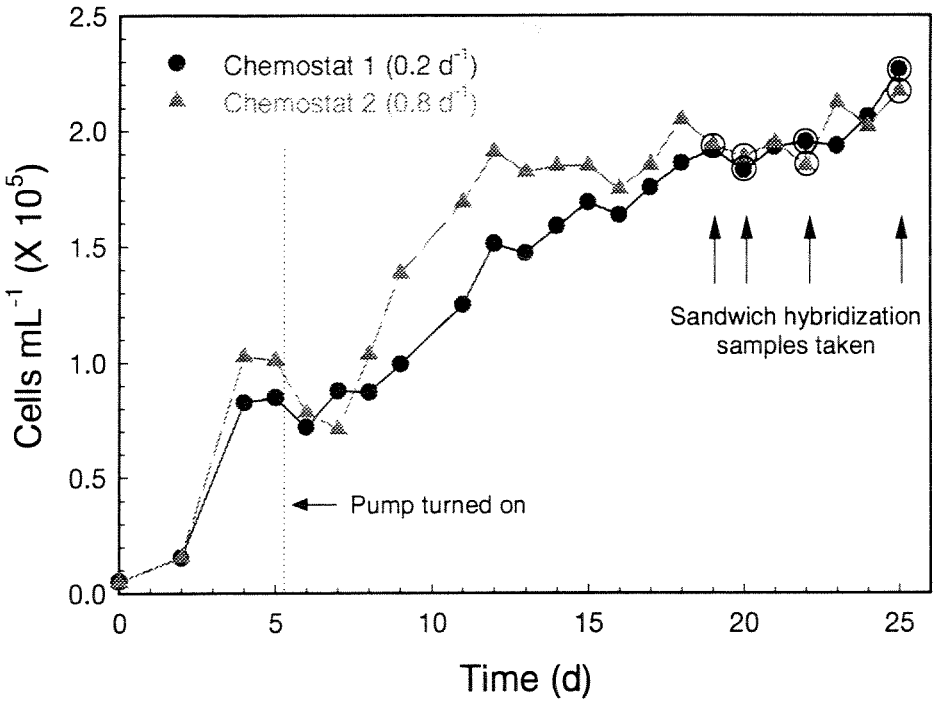
RESULTS

The rRNA-targeted probes, developed for Californian strains of *Pseudo-nitzschia* species, are also capable of strongly labelling *P. multiseries* and *P. pungens* strains isolated from eastern Canada. There was no visible difference in the rRNA whole-cell labelling intensity of *P. multiseries* clones isolated in 1993 and 1997.

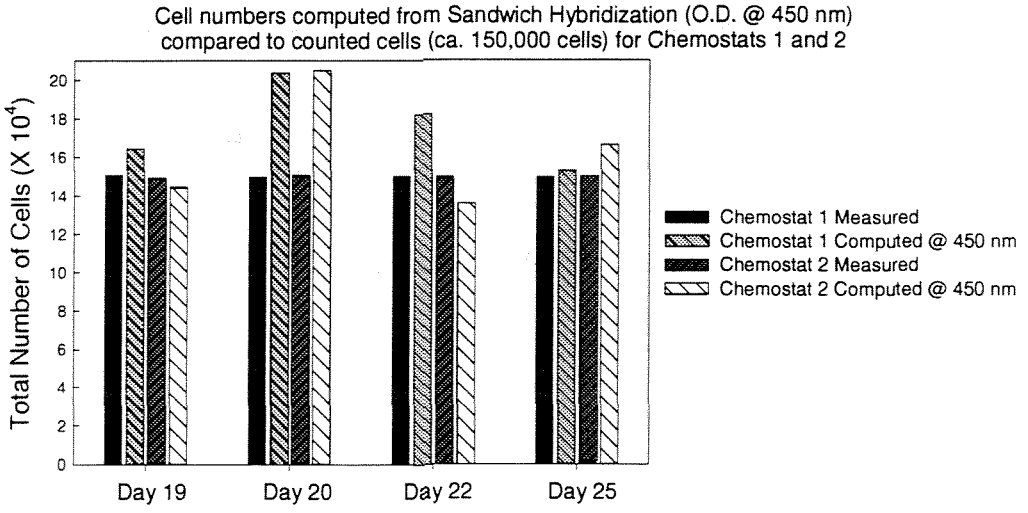
Batch culture experiments suggest that the proportion of labelled cells and labelling intensity decrease as the culture ages during stationary phase (> 18 d).

Day	CLN-1	KP-104
4	++	++
11	+++	+++
18	+++	+++
25	++	+++
32	++	++

However, cells in nature may not be found in a physiological condition akin to batch culture stationary phase. Rather, they may still be dividing, but at a growth rate reduced by the concentration of a specific limiting nutrient. We therefore grew *P. multiseries* in silicon-limited chemostat cultures at division rates of 0.2 d^{-1} and 0.8 d^{-1}.



During steady-state growth, an equivalent amount of rRNA was labelled for an equal number of cells growing at the two division rates when assessed by the whole-cell and sandwich hybridization assays (the day 20 measured vs computed difference remains unexplained).



CONCLUSIONS

1) Ribosomal-targeted RNA probes developed for *Pseudo-nitzschia multiseries* and *P. pungens* from California also strongly label these species from eastern Canadian.

2) Clones isolated in 1993 label equally well as those isolated in 1997, suggesting that the cellular RNA contents are equivalent.

3) A slight decrease in labelling of whole cells in late stationary phase batch culture suggests a decrease in cellular RNA content.

4) Cells growing at a fast growth rate ($\mu = 0.8\ d^{-1}$) exhibited an equivalent amount of labelling as those growing at a slow growth rate ($\mu = 0.2\ d^{-1}$) in chemostat culture.

5) These results suggest that the sandwich hybridization assay may quantify *P. multiseries* cells over a range of growth rates in nature.

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A HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR DOMOIC ACID SCREENING OF SHELLFISH TISSUES

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INTRODUCTION

High Performance Thin Layer Chromatography (HPTLC) is enjoying renewed interest as more and more analytical methods are developed. The method offers many advantages over conventional chromatography in areas such as cost, time saving, low waste generation and convenience. In contrast, High Performance Liquid Chromatography (HPLC) requires highly skilled personnel, has high capital costs and requires a relatively high level of maintenance. Waste generation can also be high depending on the method and procedure in used. High throughput operations also require an autosampler which can add to problems previously mentioned.

Domoic acid (DA), a marine natural product, was found to cause illness and death in 1987 (Wright et al. 1989). Since then, the Canadian Shellfish Monitoring Program has closely monitored the levels of this toxin. It has been identified in various parts of the world including New Zealand, California and British Columbia. DA has recurred sporadically in the southern Gulf of St. Lawrence and in the Bay of Fundy although the severity appears to have diminished. With the limited information now available it is impossible to establish trends or seasonality of the blooms.

In the past, a phytoplankton monitoring program was used to give advance warning of *Pseudo-nizschia multiseries*, the organism responsible for DA. The use of this information allowed a reduction in the number of samples analyzed by HPLC, with a substantial saving in laboratory time and associated costs. This program was discontinued in 1995 because of a change in focus. Since then, most samples collected for bioassay have also been analyzed for DA using HPLC with UV detection. The vast majority of these samples (>95%) have been below the detection limit of ~0.5 µg/g, with most of the positive samples occurring within a brief period of time. Under these conditions, alternative methods of analysis may prove feasible and desirable. Although HPTLC can be quantitative, this requires instruments of comparable capital costs to HPLC. The preferred method would be a quick visual determination of presence/absence with follow-up of positive samples by HPLC, perhaps located at a central laboratory with a dedicated instrument. It is within this context that we examined the use of HPTLC as a means of screening shellfish samples for the presence of DA.

MATERIALS AND METHODS

Shellfish extracts were prepared using the AOAC (1984) method. An equivalent volume of methanol was added, centrifuged and decanted resulting in a 0.25 g/mL extract. Domoic acid (DACS-1) and mussel tissue homogenate containing DA (MUS-1) were purchased from the

Marine Analytical Chemistry Standards Program of the National Research Council (Halifax, Nova Scotia). HPTLC plates used were Whatman LHP-KD silica gel 60, 10x10 cm, channeled with Linear-K preadsorbent and without fluorescent indicator. All plates were pre-washed with the final eluting solvent. Samples (20 µL) were applied manually followed by thorough drying on a hot plate at 70°C. Plates were developed for 20-40 min, producing a 5-8 cm migration distance in a saturated atmosphere. They were then dried by flowing air at 50-70°C. Post-development and visualization were achieved by various methods (Dallinga-Hannemann et al. 1995; Jork et al. 1990). Ninhydrin spray (0.5g in 100 mL butan-1-ol), followed by heating to 90°C for 15-30 min, promoted a corn-yellow colour which changed to pink in a few hours. Vanillin dip (1.0 g vanillin in 50 mL 2-propanol), followed by ethanolic KOH (1.0 mL of 1M KOH in 100 mL ethanol), promoted a yellow colour. A NBD-chloride (7-Chloro-4-nitrobenz-2oxa-1, 3-diazole) dip (0.1g in 50 mL ethanol), preceded by a sodium acetate:water:methanol (10g:20mL:40mL) solution, was dried at low temperature and subjected to 37% HCl fumes in a closed jar. A 50% paraffin solution in hexane was used to enhance fluorescence. Omission of the sodium acetate dip also resulted in good developments. DA was visible under longwave UV (320 to 400 nm) as yellow fluorescent bands. All eluting solvents were analytical grade.

RESULTS

Various combinations of solvents for chromatogram development were used, including butanol - glacial acetic acid - water (4:1:1), chloroform - methanol- ammonium hydroxide 27% (4:4:2) and isopropanol - water (8:2). In an attempt to increase separation at the target reference factor (R_f), many small changes in solvent proportions were tested. Attempts at increasing the separating power included increasing butanol to 7 parts and lowering ammonium hydroxide to 0.5 parts. None of these modifications was very successful. A substance which was always present in the tissue extracts had a very similar R_f to DA and was difficult to differentiate at moderate levels of DA (<40 µg/g). Under the conditions tested, this interfering substance had similar retention and derivitization characteristics as the amino acid tryptophan. Such a high detection limit for DA is not suitable for monitoring purposes. NBD-chloride was superior to ninhydrin in all methods tested. Because of the faint colour produced by DA with ninhydrin, the level for visual detection is usually higher. DA was usually detected at levels of 50 ng per channel using ninhydrin. Although vanillin was more pleasing to the nose, the detection levels for DA were close to those detected by developing with ninhydrin and were not pursued extensively. Using NBD-chloride it was possible to visualize DA standards at levels approaching 10 ng per channel. As the plates are able to handle fairly large amounts of material quite easily, the last step is to remove all interfering substances with a similar R_f to DA. We have yet to achieve this with a raw methanolic extract. Two possible avenues for future development are the use of reverse phase plates and ion exchange cleanup. Both offer strong chances of success with the SAX cleanup possibly reducing the detection limit to an even lower level, although the labour cost would be increased.

We believe that a HPTLC-DA method for shellfish that is fast, simple and economical is within reach. For agencies without a current monitoring program, this promising method is worthy of further study. A good argument can also be made for converting existing HPLC to HPTLC if time, operational costs, capital costs and waste generation are assessment criteria.

ADDENDUM

Since the workshop in St. Andrews, we have decided to abandon attempts at devising a procedure without cleanup. We have achieved extremely promising results with the SAX cleanup of Quilliam et al. (in press). Although the reliability and ruggedness of the method requires further evaluation, we have achieved detection limits of less than 2 µg/g tissue in naturally contaminated shellfish. We consider this level of detection as the maximum for use as a screening tool. These results will be reported in the near future.

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PHOTOPERIOD EFFECT ON THE SEXUAL REPRODUCTION OF *PSEUDO-NITZSCHIA MULTISERIES* (HASLE) HASLE, A DOMOIC-ACID-PRODUCING DIATOM

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In order to persist, many diatoms must overcome a reduction in cell size which results from vegetative divisions. Sexual reproduction regenerates maximal cell length, but is one of the least known aspects of diatom biology. In pennate diatoms, sexual reproduction usually involves pairing of two parent cells (gametangia) which come in close proximity and produce either one or two gametes each. Cell length must decrease to ca. 40% of the maximal cell size in pennate diatoms (Lewis 1984) before sexual reproduction may occur. A minimal cell size also restricts sexualization, causing these shorter populations to continue dividing vegetatively until they die (Geitler 1935; Wiese 1969; Drebes 1977). The sexual process in *Pseudo-nitzschia multiseries* (Hasle) Hasle was recently documented, when it was discovered that this was a dioecious species (Davidovich and Bates 1998). This diatom produces the neurotoxin domoic acid, responsible for the 1987 ASP incident in Prince Edward Island (PEI) (Bates et al. 1998a). The observed pattern of syngamy was Type IA2, whereby one gametangium produces two active gametes and the second produces two passive gametes (Mann 1993). Gametes fuse to form zygotes which enlarge in a bipolar manner to form auxospores. Layers of silica are deposited within the enlarged auxospores to produce the new frustules of the larger initial cells. These initial cells appear to be capable of higher levels of domoic acid production than the smaller parental cells (Bates et al. 1998b).

The goal of this study was to examine the effect of photoperiod on the success of sexual reproduction by the marine, toxin-producing diatom *P. multiseries*. Two sets of *P. multiseries* clones of discrete cell length were tested in seven photoperiod regimes. "Male" and "female" cells from two sets of clones of different size (ontogenetic age) were individually mixed and then grown under a given photoperiod. Vegetative and sexual cells were counted for a minimum of five consecutive days to estimate maximum population fecundity (proportion of gametes with respect to the number of vegetative cells) and success of fertilization, auxosporulation and reproduction (zygote, auxospore, and initial cell production, respectively) in each of nine replicates exposed to each photoperiod.

One-way ANOVA showed a significant positive correlation between the length of the photoperiod and maximum population fecundity of the longer, ontogenetically younger *P. multiseries* clones ($P < 0.05$; $R^2 = 0.58$). In this regard, *P. multiseries* is similar to the centric diatom *Coscinodiscus concinnus* W. Smith (Holmes 1966) and raphid pennate diatom *Nitzschia lanceolata* W. Smith (Davidovich 1995). Not all diatom species display this trait; i.e. gametogenesis of the centric diatom *Thalassiosira weissflogii* Grun. (Armbrust et al. 1990) was

not affected by changes in photoperiod, while a shortened photoperiod enhanced gametogenesis in the centric diatom *Stephanopyxis palmeriana* (Grev.) Grun (Steele 1965) and the araphid pennate diatom *Rhabdonema adriaticum* Kütz (Drebes 1977). Only a few studies have quantified gametogenesis or auxosporulation; in *Thalassiosira weissflogii*, >90% of vegetative cells were induced to form male gametes under selected light intensity conditions (Armbrust et al. 1990). This compares with the maximum population fecundity of ca. 25% for *P. multiseries*. In *Cocconeis scutellum* Ehrenb. var. *ornata* Grun., <2% of cells formed auxospores (Mizuno and Okuda 1985), as opposed to the 92% auxosporulation success we observed for *P. multiseries* in 16 h of light.

Cell size, which reflects population age, appears to influence the number of sexual cells produced by parental clones. Maximum population fecundity was lower in the smaller clones (a maximum of 2%, compared to a maximum of 26% in the longer clones), suggesting that these cells were nearing the minimal cell length capable of sexual reproduction.

Greatest mean reproductive success (initial cell production) occurred in 10 h of light, indicating that this photoperiod may be most conducive for sexual reproduction in nature. This photoperiod corresponds with the annual fall bloom of *P. multiseries* within PEI estuaries, the region from which these clones were isolated. However, no sexual cells have yet been observed in water column samples taken during this time. February also provides 10 h of light, indicating that sexual reproduction may be occurring at that time, although few water samples have been collected then to verify the hypothesis. It is also not known whether other components (e.g. nutrients, temperature, irradiance level) conducive for auxosporulation are present at that time.

There is a need for further investigations into the effects of wider ranges and gradual increases in photoperiod, as well as differing intensities and wavelengths of light. By understanding the photoperiod most conducive for sexual reproduction, insights may be gained into the timing of auxosporulation and the toxicity of cells in the field.

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MORPHOTAXONOMY, ISOLATION, AND CULTURE OF MARINE DINOFLAGELLATES ASSOCIATED WITH THE PRODUCTION OF SPIROLIDES

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Circumstantial evidence based upon nanoplankton species abundance in size-fractionated material harvested from the water column at aquaculture sites on the south-eastern shore of Nova Scotia has suggested a causal link between large thecate dinoflagellates, unidentified spherical "golden balls" (GB), and the occurrence of biologically active compounds known as spirolides. To establish the putative link between the presence of GB cells and spirolide occurrence in plankton and shellfish viscera from Nova Scotia, a concerted effort was initiated to determine the taxonomic affiliation of these cells and to prepare clonal isolates and cultures for physiological experiments and toxin production.

Taxonomic identification of plankton species was confirmed by Nomarski interference microscopy, scanning electron microscopy (SEM), and by calcofluor-staining to examine the thecae of dinoflagellates (1000x magnification) using UV-excitation epifluorescence. A large number of non-motile GB cells, approximately spherical in shape and golden in colour, were found in the spirolide-rich plankton fractions. The size distribution of the GB cells was roughly bimodal. Larger cells of this type, as measured (n=50) by optical micrometer, had a mean diameter of 42 µm and thus were dubbed "GB-42". The cytoplasm had a granular appearance, and some cells contained an orange carotenoid inclusion body near the periphery. All GB-42 cells emitted red autofluorescence characteristic of chlorophyll when examined under epifluorescence microscopy (Excitation: 450-495 nm BP; Emission: 520-560 BP).

Under both Nomarski microscopy and SEM, the GB-42 cells appeared to be athecate, with a rather featureless surface. They resembled pellicular cysts of *Alexandrium*, but they were larger than typical vegetative cells of *A. tamarense* occurring in Nova Scotia. Microscopic examination of empty thecae in close proximity to GB cells confirmed that they did not all belong to the same species. Among the recognisable dinoflagellate taxa in the 26-44 µm fraction with pigmentation similar to GB-42 cells, but with intact thecae, gonyaulacoid species (*Alexandrium* spp., *Gonyaulax grindleyi* [= *Protoceratium reticulatum*], *G. spinifera*, and *G. alaskensis*) were the most common. Cells of size and shape consistent with GB-42 cells were found associated with a triangular apical pore complex (APC) and a few had attached thin thecal plates, which were clearly those of *Alexandrium*. Some of these cells corresponded to *A. tamarense* (Lebour) Balech, whereas others possessed a large kidney-shaped ventral pore at the margin of the first apical (1') plate, characteristic of *A. ostenfeldii* (Paulsen) Balech & Tangen. The GB-42 cells possessed a crescent-shaped nucleus (gonyaulacoid dinoflagellate-type) located equatorially, but when probed with a fluorescent rDNA probe (courtesy C. Scholin) specific to North American strains of *Alexandrium* the results were inconclusive.

During periods of high spirolide levels in shellfish from Nova Scotian waters, size-fractionated concentrated plankton samples containing live cells were brought back on ice to the laboratory for

single cell isolations. A drop of concentrated plankton from the 26-44 µm fraction was added to autoclaved seawater and allowed to incubate overnight in small plastic Petri plates at 16°C. After overnight incubation, GB cells frequently became motile. A pooled fraction consisting largely of GB-42 cells was successfully separated from the "scratch culture" using a Beckon-Dickinson FACSort flow cytometer with detector settings and gating parameters optimised for *Alexandrium* spp. Additional pooled samples (>500 cells) were prepared by repeated manual micropipette isolations of motile cells from this cell suspension. Whether isolated by micropipette (n = *ca.* 1000) or by flow cytometry, pooled cells were found to contain spirolides.

Individual cells (n>400) from the 26-44 µm plankton fractions were also isolated to establish clonal cultures. Single cells from the "scratch culture" were isolated using a finely drawn Pasteur pipette, washed twice in a drop of autoclaved seawater on a glass well slide, and then each was placed into a separate well of a 48-well cluster plate. Cells were incubated at 16°C on a 14:10 h light: dark cycle (photon flux density: 120 µmol m^{-2} s^{-1}). Growth medium L1 (diluted 1:10) was used for cell isolation and after cultures of >50 cells were established, they were transferred to full strength L1 medium in 15-mL culture tubes. These clonal cultures were subsequently scaled up into 1-L Erlenmeyer flasks and harvested in exponential growth phase for PSP toxin and spirolide analysis.

The surviving clonal cultures (n=33) yielded the thecate photosynthetic dinoflagellates, *Alexandrium tamarense*, *Fragilidium subglobosum*, *Gonyaulax spinifera*, *Protoceratium reticulatum*, and *Scrippsiella trochoidea*. None of these motile vegetative cell cultures was found to produce spirolides in exponential growth. Many GB-42 cells are clearly derived from *A. ostenfeldii*, and most are gonyaulacoid dinoflagellates, but they do not represent a unique taxon. The detection of spirolides in cultured *A. ostenfeldii* from Denmark (Cembella et al. 1998) strongly links this species to spirolide production, but attempts to bring local strains into culture have not yet been successful.

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AUREOCOCCUS ANOPHAGEFFERENS BROWN TIDES IN NEW JERSEY

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INTRODUCTION

Brown tides of the picoplankter *Aureococcus anophagefferens* currently are one of the most important identified harmful algal bloom-related threats to fishery resources in the northeast U. S. Eastern Long Island, New York, has experienced chronic brown tides since their observation in 1985. Severe detriment to components of the biota have resulted from some of the Long Island brown tides especially earlier outbreaks (Nuzzi 1995), and also from a single brown tide in Narragansett Bay, Rhode Island, in 1985 (Tracey 1989). For example, detrimental effects included mass mortality of bay scallop, *Argopecten irradians*, in Long Island (Bricelj and Kuenster 1989), mass mortality of blue mussel, *Mytilus edulis*, in Narragansett Bay (Tracey 1989), and destruction of eel grass, *Zostera marina,* in Long Island (Dennison et al. 1989).

Early Long Island brown tides, those of 1985 and 1986, were characterized by very high intensities (*ca.* 10^6 cells mL^{-1} or more), wide geographic distributions, and long durations (*ca.* 6 mo) (Cosper et al. 1989). Some of the subsequent Long Island brown tides have been major, although not with the same combination of intensity and distribution as in the first 2 yr.

Barnegat Bay, New Jersey, may prove to be another prime brown tide site. Blooms of *A. anophagefferens* in the Barnegat Bay system in 1995 and 1997 rivaled major post-1986 Long Island blooms with regard to intensity and duration. Persistence of the species in Barnegat Bay for at least a decade and the recent blooms suggest likelihood of future brown tides there. We believe continuing documentation and study of *A. anophagefferens* bloom development in Barnegat Bay is a necessity.

MATERIALS AND METHODS

The Barnegat Bay system extends approximately 48 km along the New Jersey coast; it ranges from 2.0-6.5 km in width (Fig. 1). The bay is shallow with depths averaging 1.3 m in the northern half and 2.0 m in the southern half. Brown tide surveillance in 1995 began weeks after bloom initiation and was informative but insufficient. At the onset of the 1997 Barnegat Bay brown tide, agreement by Suffolk County New York Department of Health Services (SCDHS) to process increased numbers of New Jersey bloom samples permitted improved surveillance.

Collections in 1997 were at approximately weekly intervals during the bloom season. Primary reliance for water sampling in the bay was on collections along shore. Selection of sample sites was based on a previous phytoplankton survey in the system (Olsen 1989). Supplementary Barnegat Bay samples and samples from along the New Jersey north shore and in the Hudson-Raritan estuary were obtained, May-August, by helicopter with the cooperation of U. S. Environmental Protection Agency (EPA). Helicopter sampling sites in the bay and corresponding shore sites were as close as possible. Sample preservation (i.e., 1% glutaraldehyde and refrigeration), and immunofluorescence technique for *A. anophagefferens* identification and enumeration, were done by the methods of Anderson et al. (1993).

SCDHS processed New Jersey bloom samples supplementary to an intensive Long Island monitoring effort. As a consequence, most New Jersey samples were enumerated months after collection. Our general experience with *A. anophagefferens* samples is that cells or countable cells can decrease with long sample storage. Nine samples from the 1997 bloom were counted near the time of collection and again *ca.* 6 mo later, when most of the samples were processed. Cell concentration was basically unchanged for five of these and was decreased less than 20% in two others. However, cell concentration was lower by 37% and 56%, respectively, in two samples. Reported cell numbers may be lower in some instances, therefore, than actual bloom numbers. The Howard Laboratory initiated monthly sampling of the bay and *A. anophagefferens* enumeration in November, 1997 after substantial training by SCDHS.

BLOOM OBSERVATIONS

The first confirmed *A. anophagefferens* brown tide in New Jersey was in Tuckerton Bay in 1995 (Nuzzi et al. 1996; Mahoney et al. 1996). Tuckerton Bay comprises the southwestern-most portion of Little Egg Harbor, a southward extension of the Barnegat Bay lagoonal system (Fig. 1). This major bloom was prolonged, apparently extending from the first week in May through mid-July. When enumerated in June, its intensity was *ca.* 10^6 cells mL^{-1}. Associated with the bloom in early May, personnel at a Tuckerton, NJ, aquaculture facility, Biosphere Inc., noted high mortalities of hard clam, *Mercenaria mercenaria*, larvae and inhibition of feeding and growth of survivors in their nursery system. Full geographic distribution of this bloom was not determined. However, post-bloom peak survey and unconfirmed observation suggested that brown tide had been present extensively (i.e., an area spanning at least 32km) in the Barnegat Bay system and in adjacent Great Bay.

In 1997, available information suggests that high intensity of brown tide developed around mid-May. Growth of juvenile clams in Biosphere Inc. was severely reduced in late May. Brown tide was confirmed throughout the Barnegat Bay system and to an undetermined extent in Great Bay in June. By June 11, 4 of 8 samples from various locales contained 2 x 10^5 cells mL^{-1} or greater; the highest level was 5.76 x 10^5 cells mL^{-1}. Bricelj and Kuenster (1989) found that the lower cell concentration is the lower threshold at which adverse effects on shellfish would be expected. Greatest prevalence was in the southern half of the bay. At the end of the month, bloom level at 6 of 13 locales was *ca.* 2 x 10^{-5} cells mL^{-1} or greater; highest levels were 4-5 x 10^5 cells mL^{-1}. The bloom apparently was in pronounced decline about the third week of July. As in 1995, Biosphere Inc. again experienced feeding inhibition in their clams for a 2-mo period, and similar effects were noted in another aquaculture facility in the general area.

DISCUSSION

The first known *A. anophagefferens* blooms were regional (spanning Long Island and Narragansett Bay at least) and were initiated at the same time (May-June). Regional climatologic and, or, hydrographic change is suspected to have been prerequisite for the initial blooms (Cosper et al. 1989; Smayda and Villareal 1989). The 1995 and 1997 outbreaks in the Barnegat Bay system affirm continued regional character of the problem.

Surveys for *A. anophagefferens* throughout the northeast region in 1988 and 1990 by Anderson et al.(1993) established its distribution to be from the mid-New Jersey coast to the northern New Hampshire coast. With respect to potential for brown tide, the suitability of a given location to support blooms of *A. anophagefferens* is more important than its suitability for persistence of the species as a minor component of the phytoplankton assemblage. Observation of "brown-water algal blooms" of unidentified picoplankters (similar in gross morphology and size to *A. anophagefferens*) in Barnegat Bay in 1985, 1986, and 1987 (Olsen 1989) raises the possibility that the Barnegat Bay system also experienced *A. anophagefferens* blooms in those years. Consideration beyond suspicion in this regard is unwarranted at this time, but we are certain at least that the species was widespread and occasionally reasonably abundant in New Jersey northern shore waters at the general time. Anderson et al. (1993) detected it in 1988 (maximum concentration in September: 1.4×10^5 cells mL^{-1}) and later in several Barnegat Bay locales, in various north shore locales, and in Sandy Hook Bay. If the confirmed 1995 and 1997 Barnegat Bay blooms alone are considered, then the Barnegat system ranks with Narragansett Bay in having long term relatively low incidence of the picoplankter, but having one-two blooms in a decade. If, as suspected, brown tide occurred in Barnegat Bay and some adjacent waters in 1985-1987, then the Barnegat system would rank much closer to eastern Long Island with respect to bloom suitability.

Effects of the Barnegat Bay blooms on the natural flora and fauna unfortunately were not determined. Bloom-associated detriment to cultured shellfish exposed to 1995 and 1997 blooms, however, suggest that New Jersey brown tide can result in effects similar to some experienced in other northeast U. S. brown tide-affected regions.

Surveillance over the winter of 1997-1998 for *A. anophagefferens* in Barnegat Bay by the Howard Laboratory confirmed its presence throughout most of the bay to mid-May (the most recent survey). Levels as high as *ca.* 30×10^3 cells mL^{-1} persisted through January 1998. Levels then declined; mid-May maximum level was only *ca.* 1×10^3 cells mL^{-1}. This does not presage a brown tide this year comparable to those of 1995 and 1997. The *A. anophagefferens* population likely has been affected in the last several months with dilution due to increased flushing, and inhibition due to lowered salinity. The area experienced approximately weekly coastal storms from mid-January through March, and precipitation 10 inches above normal for the period January through mid-May (U. S. National Weather Service data). Mid-May geometric mean salinities in the bay were 13, 18.6 and 21.5 PSU in the northern, central, and southern regions, respectively, far lower than the salinities (in excess of 26 PSU) associated with major Long Island brown tides (Nuzzi 1995).

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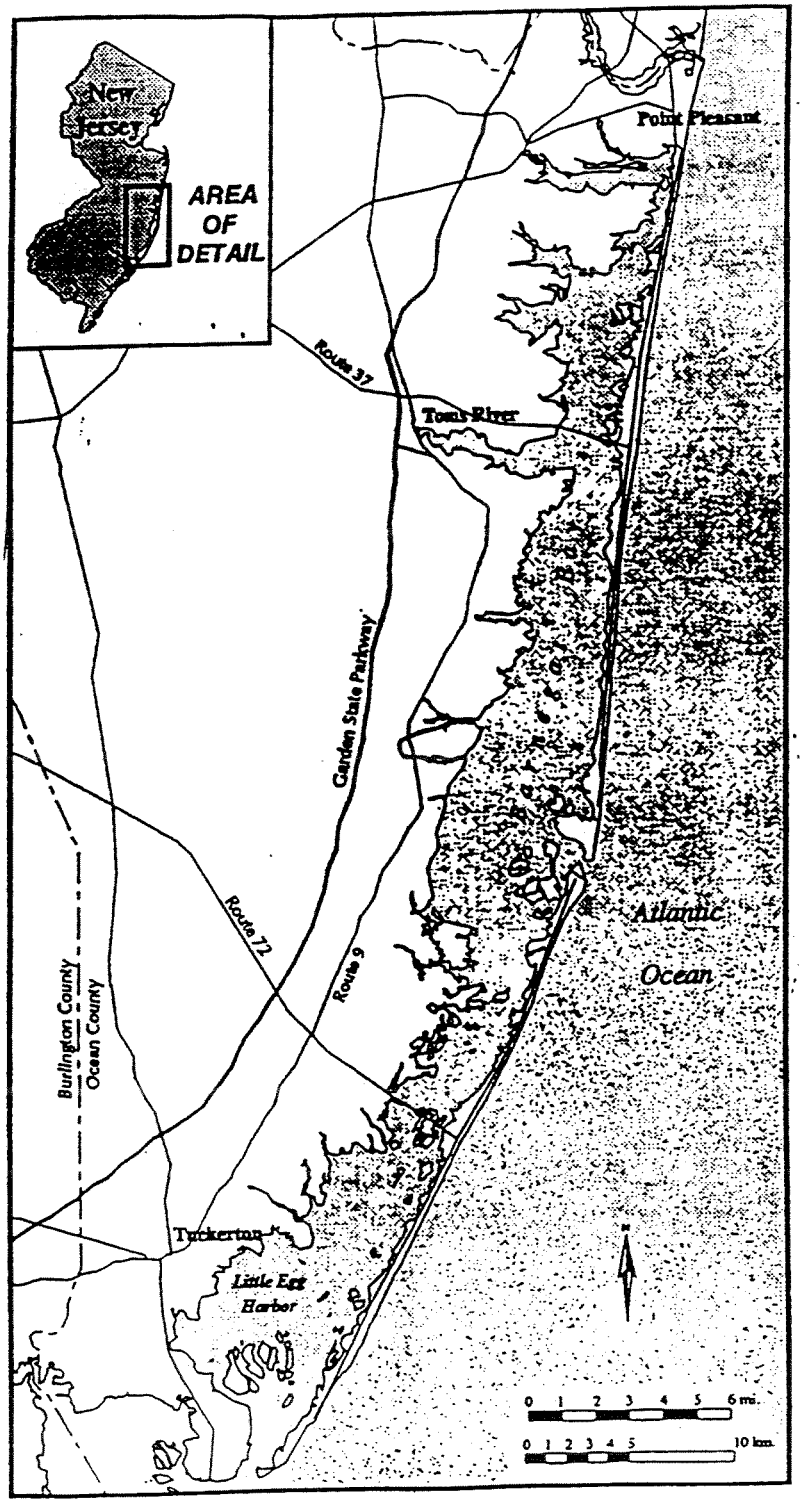

Fig. 1. Barnegat Bay lagoonal system, with insert showing its location on the coast of New Jersey

TEMPERATURE EFFECTS ON KINETICS OF PSP DETOXIFICATION IN SURFCLAMS, *SPISULA SOLIDISSIMA*

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Surfclams, *Spisula solidissima*, support an important commercial fishery in Atlantic North America and are being considered as a candidate for aquaculture in eastern Canada. They are known to detoxify paralytic shellfish poisoning (PSP) toxins relatively slowly (Bricelj and Shumway, 1998), and are also capable of extensive biotransformation of individual PSP toxins, both in the field (Cembella and Shumway 1995) and in the laboratory (Bricelj et al. 1996, Laby 1997), which may lead to an increase in net toxicity. It is therefore of particular interest to determine the role of temperature in explaining toxicity patterns of natural populations, and as a potential means of accelerating toxin elimination in this species.

Juvenile surfclams toxified in the laboratory at 11°C by exposure to a high-toxicity dinoflagellate, *Alexandrium fundyense* (strain GtCA29), were depurated on a non-toxic diet for 2.4 mo at 5, 11 and 21°C, respectively. The viscera detoxified significantly in all treatments (up to 96% toxin loss) and the detoxification rate of this tissue pool was greater (3.1 % loss d^{-1}) at 21°C than at lower temperatures (1.3 to 1.9 % d^{-1}) (Fig. 1).

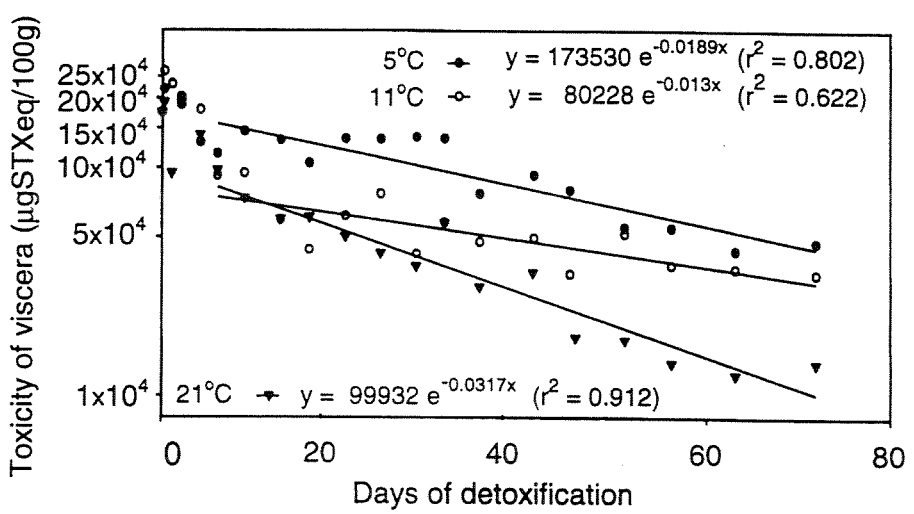

Fig. 1. Toxicity of viscera (in µg STXeq $100g^{-1}$ wet tissue weight) during detoxification of *Spisula solidissima* continuously fed a mixed diet of non-toxic diatoms at three experimental temperatures. Fitted regressions of the form $y = a\ e^{-bx}$, where y = toxicity, x = time in days, excluded data for the first 4 d of detoxification, the initial phase of more rapid detoxification. Each data point represents the mean of 2 to 3 replicate samples, each composed of pooled tissues from 3 surfclams.

In contrast, net toxicity of non-visceral tissues remained constant or increased during depuration. The time required for other tissues to exceed the toxin burden of viscera due to toxin exchange between the two tissue compartments was also directly related to temperature (= 1 and 8 wk at 5 and 21°C, respectively) (Fig. 2).

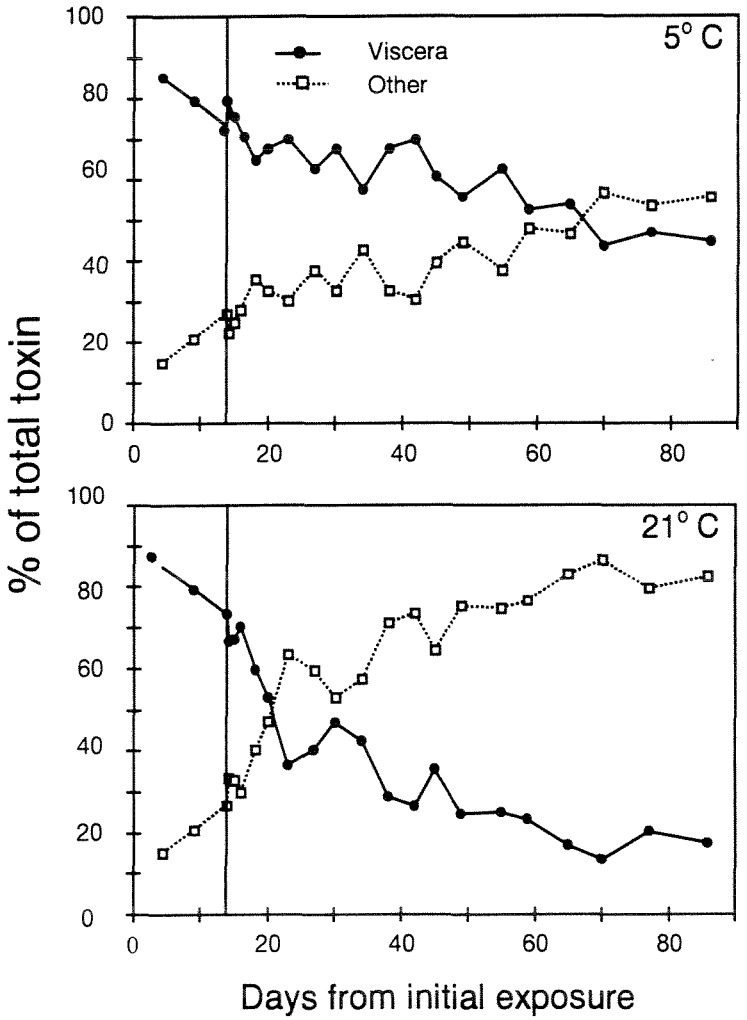

Fig. 2. Mean percent contribution of viscera and other tissues to total toxin body burden (in µg STXeq) of *Spisula solidissima* at 5 and 21°C. Vertical line marks the beginning of detoxification.

Low-potency C1+2 toxins, which contributed 52% to the molar toxin concentration in dinoflagellate cells, were rapidly converted to decarbamoyl gonyautoxins (dcGTX2+3) in clam tissues, where they comprised only 0.5% of total toxins within 4 d of toxification. This contrasts with findings for natural surfclam populations, which retained C toxins for up to 3 mo and showed no detectable level of dcGTX (Cembella and Shumway 1995). Since the conversion of C toxins is so rapid in surfclams, it is unlikely that temperature differences can explain this discrepancy between field and laboratory populations. In the present study, the relative molar concentration of saxitoxin (STX) and dcSTX increased in both tissue compartments with increasing temperature. The absolute molar concentration of STX and dcSTX in other tissues also increased during detoxification, and the magnitude of this increase was positively related to temperature. This

indicates that temperature-dependent accumulation of these toxins via transformation, selective transfer from viscera, and/or selective retention in other tissues exceeds their elimination rate. Overall, however, toxin bioconversion had little effect on net detoxification rate of viscera or in explaining the lack of detoxification of other tissues. This was indicated by the fact that net detoxification rate based on toxicity units (in µg STXeq) in viscera, did not differ significantly from that based on toxin concentration (in nmoles). This probably results from a cancelling effect, i.e. some conversions led to an increase in toxicity (e.g. STX accumulation), whereas others led to a decrease in toxicity (e.g. decarbamoylation of STX to dcSTX, and epimerization from GTX3 to GTX2).

In conclusion, a ΔT of 16°C had a relatively small effect in accelerating toxin elimination from total (whole) surfclam tissues. This is because temperature increases overall metabolism and thus the transfer rate of PSP toxins from the viscera to other tissues, and the latter, while contributing ca. 83% to total tissue mass in surfclams, do not readily detoxify in *S. solidissima*, regardless of temperature. Therefore, temperature manipulation is not recommended as a management tool to enhance detoxification in this bivalve species.

ACKNOWLEDGMENTS

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COPEPOD GRAZING OF TOXIC *ALEXANDRIUM* SPP.: DOES THE PRESENCE OF TOXIN AFFECT PARTICLE SELECTION?

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Paralytic Shellfish Poisoning (PSP) toxins, which are produced by certain species of the genus *Alexandrium* and a few other marine dinoflagellates, are inimical (even lethal) to vertebrates such as fish and humans. Nevertheless many invertebrate species, particularly suspension-feeding bivalve shellfish, can graze on such toxic phytoplankton with little or no observable effect. Previously reported work has demonstrated that various copepod species may also graze upon *Alexandrium* spp. at reasonably high rates and accumulate toxins without suffering mortality or observable impairment. Why then are toxins produced, if they are not lethal to principal consumers of the dinoflagellates, such as zooplankton and bivalve shellfish? If PSP toxins may be detected by chemosensory means (i.e. "tasted") by grazers such as copepods which are capable of selective feeding, then toxin production by *Alexandrium* spp. may provide some measure of chemical defense against predators.

Experiments were performed to determine whether the presence of PSP toxins affects selection of particles by grazing copepods. UV- fluorescent vital stains were used to label both toxic and non-toxic cultured strains of *Alexandrium* spp. (*tamarense/fundyense*) dinoflagellates, which were offered in mixtures at equal concentrations to adult females of three species of copepods: *Acartia tonsa*, *Centropages hamatus*, and *Eurytemora herdmani*. Another set of experiments offered these copepods either toxic or non-toxic *Alexandrium* spp. in mixtures with three non-toxic alternate dinoflagellate species (*Lingulodinium polyedrum* = *Gonyaulax polyedra*, *Gonyaulax cochlea*, *Prorocentrum micans*) at equal concentrations (as carbon per liter). Finally, the copepod species were tested for their ability to graze on monocultures of either toxic or non-toxic *Alexandrium* spp., to observe effects of toxin ingestion when selection is not an option.

Results indicate that these three species of copepods are capable of discriminating between toxic and non-toxic strains of *Alexandrium* spp. In mixtures of toxic *Alexandrium fundyense* and non-toxic *A. tamarense*, all three copepod species preferentially grazed non-toxic cells. Although the copepods were capable of discrimination based on toxin content, the degree to which toxic cells were avoided varied among species. *Centropages hamatus* and *Eurytemora herdmani* included more toxic cells in their diets than *Acartia tonsa*, probably owing to their greater tolerance for PSP toxin body burden. In mixtures of several dinoflagellate species, non-toxic *A. tamarense* cells were grazed at the highest rates of all available particles, indicating high relative palatability. Toxic *A. fundyense* cells in mixtures with three alternate species were grazed at significantly lower rates than non-toxic *A. tamarense* in the previous treatments. Although toxic *A. fundyense* cells were also among the least palatable particles in mixtures, in some cases they were consumed at rates not significantly different from rates on alternate prey species. Once again

A. tonsa showed strong avoidance of toxic *Alexandrium* spp. cells, whereas *C. hamatus* and *E. herdmani* were more willing to include toxic cells in their diets. When monocultures of either non-toxic *A. tamarense* or toxic *A. fundyense* were used as the sole food source, *A. tonsa* and *C. hamatus* exhibited significantly lower grazing rates in the toxic treatment relative to the non-toxic, whereas *E. herdmani* grazed either cell type at approximately equal rates. Only *A. tonsa* showed a strong negative reaction to ingestion of toxic cells; the copepods exposed to toxic *Alexandrium* spp. were clearly impaired and had lost nearly 20% of their body carbon, indicating starvation. *C. hamatus* and *E. herdmani* never showed signs of distress or impairment, despite moderate rates of toxic cell ingestion, indicating a higher tolerance to PSP toxins than *A. tonsa*. PSP toxin production appears to confer some protection from grazing pressure on *Alexandrium* spp. dinoflagellates in mixed assemblages of phytoplankton, rendering cells much less palatable than if no toxins were produced. Copepods (and perhaps other zooplankton species) apparently can discriminate between particles on the basis of PSP toxin content, selectively ingesting non-toxic cells. This probably contributes to the ability of toxic *Alexandrium* spp. to successfully compete with other algal species and thus form blooms.

SPATIAL DISTRIBUTIONS OF PSP WITHIN THE QUODDY REGION OF THE BAY OF FUNDY, MEASURED IN THE GIANT SCALLOP, *PLACOPECTEN MAGELLANICUS*

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There is growing interest in the culture of the giant scallop on the east coast of North America and several culture operations are currently underway. One impediment to the development of an accurate business plan is the uncertainty, due to phycotoxins, as to whether more than the adductor muscle (i.e. roe, whole animal) can be sold to boost the profitability of the grow-out operation. The Quoddy region in the Bay of Fundy is known for its high levels of PSP in some areas as well as the strong mixing of the waters due to its large tidal amplitude. Therefore, a study was initiated in the late summer of 1996 and 1997 to investigate how the PSP concentrations in scallops varied spatially within the region.

Samples of five scallops each were collected by diver during early September at 11 sites (10 in 1997) within the study area and returned to the lab where the digestive glands, adductor muscle and the remaining viscera were dissected and individually frozen at -80°C. PSP toxins within the tissue samples were determined by standard mouse bioassay. Additionally, at each of the sites, plankton samples from the water column and sediment samples were also taken, preserved and analysed later for algal species counts and *Alexandrium* cyst densities respectively.

Results for both years of the study indicated substantial variation in PSP levels in the scallops among the study sites. Passamaquoddy Bay generally had the lowest PSP levels with whole body budens of PSP below the legal limit of 80 μg/100g. The ranking patterns of the sites were consistent between the two years. There was also a good correlation between PSP concentrations in the viscera and the digestive gland. However, there were poor correlations in the PSP concentrations within the animals and the presence of *Alexandrium fundyense* cells in the water column or cysts of *A. fundyense* in the sediment. The implications of these data will be discussed.

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ECOHAB-GOM: THE ECOLOGY AND OCEANOGRAPHY OF TOXIC *ALEXANDRIUM* BLOOMS IN THE GULF OF MAINE.

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A 5-year program called ECOHAB-GOM was initiated to address several fundamental issues regarding *Alexandrium* blooms in the Gulf of Maine: 1) the source of the *Alexandrium* cells that appear in the freshwater plumes in the western Maine coastal current (WMCC); 2) *Alexandrium* cell distribution and dynamics in the eastern Maine coastal current (EMCC); and 3) linkages among blooms in the WMCC and the EMCC. Utilizing a combination of numerical modeling, hydrographic, chemical, and biological measurements, moored and drifting current measurements, and satellite imagery, the project will characterize the structure, variability and autecology of the major *Alexandrium* habitats in the Gulf of Maine.

In the western Gulf, *Alexandrium* blooms and patterns of PSP have been linked to a coastal current or plume of low salinity river outflow (the WMCC). One major project goal is to investigate an area near Casco Bay implicated as the major "source region" for the toxic cells that populate that coastal current. Field surveys will elucidate the biological, chemical, and physical processes that control bloom initiation and development, the delivery of cells from that source region into the WMCC, and the manner in which late-season, localized blooms are retained there to re-seed future blooms with cysts. The second major set of objectives is to characterize the linkage between toxic blooms and the EMCC, to investigate the role of tidal mixing, frontal systems, and upwelling/downwelling in *Alexandrium* dynamics, and to define the linkage between EMCC *Alexandrium* populations and those in both the WMCC (downstream) and the Bay of Fundy (upstream). A unique set of "process" studies will focus on discrete blooms or patches of cells and quantify such parameters as in situ growth rates and grazing losses of *Alexandrium*, the nutritional physiology, vertical migration behavior and transport of this species, the partitioning of toxins within the food web, and the extent to which mixotrophy supplements photosynthesis. A hierarchy of coupled physical-biological models will be used together with ECOHAB-GOM data for investigation of: 1) detailed structure within each habitat; 2) interconnections among habitats; and 3) the role of the larger Gulf-scale circulation in the long-term maintenance of *Alexandrium* populations in the region. Advances in our understanding of the underlying mechanisms controlling the population dynamics of *Alexandrium* will be distilled into simple predictive models of PSP toxicity which can be driven by a relatively small number of observable parameters. ECOHAB-GOM is thus a combined modeling/observational program, utilizing the most current and innovative technologies in an approach commensurate with the multiple scales and oceanographic complexity of PSP phenomena in the Gulf of Maine. More details on this project, including an update of cruise activities and results can be found at the project web page at: http://crusty.er.usgs.gov/ecohab/

ECOHAB-GOM Principal Investigators and their affiliations are: David W. Townsend (University of Maine), James H. Churchill (Woods Hole Oceanographic Institution), John J.

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HARMFUL ALGAL BLOOMS OFF NORTHERN WASHINGTON STATE, USA: PRELIMINARY RESULTS

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INTRODUCTION

This paper presents preliminary results from two cruises in the Olympic Coast National Marine Sanctuary (OCNMS) off the northern Washington coast. Our purpose during the cruises was to determine the distribution and abundance of potentially toxic phytoplankton species and the environmental factors affecting them.

Washington coastal waters are influenced by an eastern boundary current, the California Current System. This is a broad, shallow, slow moving current that flows towards the equator and brings cold, low salinity, highly oxygenated, nutrient-rich subarctic water to the region. Prevailing winds and currents show strong seasonal variation. There is strong wind forcing over the narrow continental shelf and upwelling and downwelling play dominant roles in biological production. The western boundary of the Sanctuary roughly follows the shelf break and there is a series of offshore canyons near the shelf break as well. Most of the coastal land is governed either by Native tribes or is in the Olympic National Park. There are only a few small towns, hence little or no coastal eutrophication.

MATERIALS AND METHODS

Data were taken during two cruises: June 27-July 9, 1996 and July 7-20, 1997. Transects were occupied from S to N, usually at night and with each transect taking ca. 8-10 h to complete. Temperature and salinity were obtained with a CTD; phytoplankton, nutrient, and some salinity samples were collected at 4-6 depths in the upper 50 m of the water column at each station using Niskin bottles attached to the CTD rosette. Representative wind data for the region were obtained from the National Data Buoy Center (NDBC). Wind patterns off the Washington coast are relatively large-scale so that data at any shelf location can be used to provide general information on wind direction along the coast (Hickey 1989; Hickey et al. 1998). Phytoplankton samples were preserved with formalin (ca. 1% final concentration) and analyzed for species presence and abundance using the Utermöhl inverted microscope method (Hasle 1978a, b). *Pseudo-nitzschia* species identifications were confirmed using scanning electron microscopy on selected samples collected by vertical net tows (20 µm mesh) in the upper 10 m. Nutrient samples were frozen and later analyzed using standard autoanalyzer methods (Whitledge et al. 1981). Salinity was determined using a Guildline Autosal salinometer in the laboratory to verify the calibration of the CTD conductivity sensor.

RESULTS

In both years, warmer, fresher water occurred to the south and colder, saltier water occurred to the north in the survey area (Fig. 1). Time series of wind vectors show that wind changes speed and direction over time periods shorter than the cruise duration (Fig. 2). In both years, southern transects were sampled during periods of northward winds, more northern transects were sampled during a period of southward winds interrupted on several occasions by wind "relaxation" or by weak northward winds. Along the Washington coast, northward winds are associated with downwelling conditions and onshore and northward surface flow over the shelf; southward winds are associated with upwelling conditions and offshore and southward surface flow (Hickey 1989). Thus the warmer, fresher water observed at southern locations in both years is likely a result of northward and onshore movement of water from the coastal estuaries south of the Sanctuary (Fig. 2). The colder, saltier water observed north of La Push each year was likely a result of the prevalence of upwelling during that portion of the survey (Fig. 2). A break between the warmer, fresher plume to the south and the colder, saltier water to the north was seen between Kalaloch and La Push each year. Temperatures were 1-3°C warmer and slightly fresher in 1997 than in 1996, but still within the range of natural variability reported previously (Landry et al. 1989). Also, nutrient concentrations were generally higher in the north than in the south with phosphate ranging from <0.1-2 µM, silicate from <1-45 µM, nitrate from 0-13 µM, and ammonium usually <1 µM (data not shown).

However, wind patterns changed rapidly over short time periods within the time frames of the cruises. Hourly wind speeds and directions are shown in Fig. 2, along with the calculated E-W and N-S wind components. Of particular interest are the short, 1- to 2-d intervals of low winds that occur after several days of sustained upwelling-favorable (southward) winds. These relaxation intervals provide stable conditions for the phytoplankton to utilize upwelled nutrients and high light to grow rapidly and attain high biomass on the shelf (3-20 µg chl a/L in the upper water column). Conversely, during periods of downwelling, nutrient levels and supply rates are lower and offshore, and "blue" water is more likely to be found over the shelf. Phytoplankton biomass is generally lower (0.1-1 µg chl a/L) and conditions are not as favorable for rapid growth.

Figure 3 shows the surface concentrations of *Pseudo-nitzschia* spp. and *Alexandrium catenella* during each cruise. *Pseudo-nitzschia* spp. were more abundant in the colder, saltier water in the north, but different species were dominant in the two years. *P. pungens* was most abundant in 1996 with higher concentrations present close to shore, while *P. pseudodelicatissima* was dominant in 1997 with higher concentrations occurring offshore. Both of these species are presumed to produce domoic acid (Martin et al. 1990; Martin et al. 1993; Trainer et al. 1998). Highest cell concentrations were generally in the upper 10 m of the water column, declining markedly below 20 m depth. *Pseudo-nitzschia* spp. were found in 85% of the samples in both years, but were usually <10% of the total phytoplankton population. *Alexandrium catenella* was never abundant, being seen in only 10% of the samples and then usually as fewer than 10,000 cells/L. It was more numerous in the north in 1996 and scattered over the whole area in 1997. Higher concentrations of both species were present in 1997.

DISCUSSION

It has long been assumed that blooms over the continental shelf are the source of toxins in shellfish populations on Washington coastal beaches and in estuaries, but there has never been any positive evidence to support this assumption. To protect the public, the Washington Department of Health routinely closes the coast from Dungeness Spit (on the Strait of Juan de Fuca opposite Victoria, B.C.) to the mouth of the Columbia River from 1 April to 31 October for the harvest of all shellfish except razor clams because of the threat of PSP. This blanket closure has been issued each year since 1942 when three people died after eating clams and mussels harvested in the area. The Health Department tests mussels in cages at three sites in the coastal estuaries and local Native tribes have some shellfish samples tested during the closure period, but there is still little information on toxin levels in shellfish along that long stretch of coastline.

When domoic acid first appeared in razor clams on the Washington coast in 1991, there were no offshore phytoplankton samples available and none were collected. Since 1995, we have collected twice monthly from five coastal beaches and rarely see *Pseudo-nitzschia* or *Alexandrium* cells in our samples. The few *Pseudo-nitzschia* blooms we have seen on the beaches were short-lived, lasting only a few days, likely representative of the dynamic ocean circulation (in particular, episodic upwelling and downwelling over the adjacent shelf) in the area. Razor clams did not become toxic during these short events. A major PSP outbreak occurred in coastal estuaries in November-December 1997. Oysters in the estuaries were affected with high PSP levels ranging from 286-341 µg toxin per 100 g shellfish, but razor clams on beaches next to and between the estuaries were not affected. Chains of *Alexandrium catenella* were reported in beach samples collected north of the estuaries (M. Lesoing, pers. comm.).

Blooms of other phytoplankton species have also been reported from the Washington coastal area. In August-September 1994 and 1995, blooms of *Ceratium furca, C. divaricatum,* and *Prorocentrum micans* occurred in coastal waters. These species do not produce toxins, but the meat of razor clams became pink and liquid. Oysters stored in jars also turned pink, leading consumers to think the shellfish were toxic and causing economic losses for the oyster growers. A major bloom of *Heterosigma* occurred on the coast off Kalaloch in the fall of 1996. The duration and effects of this bloom are not known, and there were no reports of dead fish.

Thus, it is evident that major blooms of HAB species do occur in Washington open-coast waters, but their frequency and duration are not known. Questions remaining to be answered include: Where and when do the blooms occur? What physical and biological processes regulate their population dynamics? How are the blooms transported to coastal beaches and estuaries? What is the relationship between toxic blooms and toxin accumulation in coastal shellfish? With the advent of the Sanctuary-sponsored cruises, we are gradually accumulating both HAB and hydrographic information that will help us understand the dynamics of these coastal blooms.

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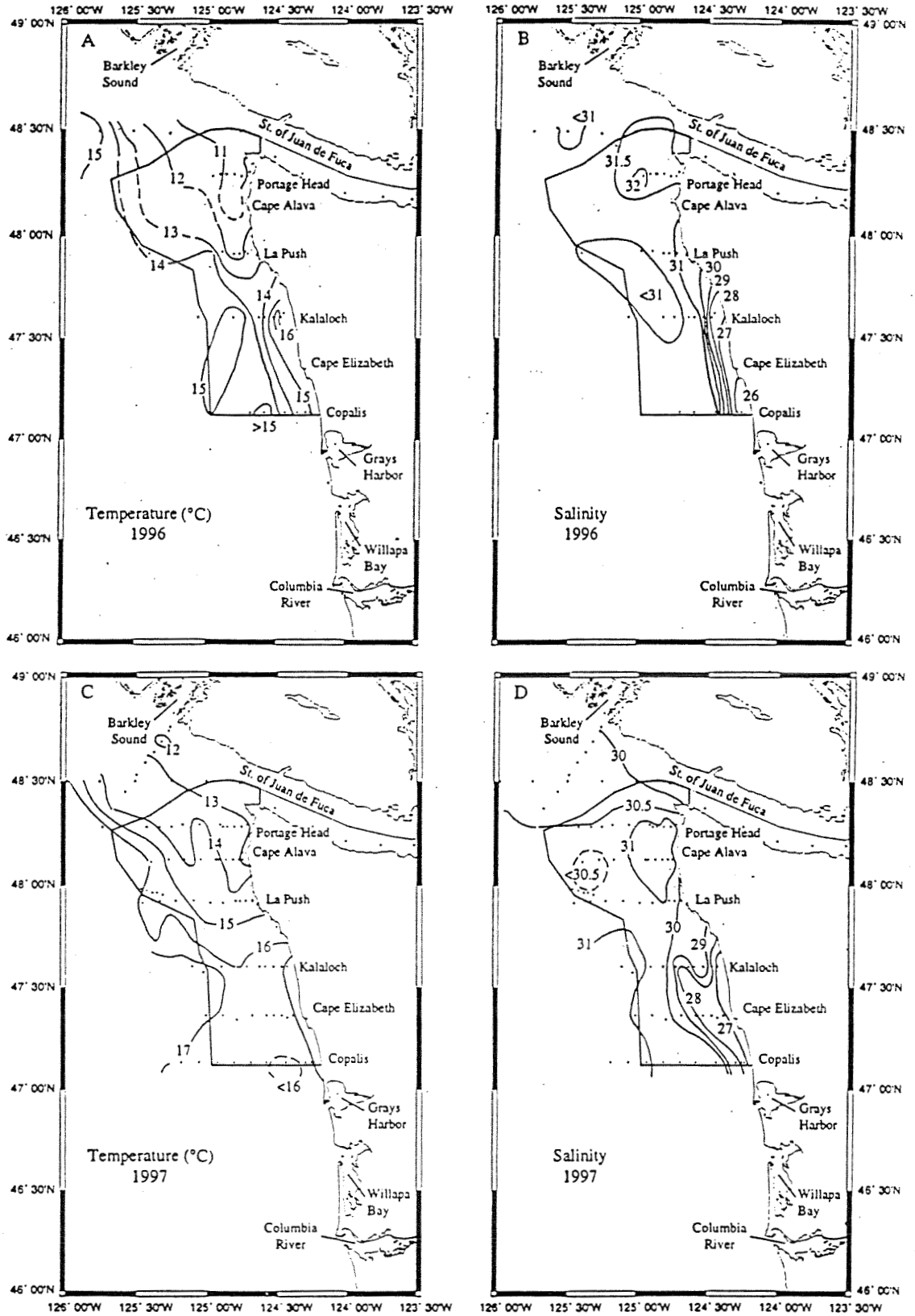

Fig. 1. Environmental conditions of surface water during cruises: (A) temperature 1996; (B) salinity 1996; (C) temperature 1997; (D) salinity 1997.

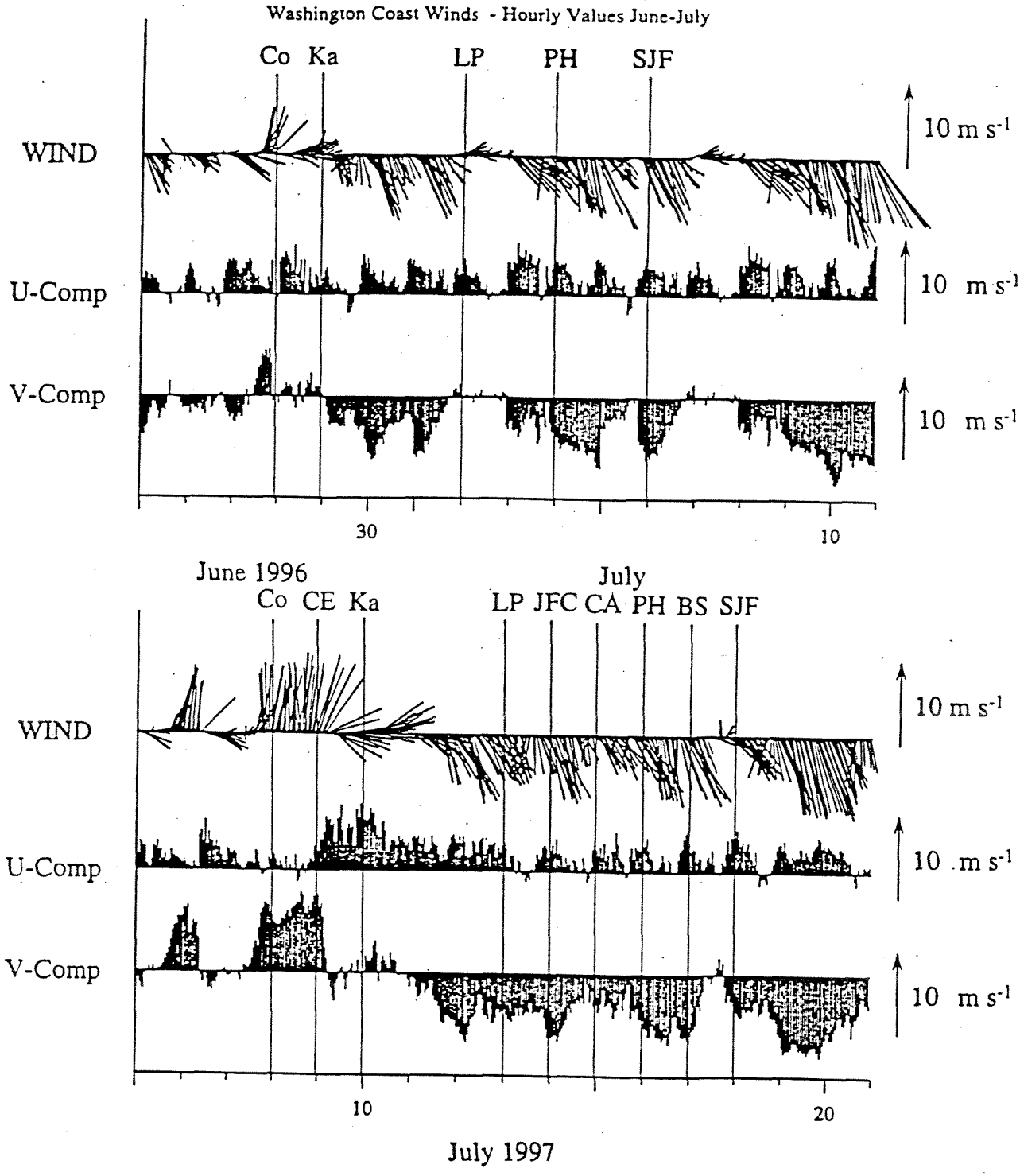

Fig. 2. Time series showing representative regional winds off the Washington coast for each survey. Wind vectors point in the direction to which the wind blows. Onshore-offshore (U) and north-south (V) components are positive onshore and northward, respectively. Northward winds are associated with downwelling conditions, and onshore and northward surface layer flow; southward winds are associated with upwelling conditions, and offshore and southward surface layer flow. Dates on which particular transects were sampled are indicated on the time axis of each plot. Location abbreviations: Co - Copalis Beach; CE - Cape Elizabeth; Ka - Kalaloch; LP - La Push; JFC - Juan de Fuca Canyon; CA - Cape Alava; PH - Portage Head; BS - Barkley Sound; SJF - Strait of Juan de Fuca.

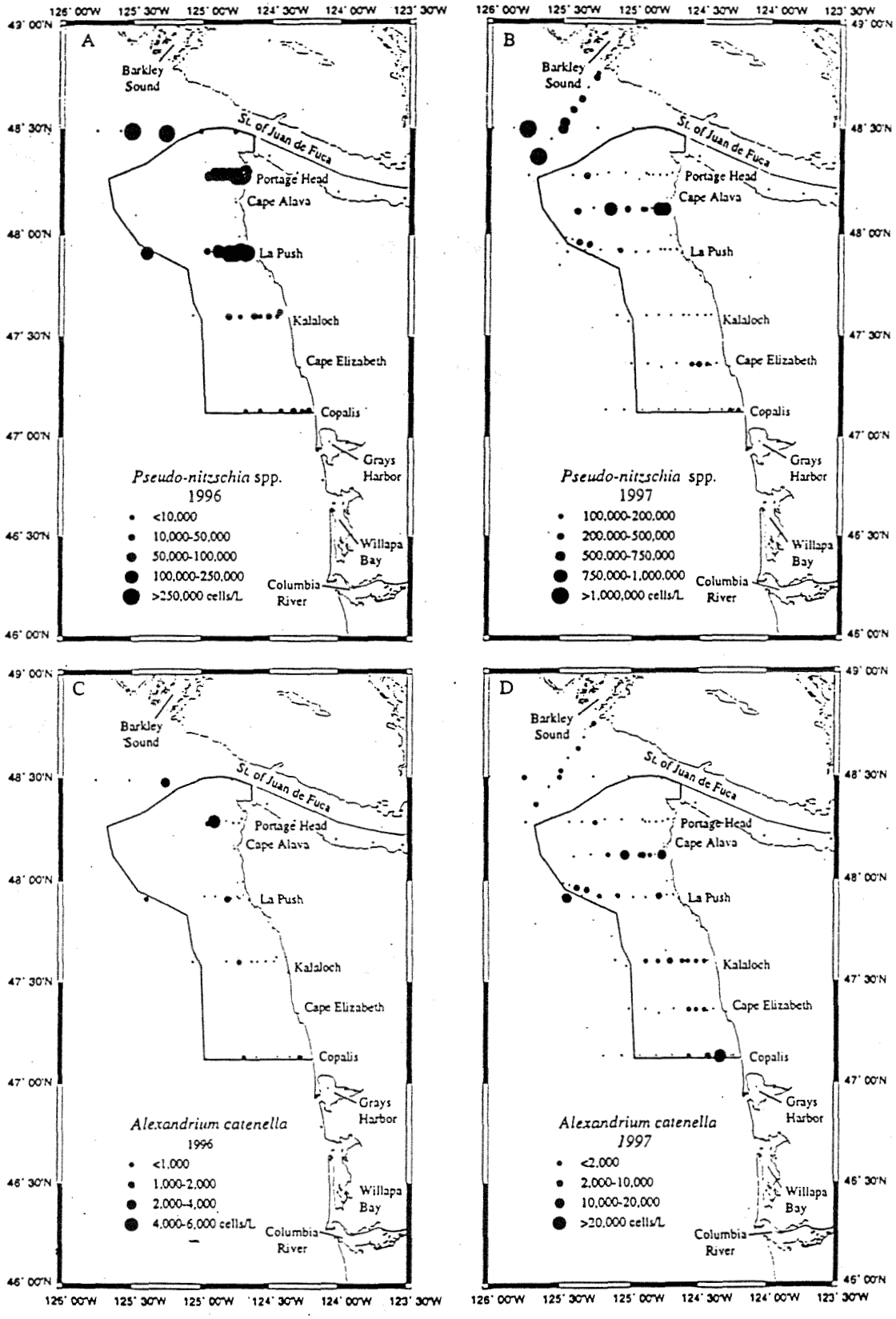

Fig. 3. *Pseudo-nitzschia* spp. and *Alexandrium catenella* distributions and abundances; (A, C) 1996; (B, D) 1997. Scales associated with dot size are different for the two years.

THE BIOTOXIN MONITORING PROGRAMME FOR ENGLAND AND WALES

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The European Shellfish Hygiene Directive (91/492/EEC) requires 'the periodic monitoring of live bivalve mollusc relaying and production areas in order to check the possible presence of toxin-producing plankton....and biotoxins in live bivalve molluscs'. The current monitoring programme in England and Wales is biased to cover commercially active classified shellfish harvesting areas and is split into two parts: the collection and examination of water samples for toxic plankton, and direct testing of shellfish flesh for biotoxins by mouse bioassay and High Performance Liquid Chromatography. These two programmes are undertaken at independent sites, with the exception of a small number of sites with recent histories of toxic algae problems which are monitored under both programmes. If the number of potential toxic algae in water samples exceeds the action level (Paralytic Shellfish Poisoning (PSP) = presence *Alexandrium* spp. cells; Diarrhetic Shellfish Poisoning (DSP) = 100 *Dinophysis spp./Prorocentrum lima* cells/L; Amnesic Shellfish Poisoning (ASP) = 150,000 *Pseudo-nitzschia* spp. cells/L) then shellfish samples are collected from the area for inclusion in the shellfish flesh biotoxin monitoring programme. A shellfishery closure will not be sought until two consecutive weekly samples have exceeded the biotoxin action level (PSP = 80μg STX equiv/100 g flesh; DSP = presence by mouse bioassay; ASP = 20μg Domoic acid/g). A shellfishery closure is usually obtained voluntarily through co-operation of commercial shellfish collectors in the area. A ban is not lifted until two consecutive weekly samples fall below the action level.

Currently, investigation is underway to establish negative screens to the mouse bioassay for PSP and DSP for use in the England and Wales monitoring programme. The present monitoring programme is being shadowed at several sites using the Ridascreen Saxitoxin ELISA kit for PSP detection and a cytotoxicity assay using KB cells for DSP detection. Retrospective testing will also be performed using a cytotoxicity assay for PSP and a protein phosphatase inhibition assay for DSP.

A bench top VG Platform (Micromass, Manchester, UK) with an electrospray ionisation source interfaced with a Hewlett Packard 1050 liquid chromatograph (Hewlett Packard, Bracknell, Berkshire, UK) has been deployed for the detection and quantitation of domoic acid (DA). Separation of DA has been achieved with an ODS(2) analytical column, a formic acid and aqueous acetronitrile mobile phase and with 50 μL/min of the column effluent entering the ionisation source. The source temperature and cone voltage was 130 °C and 30 V, respectively. The instrument demonstrated linearity over a range of 1-50 ng on-column.

A full scan spectrum of DA was characterised and dominated by the protonated $[M+H]^+$ molecule i.e., m/z 312. Determination of DA from the certified reference material MUS-1B

(National Research Council Canada) has resulted in a mean recovery of >90% (n=5). The limit of detection for real sample extracts has been determined at 0.5 μg/g indicating that the technique is sensitive as well as robust for routine monitoring purposes.

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PLANKTONIC ORIGIN AND SPATIO-TEMPORAL DISTRIBUTION OF SPIROLIDES AT NOVA SCOTIAN AQUACULTURE SITES

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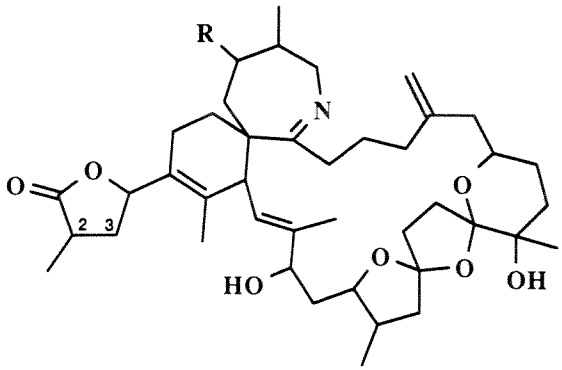
A group of novel biologically active macrocyclic compounds has been isolated from scallop and mussel viscera harvested from aquaculture sites on the eastern shore of Nova Scotia. Four of these toxic macrocycles have been structurally characterized as spirolides A, B, C and D (Hu et al. 1995); two other derivatives, the biologically inactive spirolides E and F, appear to be degradation products in shellfish (Hu et al. 1996). Several of the spirolides have been identified recently in plankton fractions (Fig. 1). The epidemiology and spatio-temporal distribution of spirolides are consistent with a seasonal planktonic origin, but the exact biological origin has remained elusive over the last few years (Cembella et al. 1998). Since the symptomology first became apparent, spirolide activity in shellfish (indicated by rapid mouse deaths upon intraperitoneal [i.p.] injection of lipophilic extracts) is usually confined to late spring-early summer (May to July). For example, in 1996, unusually rapid mouse deaths after i.p. injections of lipophilic extracts of scallop hepatopancreas (3 of 3 deaths in <7 min) were first recorded at the end of May.



	R		*MW*
A	**H**	$\Delta^{2,3}$	**691.5**
B	**H**		**693.5**
C	**CH_3**	$\Delta^{2,3}$	**705.5**
D	**CH_3**		**707.5**

Fig.1 Chemical structures of spirolides found in plankton size-fractions from Nova Scotia.

Spirolide events invariably occur after the decline of the spring diatom bloom and when dinoflagellates are dominant in the upper water column. During periods when maximum spirolide levels were detected in shellfish, near surface phytoplankton samples always contained a high relative abundance of golden-pigmented spherical cells ("golden balls"). These cells lacking surface features have also been found in the viscera of scallops and mussels (C. Carver, pers. comm. and A. Cembella, pers. obs.). Harvests of the plankton community by net tows (20 µm) indicated dominance by large thecate dinoflagellates (e.g., *Gonyaulax* spp., *Alexandrium* spp., *Scrippsiella trochoidea*, *Protoperidinium brevipes*, *Dinophysis* spp.), tintinnids, and the unidentified spherical cells.

To determine the origin of spirolides, patterns of cellular toxicity, and the spatio-temporal distribution of the causative organisms, large-volume samples (>4 x 10^3 L) were pumped and fractionated from surface waters (3 m depth) at key shellfish aquaculture sites on the eastern coast of Nova Scotia. Planktonic material was concentrated by pumping into a 20 µm mesh net, followed by size-fractionation through a stacked series of Nitex screens (95, 76, 56, 44, 26, 21 µm). In late spring through early summer (1994-97) at Graves Shoal and Ship Harbour, N.S., spirolides were typically in highest concentrations in the 21-56 µm size-fractions and their occurrence was circumstantially related to the abundance of large "golden balls" (GB) with a mean diameter of 42 µm (dubbed GB-42 cells). Physical data (temperature, salinity, σ_T) from the water column collected at Graves Shoal in 1996 indicated a deepening of the pycnocline when spirolide levels peaked in suspended-cultured shellfish.

Size-fractionated plankton concentrates were extracted in 100% MeOH and analyzed for spirolides by liquid chromatography coupled with mass spectrometry (LC-MS). Spirolide profiles were similar among size-fractions over time within a site, but the composition was different between Graves Shoal and Ship Harbour. At Graves Shoal, the primary spirolide components were B, D and an isomer D2, with A, C, and another isomer C2 as minor constituents, whereas at Ship Harbour, all size-fractions were rich in spirolides A, A2 and B. Spirolide occurrence in particular size-fractions from Graves Shoal was highly correlated with the abundance of *Alexandrium* spp. (r^2 = 0.93) and GB cells (r^2 = 0.83). Strong circumstantial evidence suggests that GB cells are the spirolide source in plankton and that the larger cells (GB-42) are primarily gonyaulacoid dinoflagellates, many of which are referable to *Alexandrium ostenfeldii*.

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AQUACULTURE AND BLOOMS OF *ALEXANDRIUM FUNDYENSE* AND *PSEUDO-NITZSCHIA PSEUDODELICATISSIMA* IN THE SOUTHWEST BAY OF FUNDY

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The southwest Bay of Fundy aquaculture industry began in the early 1980's and today has about 80 active sites. As a result of the industry's early rapid growth, a phytoplankton monitoring project was initiated in 1988 in order to: study the impact of aquaculture on the phytoplankton community, act as an early warning for industry of potentially harmful species, determine patterns, trends and establish baseline data for phytoplankton populations.

Although more than 200 species of phytoplankton have been observed in the Bay of Fundy, there are very few that have been implicated in harmful algal bloom (HAB) events. Those with the greatest economic impacts to the local area are blooms of *Alexandrium fundyense* and *Pseudo-nitzschia pseudodelicatissima*, organisms responsible for causing paralytic shellfish poisoning (PSP) and domoic acid (or amnesiac shellfish) poisoning (ASP), respectively. Industries directly affected include shellfish industries such as soft-shell clam and blue mussel.

Earlier records from 1980 indicated concentrations of *A. fundyense* in excess of 1 million cells•L in an area extending through the central Bay of Fundy and historical shellfish toxicity data shows higher than average toxicities detected in the mid 1940's, early 1960's and late 1970's. Blooms occur annually with an early peak in late May or early June and a larger bloom in mid to late July. Since 1988, highest concentrations of *A. fundyense* cells were observed at Deadmans Harbour during 1989 with 1.62×10^5 cells•L. In contrast, Lime Kiln Bay, where many aquaculture operations are located, had highest concentrations of *A. fundyense* observed during 1993 (5.2×10^4 cells•L).

Each year *P. pseudodelicatissima* first occurs in May/early June and a larger late bloom has been observed in early August. Concentrations exceeding 1 million cells•L were detected during August 1988 inside Passamaquoddy Bay and 1995 outside Passamaquoddy Bay. These dense blooms resulted in domoic acid being detected in shellfish from adjacent areas during these two years.

Data suggests that the advent of aquaculture does not appear to have caused increased or more intense blooms of *A. fundyense* and *P. pseudodelicatissima* in the Bay of Fundy.

SPATIO-TEMPORAL DISTRIBUTION OF *PROROCENTRUM LIMA* IN COASTAL WATERS OF THE GULF OF MAINE: A TWO-YEAR AGENDA

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The objectives of this project, funded by the U. S. Sea Grant Program on non-indigenous species, are (1) to determine the spatio-temporal distribution and abundance of the dinoflagellate *Prorocentrum lima* along the coast of Maine, at 10 stations where mussels are harvested, and (2) to determine the structure of microalgal communities epibiotic on mussels exposed to two types of hydrodynamic conditions. This second objective is intended to provide community diversity and characteristics of species abundance relations for the purpose of comparing pre- and post-invasion epibiotic communities. Given that the usually benthic *P. lima* has not been reported in Gulf of Maine coastal waters, that it has been associated with a diarrhetic shellfish poisoning (DSP) incident in Nova Scotia in 1990, and subsequently observed in a plankton tow near Georges Bank in 1994, it is considered a potential undesirable invader of commercial shellfish beds. The rationale for the introduction of this toxin producer, given its presence in nearby potential donor regions, is based on the numerous possible vectors of dispersal between the regions and Maine coastal waters (water circulation, drift algae, ballast water and hull fouling). Sampling of mussels and epibiota will occur from March through October in collaboration with the State of Maine Department of Marine Resources within their shellfish sampling program for PSP monitoring along the coast.

CONTROL AND MITIGATION OF ADVERSE EFFECTS OF HARMFUL ALGAE

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Adverse impacts of harmful algal blooms (HABs) on human health, traditional and aquacultured fisheries, and marine ecosystems are increasing, with devastating socio-economic impacts on coastal communities. Research and development for their control and mitigation are therefore essential and may include some of the following approaches. A PSP-toxicity test for molluscan bivalves at aquaculture and harvest sites would allow for safer, more economic harvest practices. Protocols for the depuration of phycotoxins in commercial plants are needed; domoic acid clearance from specific commercial species is feasible. Application of remote sensing devices is needed for continuous monitoring, forecasting and movement of HABs. Training of on-site farm personnel in sampling and identification of harmful algae would assist in early bloom prediction. Public education and awareness of biotoxins would reduce risks to human health. Increased use of floating barges, flupsies, to culture bivalves and future selection of shellfish and finfish growing sites relatively free from annual HABs, would reduce the adverse impacts. HAB avoidance at fish farms can be managed by moving fish pens away from encroaching blooms. Stationary pens can be made deeper or can be constructed in a manner that allows for lowering of the pens during a surface bloom. Perimeter tarping of pens allows for upwelling of deeper, colder water, either by aeration or using air-lift or hydraulic pumps. Application of these techniques prevents advection of surface blooming algae into the pens, reduce any anoxic conditions caused by the algae, and inhibits growth of many algae by lowering water temperatures. De-stratification of the water column by vertical convection also inhibits growth of harmful flagellates that require calm stratified water for growth. Newly developed, self-contained bag-culture systems for fish culture are environmentally friendly and negate many of the problems associated with harmful alage. Use of therapeutic agents in fish diets has still to be fully evaluated. Application of chemicals, flocculants, viruses, bacteria and parasites to tarped fish pens may be feasible, but the ecological consequences of chemical and biological control have yet to be evaluated. Polyculture may have merit in biological control.

THE POTENTIAL FOR SOFT-SHELL CLAM (*MYA ARENARIA*) HARVEST IN THE UPPER LETANG HARBOUR: A RESOURCE VALUATION

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INTRODUCTION

The Letang Harbour is a marine tidal inlet situated on the southern shore of NB in the Bay of Fundy (Fig. 1). The climate can be classified as cold temperate, having distinct seasonal changes. The Letang Harbour receives little fresh water, tidal ranges during spring tides can exceed 8 m, and the estuary has a mean depth of 8 m at low water during spring tides (Strain et al. 1995). The combination of high flushing rates and little freshwater input results in the complete mixing of harbour waters at all times (Strain et al. 1995).

The Letang estuary has been the site of many fishery-based industries including scallop dragging, lobster fishing, finfish fisheries (herring weirs), fish processing and soft-shell clam harvesting. Other anthropogenic influences include the Blacks Harbour sewage treatment facility that presently discharges treated effluent at Sturgeon Cove and a paper mill (Lake Utopia Paper Ltd.) that discharges treated effluent within the ULH impoundment and is released at a control dam (south of Highway no.1 causeway) from a steel flap that opens only at low tides. Today, the salmon aquaculture industry dominates the Letang Harbour, and soft-shell clam harvest has ceased since the mid-nineteen seventies because of elevated coliform counts in seawater and clam samples (Strain et al. 1995). Formerly, there was an extensive clam fishery, designated as one of the most productive soft-shell clam habitats in Charlotte County as it contained 30 major clam flats, in the Letang Harbour that employed 100 seasonal clam diggers (Wildish 1983).

Given the uncertainty of the natural resource and health of the ULH, the area remains closed for digging. Surprisingly, the million-dollar industry has been operating with few clam flats opened for digging. It is therefore crucial to establish new soft-shell clam growing areas as this would relieve potential overharvesting pressures of presently opened or conditionally opened clam flats, maintain and create employment opportunities, protect its economic significance to the surrounding communities, and better manage the natural resource. The objective of this study was to determine the availability and value of soft-shell clams of the ULH (and Charlotte County), by evaluating various water quality parameters and the overall safety of clam consumption.

METHODS

Study Areas

Review of past literature and discussion with clam diggers have identified five major growing areas of ULH (Fig. 1): 1) the control dam growing area (CD) located 400 m southwest of Highway no. 1 on the western side of the estuary (which contains three clam flats), 2) Trainors

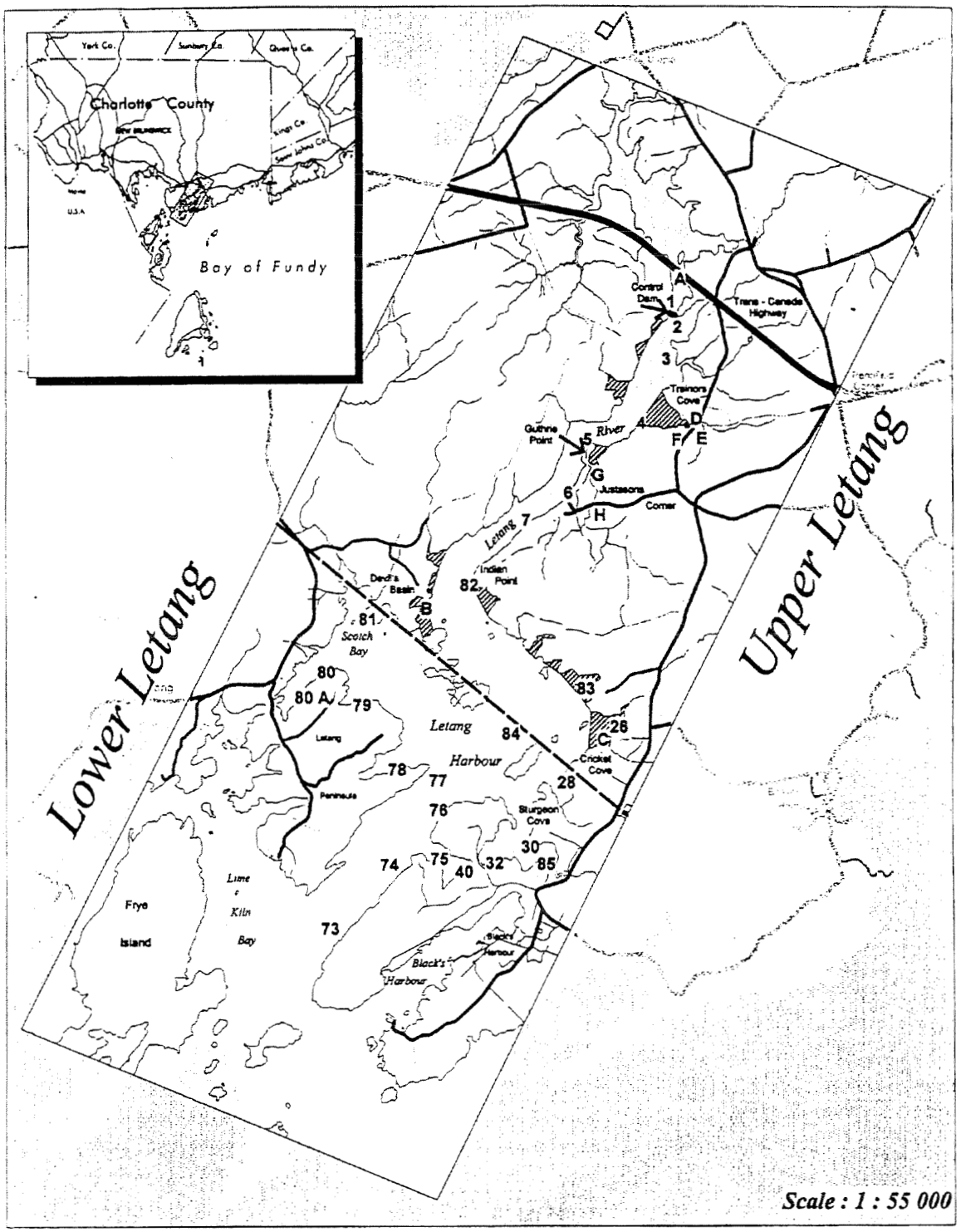

Fig. 1: Map of study area located in the Bay of Fundy, New Brunswick, Canada. Numbers and letters in bold represent sample stations of 1997 field surveys. Stations 1 to 85 were sampled for faecal coliform of coastal surface waters. Stations 4, 6, 82, 83, and 84 were also sampled for nutrient and dissolved oxygen levels. Station A was sampled for organic constituents of paper mill effluent. Stations B and C were sampled for faecal coliform levels of soft-shell clam tissues. Stations D to H represent drainage ditches and streams sampled for faecal coliform of surface waters. Slanted line patterns represent predicted growing areas of ULH.

Cove growing area (TC) located 3 km southwest of Highway no. 1 on the eastern side of the estuary, 3) Guthrie Point growing area (GP) located 5 km southwest of Highway no. 1. on the eastern side of the estuary, 4) Devil's Basin growing area (DB) 10 km from Highway no. 1 on the western side of the estuary, and 5) Cricket Cove growing area (CC) (extending to Indian Point) located 10 km from Highway no. 1 on the eastern side of the estuary (northwest of Blacks Harbour). CC was the only area examined within this extensive growing area. Study sites were chosen within each growing area as representative sites for soft-shell clam harvest. Geographical profiles, number of residences and industries, drainage pipes, culverts and other anthropogenic influences were mapped and recorded.

Population Surveys

Population surveys were conducted to determine clam availability, size class distributions and biomass of the soft-shell clam. Initial surveys were conducted to determine the overall distribution of clams within the growing area. Transects were positioned from the high water to low water levels and were sectioned into three zones: high tide, mid-tide, and low tide. Landmark objects and/or compass bearings were recorded for each transect.

Quadrats (0.1 m^2) were randomly selected within each zonation (sample plots=3-8). Duplicate samples were collected when possible. All the sediment was removed within the quadrat to a maximum depth of 30 cm. Samples were bagged, tagged, and sieved with the use of a wooden tray with a 5-mm mesh. Length (measured to the nearest 5 mm) and number of clams from each quadrat were recorded. Length/percent frequency graphs were plotted for each study site.

Length and weight of clams were also recorded at Trainors Cove in August 1995. Regression analyses were used to determine an equation that best estimates clam weight of size classes for the ULH (ECW 1995, unpublished data). Biomass was determined for legal-sized clams (~45 mm) for candidate sites.

Nutrients and Dissolved Oxygen

Total phosphorous (P) and nitrogen (N), dissolved nitrate (NO_3) and oxygen (O_2) were measured from August to October 1997 at five stations in the Letang Harbour. Five water samples (500 mL) were collected from each site during the high tide (Fig. 1). Analyses were conducted by Environment Canada (Université de Moncton, Moncton, NB, Canada).

Other Pollutants

Two water samples (4-L) were collected from the impoundment between the Highway no.1 causeway and the control dam (Fig. 1). Samples were analysed for organic compounds of treated paper mill effluent by Fisheries and Oceans Biological Station (St. Andrews, NB, Canada).

Bacterial Contamination

The most-probable-number (MPN) method was used to measure faecal coliform bacteria of coastal surface waters and tributaries of the Letang Harbour in accordance with the Canadian Shellfish Sanitation Program (CSSP-Fisheries and Oceans Canada and Environment Canada 1992). Fifteen water samples at twenty-six stations were collected from June to October 1997 (Fig. 1). Water samples were analysed by Environment Canada [mobile laboratory, Biological Station (Fisheries and Oceans Canada), St. Andrews, NB, Canada]. Faecal coliform levels were also determined for clam tissue samples at candidate sites (Fig. 1) by MPN method. One sample of soft-shell clams (25 clams) was collected and transported to the Food, Fisheries and Aquaculture Department of the Research and Productivity Council (RPC) for analyses (Fredericton, NB, Canada).

Resource Valuation

A resource valuation plan (cost-benefit analysis) was developed to evaluate the potential economic benefit of candidate sites for harvest in ULH in accordance with Eastern Charlotte Waterways (ECW) et al. (1995). The sustainable harvest of soft-shell clams for the model was suggested to be 10-15% (Robinson, pers. comm. 1997). A 10% harvest of the total biomass for candidate sites was selected for the resource valuation model. A 5- and 12-mo model for soft-shell clam harvesting was developed for Charlotte County to determine net revenues for the harvest. The harvesting rate of clam diggers for Charlotte County was also estimated.

Statistical Analyses

Nutrients and dissolved oxygen data collected in 1997 was analysed using Tukey's procedure for multiple comparison of means following a one-way analysis of variance (1-way ANOVA) (Steel and Torrie 1980). Tukey's procedure for multiple comparison of means was used to determine where differences occurred among treatment groups and, if data did not meet the appropriate criteria, the Kruskal-Wallis Test (Chi-square approximation), a nonparametric test for multiple caparison for unequal sample sizes, was used on the data set. Homogeneity of variance was determined by the use of the F_{max}-distribution. The SAS statistical package (SAS Institute Inc., Cary, NC, USA), Version 4.0 was used for all statistical analysis.

RESULTS

All sites were relatively clean, with few anthropogenic impacts. Few drainage ditches flowed onto or near study sites, many of which were of seasonal flow. There were fewer than 25 residences along ULH, all but two located on the harbour's eastern side. The drainage pipe of Blacks Harbour's sewage treatment facility, located at Sturgeon Cove, was the only discharge pipe located near or in ULH.

Initial site surveys of the growing areas (visual inspection of clam holes and clam digging) indicated the presence of soft-shell clams at all five locations. Soft-shell clams were concentrated within the mid-tidal ranges, but not isolated to this region. Variability was estimated to be 50%

between duplicate plots. Length/percent frequency plots indicate a good size-class distribution (5-70 mm), with only DB and CC having legal-sized clams (~45 mm). DB and CC were designated as candidate sites for soft-shell clam harvest. Biomass was estimated to be 14,261 kg and 29,978 kg respectively.

Nutrient levels (P, N, NO_3) increased as the sample stations approached the control dam, however, results were not significantly different ($p<0.2382$). Dissolved oxygen decreased as the sample stations approached the control dam, but results were not significantly different ($p<0.9704$).

Lignosulphonate, a molecule formed from the binding of lignin molecules and sulphur derivatives, was identified as a major component of final paper mill effluent discharged into the Letang Harbour. It was visually detected in water 400 m from the control dam (dark brown in colour).

Faecal coliform for surface harbour waters for all but sample station 28, site of Blacks Harbour's wastewater treatment facility discharge pipe, met the appropriate criteria in accordance with CSSP. Tributaries and drainage ditches that flow onto clam flats that were monitored also showed acceptable faecal coliform levels. Faecal coliform of clam samples collected from DB and CC (<20 and 40 MPN/100 g tissue, respectively) were comparable to a site that was conditionally opened at the time of sample collection, Pocologan Harbour (<20-90 MPN/100 g tissue). Levels met the appropriate criteria in accordance with CSSP (#230 MPN/100 g tissue).

The resource valuation of soft-shell clams available for harvest in the ULH suggests that DB and CC approximate one month of employment (Table 1a). The economic benefit for the soft-shell clam industry in Charlotte County was estimated between $1.6-2.9 million (Table 1b). The present harvest rate for clam flats for Charlotte County clam flats was estimated at 13% (Table 1b).

DISCUSSION

Examination of approximately one fifth of the potential soft-shell growing areas of ULH suggests a resource that is economically beneficial to Southwest New Brunswick (SWNB). Although anthropogenic impacts have been reduced in the last decade, faecal coliform counts are limited. Nutrient and oxygen levels are all within acceptable Canadian water quality standards (McNeely et al. 1984). There is little documentation of other anthropogenic influences that could pose a threat to the clam fishery except for the increased use of aquacultural biocides and the potential impact(s) of lignosulphonate, a major component of Lake Utopia Paper Ltd. treated effluent. Overall, our study supports the re-establishment of soft-shell clam harvest within ULH given continued and additional monitoring.

Soft-shell clams at study sites were concentrated, but not isolated to, the mid-tidal zone. Variability was estimated to be 50% between duplicate plots. Results indicate a good distribution of size classes (5-70 mm). Results closely resemble the survivorship curve of the soft-shell clam

Table 1a: Resource valuation (cost-benefit analysis) of soft-shell clams for the ULH, NB, Canada.

PARAMETERS	STUDY SITES		
	Cricket Cove	Devil's Basin	Total
Harvestable area (m^2)	80,410	55,670	136,080
Sustainable harvest (10 %)	0.1	0.1	0.1
Annual average price ($/kg)	2.2	2.2	2.2
Biomass of clams (kg)	29,978	14,281	44,259
Gross harvesting benefit ($)	6,595	3,142	9,737
Sustainable level (kg)	2,998	1,428	4,425.9
Number of harvesting days (n=130kg/day)	23	11	34
Harvesting benefits/m^2	0.082	0.056	0.072
Kilogram/m^2	0.0373	0.0257	0.0325

Table 1b: Resource valuation (cost-benefit analysis) of soft-shell clams for Charlotte County, NB, Canada.

PARAMETERS	12-MONTH MODEL	5-MONTH MODEL	SOURCE
VARIABLE COSTS			
Available harvested area per year (m^2)	1,000,000		ECW Inc.
10% Sustainable harvesting (kg)	32,524		
Average number flats a digger works per year	3		Charlotte Shellfish Inc.
Average number of diggers per flat	20		Charlotte Shellfish Inc.
Average flat size/area (m^2)	38,000		ECW Inc.
Equivalent harvested areas per year (m2)	2,280,000		
Average amount harvested per digger (kg/day)	130		Charlotte Shellfish Inc.
Number of tides worked daily (1-2 tides daily)	1.5	1.5	Charlotte Shellfish Inc.
Number of working days per year	360	150	Charlotte Shellfish Inc.
Average number of working days per year	540	225	
Actual level of harvesting (kg)	70,200	29,250	
Harvesting rate (%)	13	13	
Recommended sustainable harvest (%)	10-15		DFO
Average distance (return) travelled (km)	60		Charlotte Shellfish Inc.
Travel cost ($/km)	0.25		Res. Valuation Guidebook
Number of diggers per vehicle (1-2 diggers)	1.5		Charlotte Shellfish Inc.
Transportation costs per year ($)	5,400	2,250	
FIXED COSTS			
Number of hacks utilized by digger in a year	3		Charlotte Shellfish Inc.
Unit price for hack ($)	50		Charlotte Shellfish Inc.
Number of creels utilized by digger in a year	2		Charlotte Shellfish Inc.
Unit price for creel ($)	20		Charlotte Shellfish Inc.
Total annual fixed cost per digger ($)	190		
Total yearly costs of harvesting per digger ($)	5,590	2,440	
Total Yearly revenues harvested per digger ($)	154,440	64,350	
All diggers (use average number per flat)	20		Charlotte Shellfish Inc.
Total yearly costs of harvesting - all diggers ($)	111,800	48,800	
Total harvest revenues - all diggers ($)	3,088,800	1,287,000	
Net harvest revenues - all diggers ($)	2,977,000	1,238,200	
Charlotte County clam value ($)	2,363,000		DFO (1995 data)

that suggests greater numbers of spat or juvenile clams and fewer adults as time progresses (Dame 1996). At high tides, clams are exposed to influences such as freshwater runoff and desiccation that reduce recruitment and/or survivorship, and at the low tide range, are exposed to many more predators that can also affect recruitment and survivorship (Dame 1996). GP and CD growing areas showed limited clam resources with clams either below the detection level of the sampling technique or sites of poor or new recruitment. Illegal digging is pronounced at all sites and may attribute to low levels or lack of legal-sized clams in these regions. Both areas are mud flats, and the equipment available for surveying was not designed to sieve muddy sediment. CC and DB are candidate sites for soft-shell clam harvest as they have sufficient legal-sized clams to maintain a small, sustainable harvest.

Nutrient and dissolved oxygen levels for the ULH did not exceed existing Canadian and NB water quality guidelines (McNeely *et al* 1984). Nutrient levels in the ULH have been explained by anthropogenic influences and inflow from the surrounding watershed (eg. streams, rivers, etc.) (Strain and Clement 1996).

Surface coastal waters, tributaries and clam tissue samples all showed low faecal coliform counts. However, there is inadequate baseline data to provide a better, more thorough understanding of faecal coliform levels in the harbour. There is little information about faecal coliform counts during moderate to high (~15 mm) rainfall events that is vital for the determination and establishment of safe guidelines for clam digging. A monitoring program must be in place to provide annual baseline information of the Letang Harbour that will, in turn, help establish safe water and product quality criteria for soft-shell clam harvest. A 1995 phone survey by ECW of a sample population of the surrounding community determined that 70% of the population had some type of septic treatment, with 65% of them properly maintained. Increased sampling efforts can act as a preventive measure as this can help isolate and remediate trouble areas prior to extensive environmental impacts. There are fewer than 25 residences no closer than 30 m from the shoreline and one wastewater treatment facility. Faecal coliform counts are correlated to rainfall, and it is important to gather data during various rainfall events. Surveys conducted in 1995 and 1997 in accordance with the CSSP, are incomplete as the summer offered little precipitation (Menon and Roberts 1995, Richard pers. comm. 1997). Without appropriate rainfall data during these water quality surveys, criteria for openings/ conditional openings are difficult to establish.

Continued incentives should be exercised to minimize paper mill effluent discharge and to reduce toxicity as treatment solutions become readily available and affordable. Lignosulphonate is a molecule that is formed from the binding of lignin, natural wood extract, and sulfite involved in the pulping process. The molecule is very inert, does not readily undergo bacterial breakdown and has been detected as far as the mouth of the estuary (Wildish, pers. comm. 1997). However, bioaccumulation, developmental and reproductive impacts of this compound in organisms are still unknown. Its dark brown colouration in the water column does, however, reduce the amount of light that can normally penetrate the water column and reduces primary production. Decreases in productivity can result in reduced growth rates of molluscs and other heterotrophs. Future studies should examine the impact of lignosulphonate on organisms and the productivity of the estuary. The most conservative value for a sustainable harvest of soft-shell clams in the Letang Harbour should be considered given the lack of growth rate information.

The resource valuation suggests a small but significant economic gain for establishing the ULH as a conditional site for soft-shell clam harvest. CC and DB account for a combined month of soft-shell clam harvest (Table 1a). Also, further analyses of the data suggest that the present harvesting rate is 13% (Table 1b), a value that is within the sustainable harvest for soft-shell clams (Robinson, pers. comm. 1997). Furthermore, two models have been devised that demonstrate the net revenue for a typical 5- and 12-mo harvest. Net harvest revenues were estimated to be $1.24 million and $2.98 million respectively (Table 1b). Statistical information for the 1995 harvest of soft-shell clams in Charlotte County suggested a net annual revenue of $2.36 million. Additional and better estimates of resource valuation parameters are necessary to improve the accuracy of the model. This is the first study to examine the economic impact not only for ULH, but for the soft-shell clam industry of Charlotte County.

In conclusion, this study supports the potential reestablishment of soft-shell clam harvest within ULH given continued and additional examination of faecal coliform and paper mill effluent. In addition, aquacultural biocides should also be addressed in future investigations as there is little known about their persistence in the marine environment. This study will serve as a protocol for future sanitary surveys of similarly impacted coastal waters.

ACKNOWLEDGEMENTS

Thanks to ECW for the tremendous experience I gained developing this research project; ECW staff, industry, the University of New Brunswick (Saint John Campus), and Federal and Provincial Agencies for sample collection, analyses of sample materials and suggestions; Mr. T. Kennedy of Strait North Solutions for map design; ECW MapInfo Professional computer resource; and Dr. T. Chopin for the review of the résumé. Analyses were reviewed and critiqued by Mr. Yves Bourassa (Economic Division, Environment Canada, Hull Quebec) and Dr. Neil Ridler (Department of Arts, University of NB, Saint John, NB)

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ESTABLISHING SAFE LEVELS OF SEAFOOD TOXIN INGESTION: THE HEALTH CANADA PERSPECTIVE

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Ensuring that the Canadian public has access to safe, nutritious and wholesome food is the mandate of the Food Directorate of the Health Protection Branch of Health Canada. Staff of the Bureau of Chemical Safety in this Directorate are responsible for assessing the potential risk to Canadians of dietary exposure to a wide variety of natural and man-made chemical substances.

Together with the provisions of the Food and Drugs Act and Regulations, the Bureau of Chemical Safety assesses the use of chemicals in foods. These recommendations are incorporated as guidelines or regulations which are applied for all food items offered for sale in Canada. Since the Food and Drugs Act does not apply to sportfish, foods which are gathered or collected recreationally or native foods, the recommendations of the Bureau are given as advice to other government bodies (federal, provincial/territorial, local) and agencies.

The purpose of this presentation was to provide an overview of the procedure and rationale followed in the preparation of a health hazard assessment for seafood toxins. A similar procedure is followed in the assessment of other natural toxins (plant, fungal and identified microbial chemicals), man-made chemicals in the environment and pre-market evaluation of food additives and food packaging materials or chemicals which may come into contact with food at some point during food preparation, processing or storage. It should be noted that most health hazard assessments consider the daily or weekly lifetime exposure to a chemical rather than the single or daily acute exposure situation which is the case for most seafood toxins, such as domoic acid [amnesic shellfish poisoning (ASP)] and paralytic shellfish poison (PSP).

HEALTH HAZARDS AND ASSESSMENTS

Health hazards are identified through numerous means. A request to assess a potential hazard may be initiated by Health Protection Branch personnel, the Canadian Food Inspection Agency and other government departments or agencies, industry, consumer groups or associations, or individuals. Identification of a potential hazard may occur through the examination of adverse reaction reports, monitoring reports, epidemiological investigations, scientific literature, toxicological studies or through contacts with other countries or scientific organizations.

When the identity of a potentially harmful chemical in food is known, the Chemical Health Hazard Assessment Division of the Bureau of Chemical Safety conducts a health hazard assessment. In cases where the chemical's identity is not known or unclear, but it is of microbial origin, the assessment is the responsibility of the Bureau of Microbial Hazards of the Food Directorate. Assessments conducted by the Chemical Health Hazard Assessment Division are often conducted with input from other Directorate staff including the Food Research Division, Toxicology Research Division, the Bureau of Nutritional Sciences and the Bureau of Microbial Hazards and from the National Research Council, Department of Fisheries and Oceans, universities and foreign contacts.

A health hazard assessment of a known food chemical is a scientific, multi-step process conducted by two groups of specialists. Staff toxicologists determine the toxicity of the chemical and establish a tolerable daily intake (TDI) level. Staff evaluators examine the potential human exposure to the chemical through ingestion and establish a probable daily intake (PDI) value. The TDI and PDI values are then compared, a risk management analysis conducted and recommendations are proposed.

DETERMINING THE TDI

The TDI is usually defined as the amount of a chemical that can be ingested every day during a lifetime with reasonable assurance that no adverse health effects will occur. This term is also used in cases when a single exposure to a chemical may result in acute toxicity such as occurs with several seafood toxins including domoic acid and paralytic shellfish poisoning. The TDI is based on the evaluation of laboratory animal toxicity experiments: acute (single exposure), subacute (28 day exposure), subchronic (90 day exposure) and/or chronic (lifetime exposure - minimum one year). Ideally, these experiments would have been conducted in at least two different species with the chemical of concern administered orally. Additional specialized experiments may be required for some chemicals, including pharmacokinetic, neurotoxic, reproductive or carcinogenic studies. When available, human case reports and epidemiologic studies are also evaluated. Where possible, a no observable adverse effect level (NOAEL) or a lowest observable adverse effect level (LOAEL) is determined for each experiment. The NOAEL (or LOAEL) from the most critical experiment is then considered in relation to the results from the other experiments. A safety factor which may range from 10 to several thousand is then applied to this NOAEL. It is based on intraspecies variation, interspecies variation, adequacy of the toxicological database and the severity of the adverse effects observed.

In the case of domoic acid, there is a limited amount of animal toxicity data, but there is human toxicity data based on the human exposure in Canada in late 1987. Although it was not possible to determine a NOAEL from this human data, a LOAEL of 1.0 mg domoic acid/kg body weight (bw) was determined. A safety factor of 10 (individual variation) was applied to the LOAEL to calculate the TDI of 0.1 mg domoic acid/kg bw. Based on rat exposure data, a NOAEL of 28 mg domoic acid/kg bw was determined and a safety factor of 100 (interspecies variation and individual variation) was applied resulting in a TDI of 0.28 mg/kg bw, which supports the TDI of 0.1 mg domoic acid/kg bw based on actual human exposure.

DETERMINING THE PDI

To determine the PDI, staff must first identify all foods which may contain the chemical of concern and at what concentrations it can occur. The amounts of these foods consumed must then be considered to determine the exposure level from the entire diet. The amount of food consumed is influenced by numerous factors, including age, gender, geographic location, cultural background, socio-economic status, food availability (seasonality), individual preferences and public perception. Average and high consumption rates are taken into consideration, as well as the potential exposure of specific subgroups of the population, such as children, pregnant women and the elderly. In some cases, it is necessary to estimate the dietary intake of the chemical based on local or regional consumption information. This would occur in the case of food sources which have been hunted, fished or harvested locally and which are not sold commercially.

Potential non-food sources of exposure to the chemical are also considered. In addition, certain factors inherent to the toxin may be important in determining the PDI, including seasonality and geographic location. In the case of domoic acid, the presence of the toxin may be limited to certain locations along the sea coast, with no indication of the toxin being present in the same species harvested from immediately adjacent areas. Domoic acid also tends to be present in shellfish during certain times of the year.

Fish consumption data for the determination of the PDI for seafood toxins is based on surveys conducted in Canada and the USA, and on anecdotal data. Unless more specific data are available, it is assumed that the general population consumes 22 g commercial fish product/day averaged over a lifetime. For subsistence consumers and sport fishers, the estimated consumption is 40 g fish/day. In considering the acute ingestion of mussels contaminated with domoic acid, a single serving is estimated to be 240 g fish/day or the equivalent of 24 mussels as a single meal. Based on the TDI, a recommended maximum daily intake of domoic acid was determined to be 0.02 mg/g mussel tissue. Assuming that a 60 kg individual consumes 240 g mussel tissue in a single meal, the following PDI calculation is performed:

240 g mussel tissue x 0.02 mg domoic acid = 0.08 mg domoic acid/kg bw x 60 kg bw

COMPARISON OF TDI AND PDI

The third step in the health hazard assessment process is the comparison of the TDI to the PDI. If the PDI is equal to or exceeds the TDI, various risk management options are considered. These options include the establishment of guidelines or the development of specific regulations, the restriction or ban on consumption of the food commodity originating from that locale, voluntary compliance measures by producers, the recommendation of changes in dietary habits by consumers, the initiation of education programs, or the specification of certain processing conditions to minimize or reduce contaminant levels in the food product ingested. All aspects of a recommended action are carefully considered with respect to the probable outcomes prior to implementation.

For domoic acid, the Health Canada recommendation is the closure of the harvesting of shellfish in areas where domoic acid concentrations exceed 0.02 mg/g mussel tissue and this guideline is enforced by the Canadian Food Inspection Agency. The implementation of this guideline for domoic acid, as for other seafood toxins, together with activities carried out Fisheries and Oceans Canada, the Canadian Food Inspection Agency and the National Research Council, and the active participation of shellfish producers and other interested stakeholders, continue to ensure that Canadians can enjoy the safe consumption of shellfish.

CONCLUSIONS

A health hazard assessment is conducted by Health Canada specialists using the best data available at the time. These assessments are subject to continual review and update. When required, a health hazard assessment is conducted on a case-by-case basis.

REPORTER GENE ASSAYS FOR ALGAL TOXINS

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We have modified the cell based directed cytotoxicity assay for sodium channel active (PSP, NSP, CFP) and calcium channel active phycotoxins (MTX and Pfiesteria-fish killing activity) using the c-fos-luciferase reporter gene construct. N2A mouse neuroblastoma cells and GH_4C_1 rat pituitary cells were each stably transfected with the reporter gene c-fos-luc which contains the firefly luciferase gene under the transcriptional regulation of the human c-fos response element. The characteristics of the N2A reporter gene assay was determined by dose response with brevetoxin-1 (PbTx-1). PbTx-1 (50 ng/mL) induced c-fos-luc in a time-dependent manner which was detectable at 4 h of exposure and reached a maximum at 12 h. PbTx-1 caused a saturable induction of c-fos-luc with an EC_{50} of 4.6 ± 2.2 ng/mL (n=5) and ciguatoxin 3 given at 3 pg/mL induced c-fos-luc to the same maximal extent. Saxitoxin caused a concentration dependent inhibition of PbTx-1 induction of c-fos-luc with an EC_{50} of 3.5 ± 1.1 ng/mL (n=3). GH_4C_1 cells expressing c-fos-luciferase were responsive to maitotoxin (3 ng/mL) and the fish killing activity produced by *Pfiesteria piscicida*. The effect of maitotoxin, but not the *P. piscicida* activity was blocked by nimodopine, although both increased intracellular calcium in GH_4C_1 cells. The detection of the *P piscicida* activity was sensitive (10-100 *P. piscicida* cells) and activity was identified in both toxic strains tested. These results indicate that cell-based assays for sodium and calcium channel active phycotoxins can be conducted under viable conditions using luciferase as an endpoint.

ab

APPLICATION OF rRNA PROBES AND A DOMOIC ACID RECEPTOR ASSAY TO FIELD POPULATIONS OF *PSEUDO-NITZSCHIA*

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The ability to rapidly acquire reliable, quantitative data on the abundance and distribution of harmful algal bloom (HAB) species and the toxins they produce has been a long-standing, yet somewhat elusive, goal. For those concerned with algal blooms and their negative impacts from either a regulatory or research perspective, such capabilities promise to enhance the efficiency of monitoring and inspection programs, as well as provide valuable near-real time data on HAB dynamics. We have initiated a project to develop concurrently the application of rRNA probe and receptor binding assay technologies for the rapid assessment of HAB species and their toxins using field populations (Monterey Bay, CA) of the domoic acid (DA) producing diatom *Pseudo-nitzschia* as a model. The principal aims of this study were: 1) inter-comparison of rRNA probes for several *Pseudo-nitzschia* spp. applied using whole cell and cell homogenate (i.e., sandwich hybridization) techniques; and 2) modification of a DA receptor assay for use with natural phytoplankton samples and comparison with the established FMOC/HPLC method. Results to date show that receptor assay measurements of DA generally corresponded well with HPLC values. Most discrepancies involved apparent false negative responses in the receptor assay as compared to FMOC/HPLC, although we have yet to rule out the possibility of sample matrix interference with the HPLC analyses. The two rRNA probe application methods being evaluated frequently yielded similar estimates of *Pseudo-nitzschia* spp. abundance; however, the sandwich hybridization occasionally showed a response when no cells were detected by the whole cell approach. Overall, the presence of DA appeared to correspond most closely with the sandwich hybridization data, suggesting that DA can reside in food web components not associated with recognizable cells of *Pseudo-nitzschia* spp. Possible mechanisms for the occurrence of DA in the apparent absence of *Pseudo-nitzschia* cells will be discussed.

ab

VALIDATION OF THE MARITIME IN VITRO SHELLFISH TEST (MIST™) KITS FOR MARINE BIOTOXINS

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The Maritime In Vitro Shellfish Test (MIST™) for paralytic shellfish poisoning (PSP) is a user-friendly, shippable kit for the detection and quantitation of PSP in shellfish tissue extracts, or from extracts of phytoplankton, cyanobacteria, or other types of seafood. It is available in fully quantitative, semi-quantitative and qualitative versions, which vary in cost, level of complexity and amount of information produced. All three versions of the PSP kits underwent a rigorous trial in the spring of 1997. The kits were shipped to the United Kingdom regulatory laboratory in Aberdeen, Scotland, where standard AOAC shellfish extracts were tested in the standard 3-mouse bioassay, the three versions of MIST™ kits and the neuroblastoma cell bioassay as performed by the Gallacher group in Aberdeen. Further, an AOAC trial to test ruggedness and reproducibility was started in February 1998. Available results from these validation trials will be discussed in detail. Progress on a new cell bioassay under development for diarrhetic shellfish poisoning (DSP) will also be presented.

ex ab

RECENT DEVELOPMENTS IN ANALYTICAL CHEMISTRY OF MARINE TOXINS

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Marine toxins present a significant challenge to the analytical chemist due to their wide variety of structures, ranging from very polar to lipophilic and from low to high molecular weights. This has resulted in a patchwork quilt of bioassays and chemical methods being used to monitor for toxins in plankton and in seafood. Due to the difficulty and cost of implementing many of these methods, government inspection agencies are having great difficulty meeting demands.

This paper discusses an important advance in analytical technology that can help with these problems. The technique of LC-MS, a combination of liquid chromatography and mass spectrometry, has proven to be a powerful tool for the detection and quantitation of toxins in plankton and shellfish at part-per-billion levels, the identification of new toxins, and the investigation of toxin metabolism in shellfish (Quilliam 1996). LC-MS methods have been developed for the following toxins: domoic acid and other ASP toxins; okadaic acid and related DSP toxins, including DTX1-5 toxins; pectenotoxins; yessotoxin; saxitoxin and other PSP toxins; brevetoxins; spirolides; and ciguatoxins. LC-MS is in fact the only analytical method that has been shown to be suitable for the analysis of all toxins.

LC-MS meets all the needs of laboratories involved in both monitoring and toxin research:

- universal detection capability
- high sensitivity
- high selectivity and specificity
- minimal sample preparation
- ability to deal with the structural diversity and labile nature of toxins
- separation of complex mixtures of toxins
- precise and accurate quantitation
- wide linear range
- automation
- high throughput
- rapid method development
- legal acceptability for confirmation
- structural information for identification of new toxins, analogs and metabolites

We are currently investigating whether the technique can be used in a multi-toxin analysis approach. The analysis of all toxins in one extract by one LC-MS analysis is certainly a possibility, but such an analysis would be quite long (at least 30 min/sample) due to the need to do a wide-ranging gradient elution separation. The limitations of the multi-toxin approach lie not in the LC-MS system, but in sample preparation – i.e., finding a universal extraction solvent and cleanup scheme that give good recovery for all toxins. Of course it is unlikely that all toxins would have to be monitored at once – most locations only suffer from one to two different types of toxins. We

are now focusing on developing methods for water soluble toxins such as PSP and ASP in one group and lipophilic toxins such as DSP and NSP in a second group. We have developed preliminary extraction and analysis conditions to accomplish this but further method validation is required. An advantage of this approach is the possibility of higher selectivity through additional cleanup and lower detection limits through an extra degree of pre-concentration.

The high capital cost of LC-MS systems presents difficulties for many laboratories, although the recent introduction of less expensive, easy-to-use "bench-top" instruments should help to reduce these problems. In addition, the actual cost per sample must be considered carefully when comparing different methods. Analyses by LC-MS can be very rapid (as low as 2 min in some cases) and can be totally automated, resulting in a very low cost per sample. The cost of LC-MS analyses is also low because minimal sample preparation is required, compared to other analysis methods based on complicated cleanup and derivatization schemes. This feature is also important for sample throughput, as sample preparation is usually the major bottleneck in most laboratories. An additional item of importance to some laboratories is that an LC-MS system can be used for both research and monitoring work, and for a variety of analytes, not just toxins.

An LC-MS instrument certainly does need a steady flow of samples to justify its purchase, unless it is also used for research. Obviously the LC-MS option is best suited to a central laboratory that would have the technical expertise and the workload to justify such an instrument. One of the most appealing features of LC-MS to many laboratories is the possibility that a wide range of methods could be replaced by just one instrument. Caution must be exercised here, as complete reliance on one instrument could present difficulties, as any breakdown could result in delays during a crucial monitoring program. However, our experience is that the relatively inexpensive modern LC-MS systems are very reliable and give a 90%+ up-time; service contracts on such instruments usually allow a 24-48 h repair. The bottleneck of using just one instrument can also be a problem, but with automation and high speed analyses, an LC-MS system can deal with many emergencies. A laboratory basing its entire operation on LC-MS, may wish to have 2 instruments in any case, one for routine monitoring and another for research and methods development as well as being a backup to the routine system. Of course this is a difficult proposition to sell as many institutions cannot even afford LC systems! A combination of rapid assay methods for screening and an LC-MS system in a central laboratory for research and confirmation may be the best route to follow.

Another cautionary note is that a fair amount of technical expertise is involved in operating an LC-MS system, although the newer bench-top LC-MS systems are becoming very easy to use and maintain. In our laboratory, we have many instrument users, including temporary summer student assistants. Training usually takes no more than 1-2 d. The method development work, however, is generally carried out by Ph.D. analytical chemists.

Whatever the opinions are about investing in LC-MS systems as a tool for routine toxin analysis, I think that most people will agree that this technique will continue to play a significant role in the future of toxin research.

Quilliam, M. A. 1996. Liquid chromatography-mass spectrometry of seafood toxins, p. 415-444. *In*: D. Barcelo, D. (ed.) Applications of LC-MS in Environmental Chemistry: Elsevier Science Publ. BV, Amsterdam.

REPORT OF WORKING GROUP #1

Aquaculture Insurance in the Bay of Fundy

Presenter: Terry Trecartin (insurance broker, Mitchell McConnell Insurance Ltd., Saint John, NB, Canada E2L 3Y2)

Moderator: Tom Sephton Fisheries and Oceans Canada, Biological Station, St. Andrews, NB, Canada E0G 2X0

Rapporteur: Blythe Chang Fisheries and Oceans Canada, Biological Station, St. Andrews, NB, Canada E0G 2X0

- approx. 20 attendees

INSURANCE BROKER

- represents clients (farmers) - broker buys insurance for the farmer.
- T. Trecartin is one of only 3-4 brokers for fish farms in North America.
- most farms have insurance, but some large companies self-insure.

UNDERWRITER

- pays claim

now only 2 or 3 underwriters for fish farms in world - e.g. Lloyd's of London

RISK MANAGEMENT PROCESS

- to assess applicant:
  - identify and analyze exposure
  - formulate alternative techniques
  - select best alternative
  - implement techniques
  - monitor and modify

PERILS

- basic policy
  - insures loss from pollution - mortality or loss in market value
    - malicious poisoning
    - contaminated feed
    - toxic algal/plankton blooms (unless specifically excluded)
      - on west coast, policies can stipulate the requirement for daily phytoplankton monitoring
  - don't need to identify polluter, but need to show that pollution was the cause of the loss.

Other perils can be added:

- theft and malicious acts

- predation
- storms, lightning, tidal waves, collision, structural failure
- freezing, supercooling, ice damage
- deoxygenation
- any other change in chemical composition of water

Also can add:

- disease extension (see below)
- fish stock in transit
- cages, nets, workboats, & 3rd party liability resulting from their use

For on-shore facilities (e.g. hatcheries), can add other perils:

- flooding, land slippage, fire, electrical breakdown, etc.

Could also get "pollution liability," but would be expensive (probably $50,000 or more). This would not cover illegal acts.

DISEASE EXTENSION CLAUSE

- covers mortality, but *not* loss in market value
  not exceeding 30 consecutive days (to be increased to 60 days?)
- excludes intentional slaughter (e.g. government ordered)
- once a kill order is given, coverage ceases for the identified cages
- covers any disease; includes sea lice
- treatments must be under veterinary supervision

In recent Infectious Salmon Anemia (ISA) claims, farmers received only about 44% of cage value in claims. This was because of the slow development of this disease, which meant that mortalities occurred over a period longer than 30 days. For this reason, there is some pressure to increase this period to 60 days.

BASIS OF INDEMNITY

- up to individual farmer as to how much he/she will insure fish
- different indemnity for different stages (smolts, larger fish, market fish, broodstock)
- cannot insure for more than market value

BAY OF FUNDY STATISTICS

- first policy Nov 97
- current rate 3% of value of fish in water (excludes cage damage)
- normally 25% deductible
- insurance now on a per cage basis

LOSS RATIO (claim payments / premiums)

- since 1988 average 74%. Range: 19% (1988) - 170% (1993)
- need 50% to allow underwriter to build a contingency fund
- total costs for underwriters (including claim payments, commissions, etc.) are greater than premiums. Premiums have not risen, due to current intense competition between underwriters.

CLAIMS IN THE BAY OF FUNDY

- since 1988, 356 policies (one year policies) and 124 claims - i.e. approx. 1:3 claim:policy ratio (very high)
- timing of claims: highest Dec-Mar (63% of claims)
    - seal predation, superchill
- types of claims:

Type	Frequency of claims (%)	Amount paid ($) (%)
Predation	42	32
Storm	14	13
Superchill	5	6
Disease	22	34
Equipment	17	15

- at every renewal, a new application is submitted and reviewed. If the applicant has history of repeat claims, the insurer will decide whether to insure or not, and if yes, at what rate.
- competition between underwriters is keeping premiums low
- if there was cooperation between underwriters, this would probably mean higher premiums and more stringent requirements (i.e. would not insure some sites or would require more loss prevention)
- it was noted that not enough attention is given to risk assessment, scientific input, checking of farm operations (husbandry methods, etc.).

REPORT OF WORKING GROUP #2

AOAC Mouse Bioassay for the Determination of PSP Toxins – Possible Revisions

Moderator: Gregory Boyer, Faculty of Chemistry, SUNY college of Environmental Science and Forestry, Syracuse, NY, 1320
Rapporteur: Michael Quilliam, Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford St., Halifax, Nova Scotia, B3H 3Z1

Paralytic shellfish poisoning (PSP) is caused by a group of potent neurotoxins which include saxitoxin (STX) and several STX analogs. It is a world-wide problem, posing a serious threat to public health because of its high rate of fatality. The AOAC mouse bioassay for PSP toxins (AOAC International 1995) is used in most countries to screen shellfish for the presence of PSP toxins. The procedure involves boiling 100 g shellfish tissues with 100 mL 0.1 N HCl for 5 min, adjustment of the volume back to 200 mL and the pH to 2-4, injection of 1 mL of extract into the intraperitoneal cavity of a mouse and recording of time of death. Although the mouse bioassay is quite simple and has been very effective at protecting the public from shellfish contamination for the last 40 years, it was felt by some that the time had come to examine some possible modifications that could lead to greater efficiency, better precision, and lower costs.

The Rapporteur raised an issue that is fundamental to the reporting of results. The units currently used for reporting PSP toxin concentrations (μg STX equivalent per 100 g of edible tissue) are rather archaic and quite unique in the field of contaminant analysis. This had its origins in the use of 100 g in the extraction procedure and has continued because of tradition. It was suggested that "μg STX equivalent per kg" would be a better approach. Thus, the regulatory action level would be 800 μg STX equivalent per kg of edible tissue. Some European countries have been using this for some time.

One of the suggested procedural changes was to use weighing to control the final adjustment of volume back up to 200 mL. Losses due to transfer of the homogenate from the beaker used for boiling to a graduated cylinder used for volume adjustment can lead to a negative bias. It would be easier just to weigh the extract before boiling and afterwards add enough water to return to the original weight. The availability of top loader digital balances in most laboratories would make this a feasible option to the current volumetric approach.

It was suggested that better pH control would help to improve reproducibility. In the AOAC procedure, it is recommended that the pH of the boiled extract be adjusted to 3, but a pH range of 2-4 was suggested as acceptable. However, acidosis effects can be observed in mice injected with pH 2 extracts. Therefore an emphasis on tighter control to pH 3 would be useful. Some laboratories have experimented with the use of more concentrated acid (1 N HCl) for extraction. Such a variation to the procedure has not been validated with an AOAC collaborative study. It is possible that such a modification could lead to an overestimation of toxicity, due to greater conversion of C toxins to the more toxic GTX's.

The problem of salt suppression was discussed. Sodium ions present in acid extracts of tissues can reduce IP toxicity of PSP toxins by as much as 20-50%. Low levels of PSP toxins

measured with a full strength extract (no dilution) are not very accurate. Sometimes dilution can lead to higher levels (e.g., a sample giving an initial measurement of 600 mg/kg might give a result of 800 mg/kg after a two-fold dilution!). Although the safety factors built into the regulatory action level take care of this, there can be confusion when comparing results from different techniques (e.g., HPLC) with those from the mouse bioassay. Unfortunately, not a great deal can be done about this, other than recognizing that the assay is not very accurate at low contamination levels because of this effect.

When an assay gives a death time of less than 5 min, it is necessary to repeat the assay on a diluted extract in order to get a death time in the 5-7 min range where quantitation is most accurate. Sommer's table (a dose-response curve for pure saxtoxin standard) does not lead to accurate dilutions because of the salt effects at the initial low dilution. Sometimes this results in the need for a third trial at yet another dilution. Don Richard has prepared a modified table that will permit a more successful first dilution.

The issue of sampling was discussed. The requirement to use 100 g of tissue was questioned. The original procedure had this to ensure that enough animals are sampled to avoid the substantial inter-animal variation problem. When some larger animals (e.g., geoducks) are analyzed, it was suggested that directions should be given to homogenize a sufficient number of animals. Of course the cost of sampling many large animals is an important consideration. It has been said (by one of my professors long ago) that "without a sample there can be no analysis, but with an improper sample there should not be an analysis at all!". Therefore, the number of animals required is an issue that must be studied carefully and will require consultation from a sampling expert.

As to whether 100 g of tissue homogenate needs to be boiled was an additional question. If a representative homogenate has been prepared using a sufficient number of animals, it should be sufficient to sub-sample a smaller amount such as 10-20 g for extraction. Tests done recently by Jellett Biotek and NRC have shown that there is no significant difference in extraction yield. Such procedures can simplify operations and also reduce glassware and waste.

It was suggested by Don Richard et al. in a poster that it might be possible to reduce operational costs by reducing the number of mice used in the initial screening test from 3 to 2. This is based on their observations over several years that there would have been very few, if any, instances of false negatives using such an approach. A 30% reduction in mouse costs would be significant in these times of budget reductions. This issue raised some lively discussion with some people being rather reluctant to change procedures without further studies.

It was also suggested that it is important to establish conversion factors (mg STX per mouse unit) for each operator rather than just for the laboratory, as there can be considerable variations between operators. Examining the standard deviation of each operator and making efforts to minimize it could help a great deal. Recommending techniques such as speed of injection would be helpful.

It was felt that it would be worthwhile for someone to consult with various laboratories on these and other possible improvements and then to initiate a collaborative study leading to an

improved AOAC method. M. Quilliam, who is AOAC General Referee for Phycotoxins, suggested that Don Richard would make an excellent Associate Referee and encouraged him to volunteer for the position.

REFERENCE

AOAC International: 1995. Paralytic shellfish poison, biological method, Chapter 35, pp. 21-22. In: *Official Methods of Analysis*, 14th Edition. AOAC International, Arlington, VA.

REPORT OF WORKING GROUP #3

Regulation of Marine Products for Natural Toxins

Moderator: Roland G. Rotter, Bureau of Food Regulatory, International and Interagency Affairs, Food Directorate, Health Protection Branch, Health Canada, Address Locator 0702C, Ottawa, ON, Canada K1A 0L2

Rapporteur: Katsuji Haya, Marine Environmental Sciences Division, Fisheries and Oceans, Biological Station, St. Andrews, NB, Canada E0G 2X0

In Canada, the Canadian Food Inspection Agency (CFIA) enforces regulations concerning marine toxins in seafood and seafood products. The Health Protection Branch of Health Canada sets the regulatory and guideline concentrations of toxins in seafood. The CFIA routinely monitors shellfish and shellfish products for marine toxins by mouse bioassay for paralytic shellfish poisoning (PSP) toxins and by HPLC for domoic acid (DA). If concentrations of the toxins exceed the regulatory level, the CFIA has the authority to close the fishery until the concentrations of the toxins fall below the regulatory levels. Discussion in this session included some information items, touched on several aspects of concern regarding the development of regulatory guidelines and some suggested recommendations. Following is a summary of the discussion and therefore not necessarily a comprehensive report on the topics presented.

Information Items

In the determination of regulatory levels of marine toxins, all available scientific and consumer eating habit information as possible is used. Minimum international acceptable concentrations are set in guidelines by the World Health Organization. A country can make its own evaluation, but if the regulatory level is lower than that set internationally the country must provide scientific justification. For example, Sweden and Philippines use 40 μg/100g instead of 80 μg/100g for PSP toxins.

The Canadian Government is moving towards deregulation and putting more onuses on the industry for compliance. This includes a preference to prepare guidelines versus enacting regulations. Regulations require an Act of Parliament to change while guidelines can be modified as new knowledge is developed. Also, once the level is set there are legal implications, therefore regulatory levels tend to be conservative using the best available information. It was emphasized that producers have a vested interested in safety of their product. For example, the Nova Scotia shellfish industry has been very cooperative with Provincial and Federal government research and regulatory bodies for the last 8 yr. They would like to have a simple test that will provide yes-no answer.

Under the current regulation, the CFIA, on finding higher than regulatory levels of DA, PSP or diarrhetic shellfish poisoning (DSP) in seafood, requires Fisheries and Oceans (DFO) to close the fishery. The CFIA can make recommendations to DFO but can not require them to close for other suspected illness due to the consumption of seafood. These recommendations are

based on epidemiological evidence, anecdotal information and consumer complaints. It was acknowledged that CFIA (due to procedural problems) lacks uniformity in their testing protocols between regions and that their monitoring programs are reactive. That is, sampling of shellfish is intensified only when indicator-monitoring sites suggest a potential problem.

In the United States the monitoring and enforcement of seafood safety varies with the State. DSP regulations fall under the National Shellfish Sanitation Program. In California there is a monitoring program for DA producing organisms, in Washington State DA is monitored by analyzing shellfish extracts by HPLC and Alaska monitors both DSP and DA in Dungeness crabs.

In New Zealand four toxins are of concern. There is a monitoring program for phytoplankton that samples weekly for NSP and DSP producing organisms, and samples biweekly for PSP and DA producing organisms. If any of the organisms associated with the production of marine toxins are detected, then shellfish sampling is initiated.

In the United Kingdom the main concern is for DSP and PSP in the Northeast, South Wales and Southwest coasts. Water samples are analyzed in English labs, and shellfish samples are analyzed in Aberdeen, Scotland. Sampling is conducted weekly between May and September. For the rest of Europe, DSP is the main concern in Scandinavia, Holland, Germany (most of Europe), while PSP is of concern in Brittany and Tunisia. It was noted that regulations in Europe are of concern to the Canadian Industry, as it is an export market.

Health Risk Assessments

The following discussion resulted from the question: "What are the processes and testing required in the setting of safe levels and regulatory concentrations for new toxins, for example the spriolides?" (See Rotter *et al.*, Establishing safe levels of seafood toxin ingestion: the Health Canada perspective, page 114). For the identification of the hazard and a complete assessment of the risk to health of a new toxin, a chronic study of at least two years is preferred. Such a study is critical in determining the tolerable daily intake. The estimated cost for a full toxicity study for one toxin would be $4-5 million. It is not possible for Health Canada to conduct such studies considering it is short staffed and the annual budget is $2 million. Therefore, Health Canada must rely on reports in the scientific literature. In some cases the toxins are used as pharmacological tools in neurological research, for example DA. Such studies will eventually lead to increased knowledge. In the case of emergencies, for example, the DA incident of 1987, provisional short term testing could be used. In this case, acute lethality of DA to rats and the no observable effect level (NOEL) for neurological effects in monkeys administered DA by interperitoneal injection was determined. The results from these studies were used in setting the safe level of DA in mussels and it was assumed that the human survivors who had consumed the mussels contaminated with DA were non-sensitive individuals. The regulatory level of 20 μg/g mussel is still in effect today.

The following precautions need to be considered for toxins in the identification of the hazard and risk assessment to health:

- The route of administration in toxicological studies must be considered. Oral is preferred over interperitoneal administration, as this would be the route of entry for consumers of seafood. Also one needs to be aware of the effect of the route of administration on the stability of the toxin. For example, spirolides would not be toxic via the oral route as they are unstable at low pH and would decompose in the stomach.

- The material used for testing must be considered, that is, the pure toxins versus extracts of seafood containing the toxin. For example, extracts of mussels were used in most DA toxicological studies. Extracts may contain a mixture of toxins, for example PSP and spirolides, or they contain a multitude of other chemicals.

- Interanimal variation is a concern. There are different susceptibilities between humans, and the regulations are designed to protect as many as possible. Test animals such as rodents and primates (better) are only indicative of human effects.

- Okadiac acid is implicated to be a carcinogen, as it forms DNA adducts and therefore considered a tumor promoter although it is negative in the Ames test. This is one of the toxins in DSP and okadiac acid's tumor promoting effects were not considered in setting current regulations for DSP in seafood. It is unclear how this will affect future DSP regulations.

Recommendations

- There is a need for re-evaluation of Memorandums of Understanding between government departments and to improve communication between bureaucracy and the scientific community. It was felt that because of the lack of communications between these fractions that there was too much inefficiency in responding to new toxins.

- There was a plea to make the units used for regulatory levels consistent, and the recommended unit was μg/kg.

- The regulatory level for PSP toxins in lobster hepatopancreas of 80 μg/100g hepatopancreas, and for DA of 20 μg/g in crab viscera should be revisited. That is, the assessment should take eating habits into account, for example two lobster hepatopancreas per meal instead of basing it on the levels set for whole animal homogenates of clams and mussels.

REPORT OF WORKING GROUP #4

Eutrophication - Potential Introduction of *Pfiesteria*

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Rapporteur: Stephen S. Bates, Fisheries and Oceans Canada, Gulf Fisheries Centre, P.O. Box 5030, Moncton, NB, Canada E1C 9B6

INTRODUCTION

In 1991, researchers first made a link between blooms of the newly discovered dinoflagellate *Pfiesteria piscicida* (new genus, family and species) and fish kills in the Pamlico River estuary, North Carolina, on the east coast of the USA (Burkholder et al. 1992). In the presence of large schools of finfish, this organism is thought to excrete a water-soluble toxin that narcotizes the fish and a lipid-soluble toxin that causes bleeding ulcerative lesions, sloughing off bits of epidermis that are then consumed by the dinoflagellates (Noga et al. 1996; Burkholder et al. 1998). Despite several other severe bloom-related fish kills in the interim, it was not until the summer of 1997, when fish were killed farther north in estuaries of the Chesapeake Bay, that national media attention was more strongly focused on the problem. Canadian media also became interested (e.g., McKenna 1997; Poitras 1997) and questions were raised about the possible introduction and survival of toxic stages of *Pfiesteria* in our colder Canadian waters. A Briefing Note, entitled "*Pfiesteria piscicida*, a Toxic Estuarine Alga", was subsequently prepared by Iola Price (Director, Aquaculture and Oceans Science Branch, Ottawa) and Stephen Bates (Research Scientist, Marine Environmental Sciences Division, Moncton) for the Minister of Fisheries and Oceans. At the same time, concerns were also being expressed about the accumulating evidence showing a possible link between cultural eutrophication and the occurrence of blooms of toxic *Pfiesteria* in coastal areas of the mid- and southeastern United States (Burkholder 1998); could a similar situation arise in Canada? Do Canadian waters provide the necessary conditions for an optimum *Pfiesteria* habitat: warm, poorly flushed, shallow and quiescent waters, high nutrient loadings, and the presence of schools of oily fish (Burkholder et al. 1995)? Indeed, the presence of *P. piscicida*, or any number of other recently discovered toxic *Pfiesteria*-like dinoflagellates in the "*Pfiesteria* complex" (e.g., *Cryptoperidiniopsis*) in Canadian waters could be devastating for the aquaculture and natural harvest fisheries, and human health (Glasgow et al. 1995; Burkholder 1998; Burkholder et al. 1998). This topic is relevant not only to Canada, but also to U.S. states north of Delaware, the most northerly location thus far identified for these organisms. Similar grave concerns are also being expressed by European countries.

The organizing committee of the 6th Canadian Workshop on Harmful Marine Algae felt that this would be an appropriate time to gather together the available expertise to discuss 1) possible links between eutrophication and *Pfiesteria* blooms, and 2) possible actions that Canada could undertake in order to deal with the potential presence or future introduction of *Pfiesteria*

spp. or *Pfiesteria*-like organisms into Canadian waters. The discussion was organized around the following topics:

DETERMINING THE PRESENCE OF *PFIESTERIA* OR *PFIESTERIA*-LIKE SPECIES IN CANADIAN WATERS

Are *Pfiesteria*-like species already present but unnoticed in Canadian waters?

The possibility was discussed that *Pfiesteria* and/or related dinoflagellates are already present in Canadian waters. After all, these organisms have had eons to evolve, spread, and adapt to prevailing conditions. How then, does one go about determining their presence? One could start by trying to correlate fish kills or fish with lesions with the presence of *Pfiesteria*. However, there are many causes of mass mortalities and lesions other than *Pfiesteria*. More importantly, several factors complicate the identification of *Pfiesteria* and related organisms, including its life cycle and lack of reliable detection methods, as discussed below.

Pfiesteria has a complex life cycle with at least 24 stages that include flagellates, amoebae and cysts (Burkholder and Glasgow 1997). Not all of these stages preserve well and many are cryptic (Burkholder et al. 1995). It was pointed out that at least one of the stages (the rhizoid amoeba; #4 of Fig. 1 in Burkholder and Glasgow 1997) has not been well described and that it appears quite similar to several common heliozoans. It is therefore exceedingly difficult to be certain that an unknown organism fitting the description of any one of these life stages is, in fact, *Pfiesteria*. One must isolate the organism and follow its life stages in culture, a tedious approach not easily carried out. An example was given that unidentified amoeboid forms (not belonging to *Pfiesteria*) are found in many samples collected from coastal waters of Washington state. It was therefore concluded that numerous organisms may have life stages that include amoeboid forms in the water column or sediments. These look alike "gymnodinioid" dinoflagellates may never be toxic, but their presence can cause taxonomic confusion.

What detection techniques are currently available or being developed?

No fast and reliable techniques are presently available to detect and identify *Pfiesteria* and related organisms. The approach now used is basically the same one that originally detected the presence of fish-killing stages in estuaries of North Carolina in the early 1990s, i.e., an algal or a fish "bioassay" of the water (Burkholder et al. 1995). Essentially, a sample of sediment plus overlying water is incubated in the presence of either algae (primarily cryptomonads) or fish, as food sources. If any *Pfiesteria* stages are present, the fish stimulate the toxic flagellated form(s). The bioassay using fish, however, is dangerous to human health and requires a Biohazard Safety Level 3 facility. In the presence of algae, the nontoxic form of the dinoflagellate is produced. To correctly identify the dinoflagellate forms, enough cells must be concentrated and then their outer membranes must be removed in order to examine the thecae using scanning electron microscopy. This time-consuming method requires considerable expertise and is not suitable for routine monitoring work. Consequently, the approach has not been widely used outside the areas where *Pfiesteria* and related organisms have already been found. One exception mentioned was the search (unsuccessful) for *Pfiesteria* in coastal waters of Massachusetts by incubating sediments and water in the presence of algae. Session participants ultimately criticized the use of such

bioassays because the results are not consistent; any number of difficult-to-identify mixotrophic gymnodinioid species may appear. A recent communication (Glasgow et al. 1998) concluded that the algal bioassay is not reliable because few or even none of the toxic *P. piscicida* cells present were able to be detected. Alternatively, one may survey for the presence of cysts in the sediments. So far, however, this approach has not been successful because the cysts are small and difficult to identify using microscopy.

Thus far, the approach with the greatest potential appears to be the application of molecular probes that identify DNA sequences that are specific to *Pfiesteria piscicida* (Toffer et al. 1998; Greer et al. 1998). This area of research has shown promise for other species of harmful marine algae, but progress has been slow for *Pfiesteria*. Nevertheless, it was mentioned that Parke Rublee (University of North Carolina at Greensboro) is close to completing the development of such probes. The probes must now be tested in waters other than North Carolina, with different stages of *Pfiesteria*, and with other organisms in the *Pfiesteria* "complex". It was announced that several proposals targeted at developing additional molecular probes have been funded in the second round of the ECOHAB (Ecology and Oceanography of Harmful Algal Blooms) funding program; see:

http://www.redtide.whoi.edu/hab/announcements/ecohabannounce/AO_ECOHAB98.html

and

http://www.redtide.whoi.edu/hab/announcements/pfiesteria/pfiesteriastrategy.html.

For now, it was concluded, we must continue to wait for the final development of reliable oligonucleotide probes against *Pfiesteria*. It was suggested that, in combination with these molecular probes, techniques are required to directly detect the two toxins produced by *Pfiesteria*. Although reporter gene assays are available for detecting *Pfiesteria* toxin activity (see talk by J. Ramsdell, this proceedings), analytical techniques cannot be developed until the molecular structure of the toxins is completely elucidated. Until these techniques are perfected, it was suggested that a good master's student project could still be to use the bioassay method to search for gymnodinioid species in warm shallow embayments within Canadian waters. This would provide us with the opportunity to start the learning process by becoming familiar with the distribution and taxonomy of such organisms.

RISK OF INTRODUCTION

What routes may introduce *Pfiesteria* into Canadian waters?

Even if *Pfiesteria* is not currently present in Canadian waters, what is the risk that it may be introduced, and via what routes? One possible route discussed is via ships' ballast waters, a known vector for introducing other exotic species from different parts of the world (e.g., Smith and Kerr 1992). It was pointed out that the greatest risk for harboring exotic species is not necessarily the water that is drawn into the ballast tanks but rather the bottom sediments from the mooring site that are pumped into the tank with the overlying water. This may be especially true for *Pfiesteria*, since this organism has several stages that reside within the sediments. Such sediments can inadvertently be pumped into ballast tanks if ships take on the ballast in a shallow port.

The first step in assessing the risk of introduction via ballast water is to determine if the shipping routes include ports in areas where *Pfiesteria* has been most prevalent, i.e., along the mid-Atlantic southeastern states of the USA. Assuming that the Canadian east coast is most vulnerable, it was pointed out that such information is available from the Eastern Canada Region Vessel Traffic Services database of Transport Canada. A breakdown of ports of origin has recently been compiled in order to assess the potential risks of introducing nonindigenous marine organisms into the estuary and Gulf of St. Lawrence (Harvey et al. in prep.). In 1995, 183 ships came from ports along the east coast of the USA, including the potentially risky ports of Baltimore MD, Wilmington DE, Georgetown SC, and Savannah GA. Vessels from these ports, however, reported that the majority of their ballast water was first exchanged in the "open ocean", in compliance with the Voluntary Guidelines for the Control of Ballast Water Discharges from Ships Proceeding to the St. Lawrence River and Great Lakes. Nevertheless, coastal phytoplankton species were found in the ballast waters, including 19 harmful or toxic species. Moreover, almost 50% of the ballast sediment contained mostly unidentified dormant dinoflagellate cysts, leading to the conclusion that such sediments may be an important vector for introducing exotic species. It is not known how any of the currently proposed methods for "disinfecting" ballast water and sediments (e.g., high temperature treatment) would affect the survivability of the different stages of *Pfiesteria*, especially the highly resistant cyst stages (see below). The above information is for the estuary and Gulf of St. Lawrence. Similar data can be obtained about marine traffic between ports along the mideastern USA and the Bay of Fundy, principally the port of St. John, NB.

Other potential introduction mechanisms were discussed, including birds, shellfish, and fish. For the latter, the question was raised about the possibility that certain migrating fish species could be vectors of nontoxic stages of *Pfiesteria* cells if they were ingested in one location and later excreted in waters farther to the north. Because there is no evidence that *Pfiesteria* cells are epiphytic or epizooic, it appears unlikely that they could be transported on the surfaces of plants, animals, or boats.

RISK FOR TOXIC OUTBREAKS

The potential temperature barrier

One environmental factor that may reduce the risk that introduced *Pfiesteria*-like organisms may survive is the low temperatures characteristic of Canadian winters. It may also provide a barrier for any possible slower movement northward of these organisms. The current distribution of *Pfiesteria*-like organisms along the mid- and southeastern Atlantic states suggests that they require warmer waters to survive. Field observations have documented *Pfiesteria piscicida* toxicity across a wide temperature gradient from 6 to 31°C, with most outbreaks occurring at 26°C or higher (Burkholder et al. 1995). Certainly, it is possible to achieve these warmer temperatures in Canadian waters during the summer in certain shallow estuaries. For example, open waters of Prince Edward Island estuaries generally reach summer temperatures of between 20 and 25°C (e.g., Bernard 1993); shallow embayments would be warmer than this. Conditions during the colder months of the year, when about half of the foreign ships discharge their ballast water into ports of the Gulf of St. Lawrence (Harvey et al. in prep.), would limit the

potential for the survival or proliferation of *Pfiesteria*-like dinoflagellates. It must also not be forgotten that certain strains may have a wider temperature tolerance that would allow them to adapt to colder waters. For example, in British Columbia, where low temperatures were once believed to constrain blooms of the fish-killing raphidophyte flagellate *Heterosigma akashiwo* to certain southern parts of the province, they are now problematic in the colder waters of Broughten Island, farther to the north (see talk by J.N.C. Whyte, this proceedings). Also, some of the cyst stages of *P. piscicida* may be extremely resistant to low temperatures, as they have survived freezing for at least 2 months (Burkholder, pers. commun.). Up to 20% of the cysts have also survived immersion in concentrated sulfuric acid, concentrated ammonium hydroxide, and bleach, as well as desiccation (Burkholder and Glasgow 1997).

Laboratory studies are clearly required to determine the temperature range at which the various stages of *Pfiesteria*-like species can survive. It is likely that each stage could have a different temperature tolerance. Participants indicated that these studies have already begun in at least two U.S. laboratories. In addition, proposals have been submitted to the second round of ECOHAB to study the effects of temperature, as well as other environmental parameters, on the physiology of *Pfiesteria* and related organisms.

The high nutrient load requirement

One aspect that has caused considerable debate among scientists and within state agencies in the USA is the proposed link between cultural eutrophication and the outbreak of toxic *Pfiesteria* blooms (Barker 1997; Boesch 1997; Hughes 1997). Evidence has been presented that most fish kills have occurred in phosphorus- and nitrogen-enriched estuaries, e.g., near phosphate mining, sewage outflows, fish processing plants, or poultry and hog farms (summarized by Glasgow et al. 1995; Burkholder 1998). Because *Pfiesteria* is a protozoan animal, not a photosynthetic alga, its ways of acquiring nutrients are complex, including mixotrophy, phagotrophy, heterotrophy, and even a limited degree of photosynthesis via chloroplasts that it "steals" from its algal prey (kleptochloroplastidy). Organic and inorganic nutrient enrichment may indirectly stimulate *Pfiesteria* by supporting the growth of its algal prey. Nutrients may also directly stimulate the toxicity of flagellated stages of *Pfiesteria*. It was pointed out, however, that not all outbreaks of fish-killing *Pfiesteria* occur in nutrient-enriched waters. In North Carolina, for example, about 75% of such outbreaks occurred in nutrient-enriched coastal waters (Burkholder et al. 1995); 25%, therefore, occurred in relatively less enriched environments (Hughes 1997). Apparently, then, the primary requirement for an outbreak of toxic stages of *Pfiesteria* is the presence of fish, although nutrient enrichment can exacerbate the problem.

In Canada, one may at first believe that anthropogenic nutrient inputs are not that significant, relative to the USA, given our lower and more sparsely distributed population. However, a closer examination may reveal certain geographic areas where point and non-point nutrient sources are great. Specifically, cases were mentioned in eastern Canada where poultry and hog farms presently exist near rivers, and where hog farms are proposed to be expanded. Local residents have already become aware of the controversy surrounding the link between eutrophication due to animal farms and *Pfiesteria* outbreaks in the USA, and are using this information to lobby Canadian government agencies. Questions remain, however, about the

solidness of this link and about whether or not there is a nutrient threshold above which fish kills due to *Pfiesteria* may be most prevalent.

RECOMMENDATIONS

Several recommendations were offered in order to place Canada in a proactive position regarding the potential discovery and/or inadvertent introduction of toxic *Pfiesteria* or related dinoflagellates.

- Develop an action plan to deal with such a threat. The USA was at first caught off guard by its *Pfiesteria* crisis, and this led to an unnecessary amount of disagreement and lack of cooperation among a number of the affected state governments. It also caused significant economic losses due to the over-reaction of skittish seafood consumers.

- Draw on the successful experience that Canadians already have in dealing with the 1987 domoic acid "mussel crisis" on Prince Edward Island. This includes strengthening and extending existing links among federal agencies (i.e., Fisheries and Oceans Canada, Canadian Food and Inspection Agency, Health Canada, National Research Council of Canada), provincial agencies, universities, and the aquaculture and fishing industries.

- Open lines of communication among various experts in diverse fields related to harmful algal blooms and fish, both in Canada and the USA; this could take the form of e-mail (including proprietary) list servers. Strengthen links with U.S. researchers who are working directly on the *Pfiesteria* problem, in order to remain up to date on the latest scientific advances.

- Attend workshops and training sessions organized by U.S. researchers regarding *Pfiesteria* taxonomy, new identification and monitoring techniques, new analytical methods and assays for *Pfiesteria* toxins, and standardized techniques for taking samples of water and dead fish.

- Educate the public via newspaper articles, radio and TV; establish links with trustworthy reporters. Augment existing Web pages regarding Canadian harmful algal bloom events and research. Establish mechanisms (e.g., "hot lines") for the public to communicate back to scientists regarding fish kills and harmful algal bloom events; ensure that there are "after hours" telephone numbers allowing the public to contact the appropriate scientists. Educate the medical community regarding the health impacts of *Pfiesteria*.

- Become involved in Environmental Assessments of projects that may result in significant nutrient inputs into rivers and coastal waters. Educate policy makers regarding the potential threats due to *Pfiesteria*-like species.

- Determine the locations of Level 3 Biohazard facilities in Canada in order that experimental and test systems may be rapidly set up in the event of a toxic *Pfiesteria* outbreak.

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Attached is an insert for the following report. Please attached to this report:

Martin, J.L., K. Haya (editors). 1999. Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae
Canadian technical report of fisheries and aquatic sciences 2261

Figure 1. Eastern New Brunswick

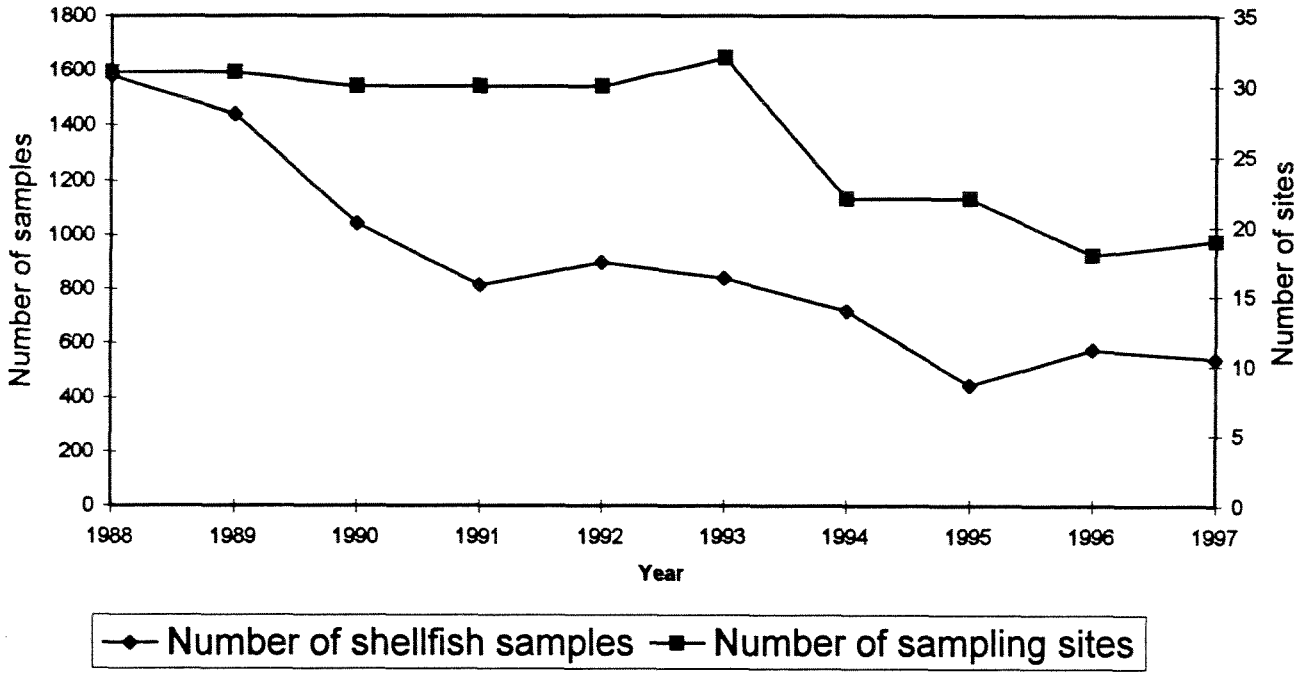

Figure 2. Prince Edward Island

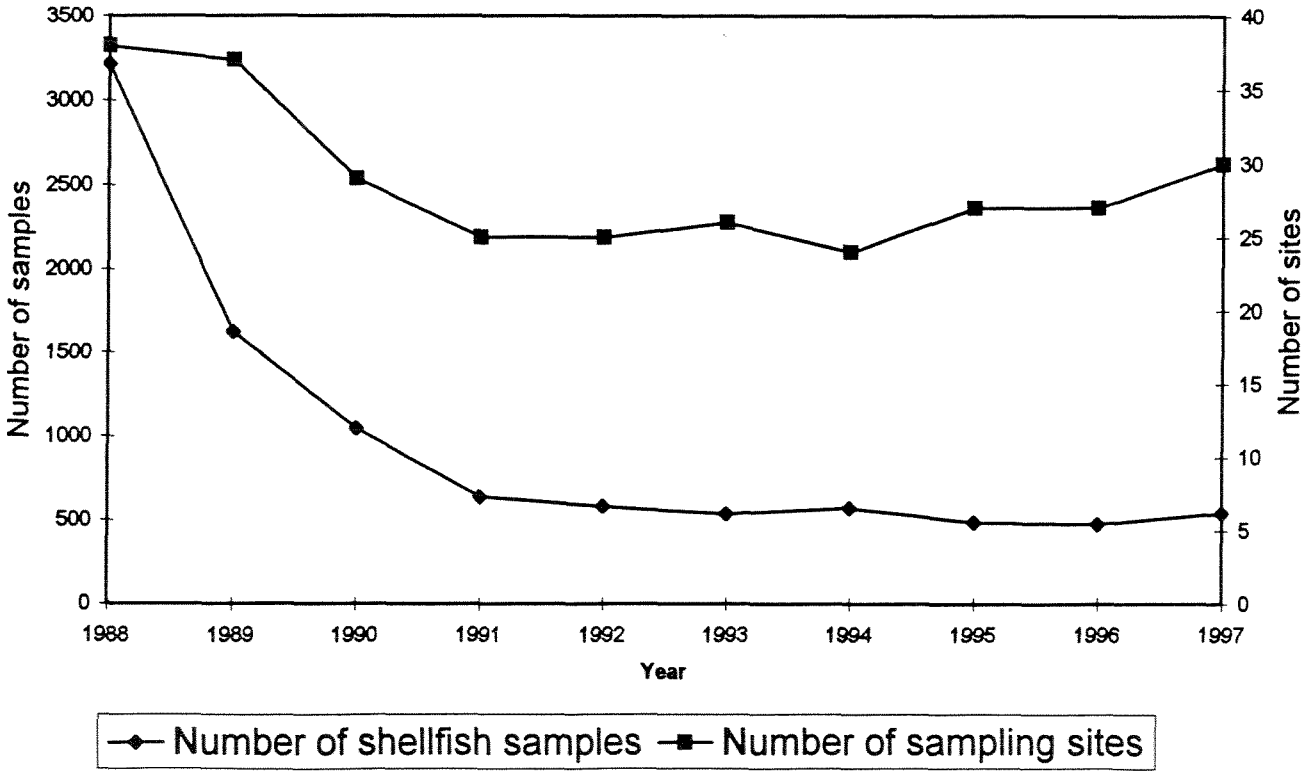

Figure 3. Gulf side of Nova Scotia

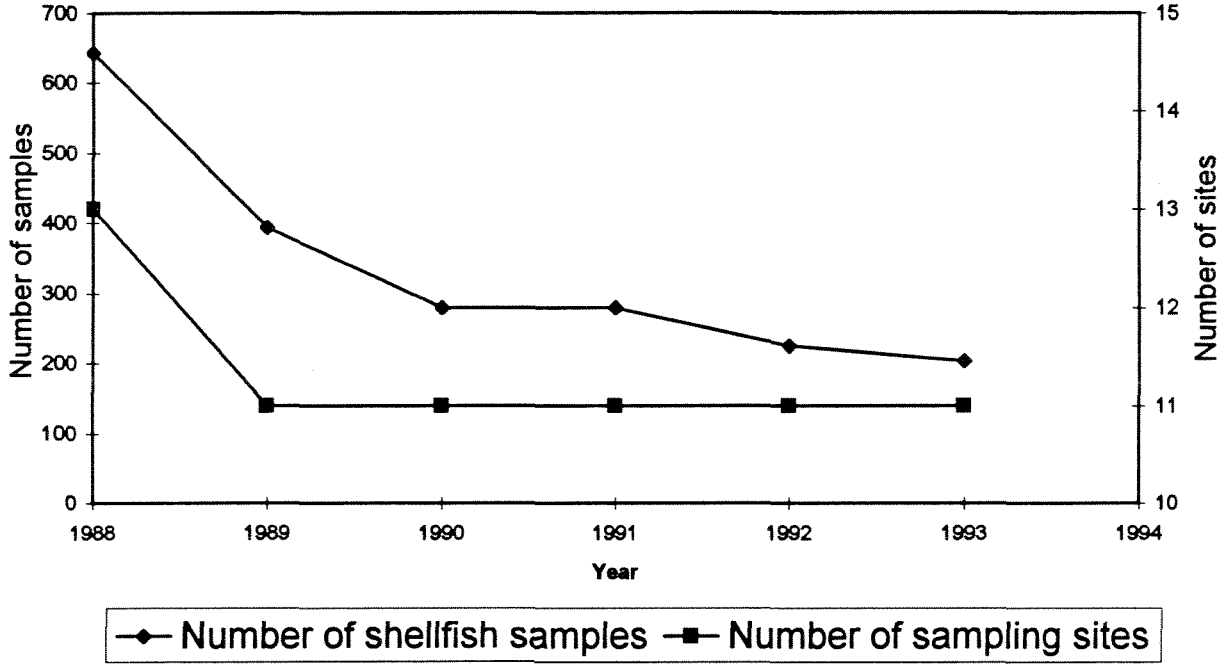

Figure 4. Southwest New Brunswick

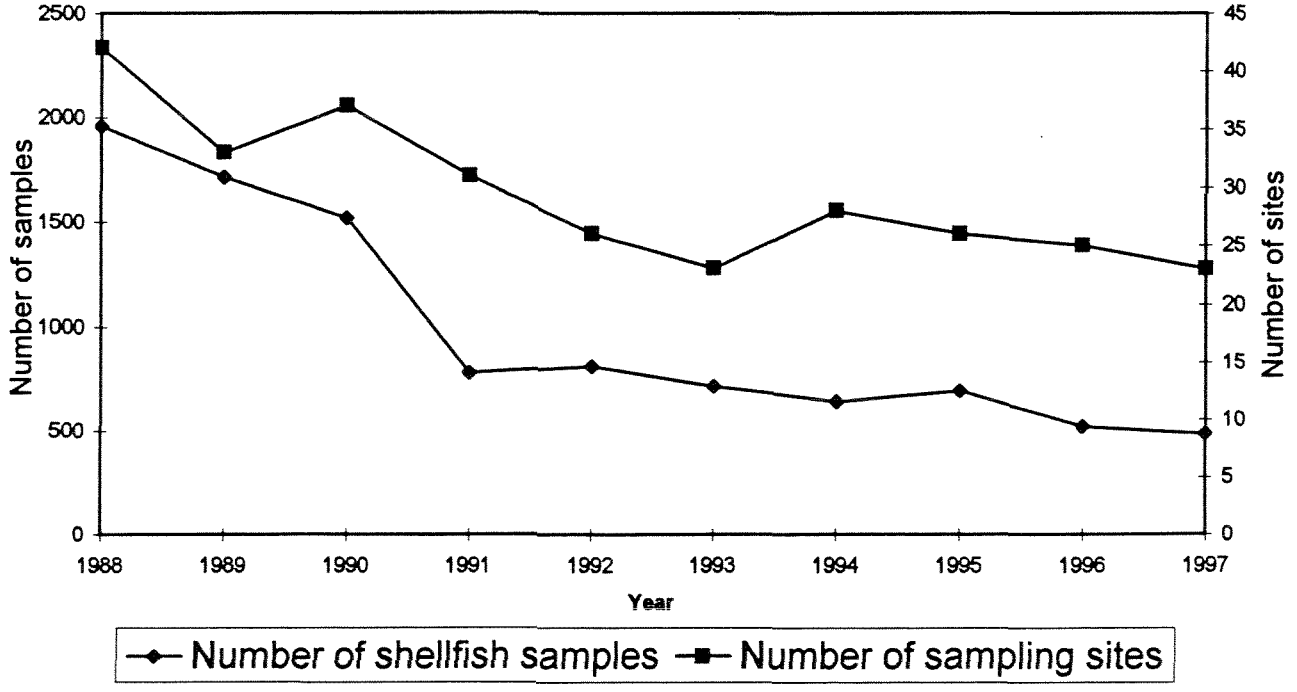

Figure 5. Total number of shellfish samples from New Brunswick and PEI

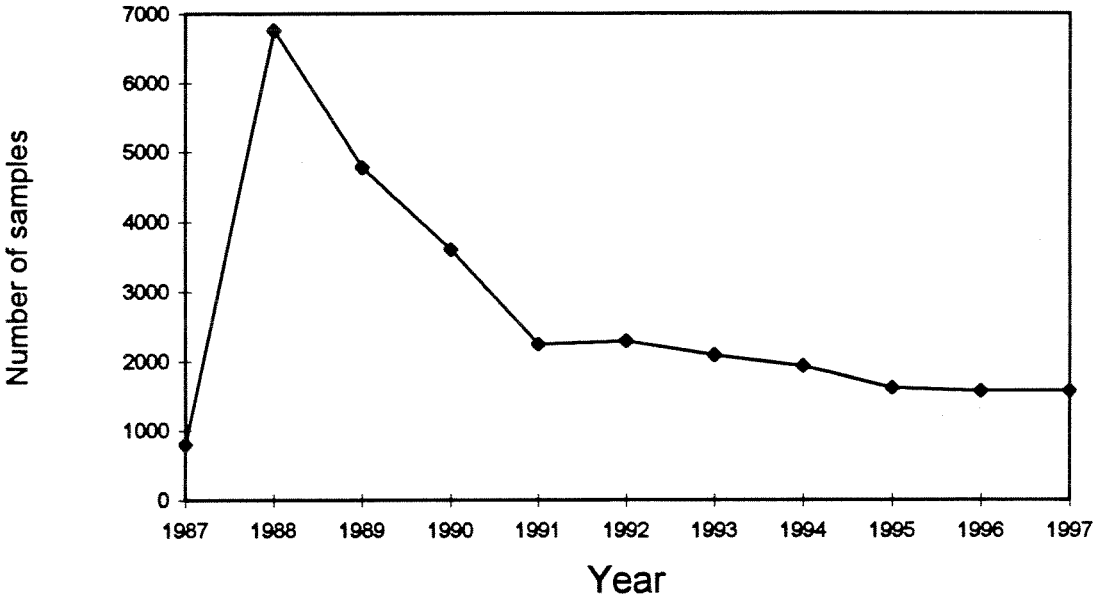

Figure 6. Phytoplankton Monitoring

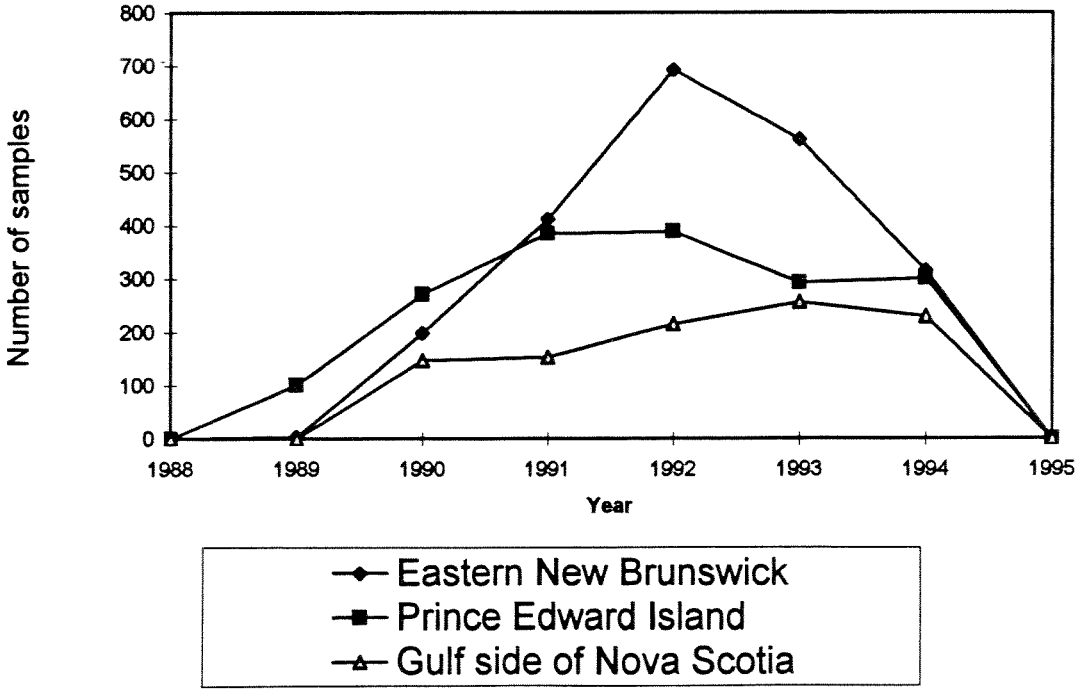

Figure 1. Mean and confidence interval (95%) for all analysts. Ranked by precision.