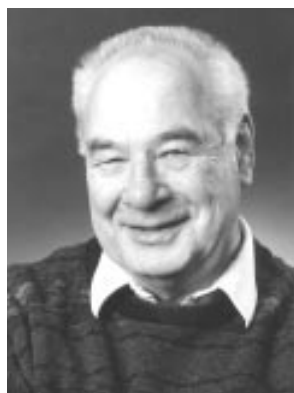

Phycotoxins: Physiology and Production

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Introduction

Phycotoxins ([phyco = seaweeds and algae] plus toxins) are a diverse group of poisonous substances produced by various aquatic plants in marine and fresh waters throughout the world. Not all aquatic plants produce toxins; and among those that do, not all, even from the same genera and species, produce toxins at all times and under all circumstances. In addition, problems with toxins occurring in shellfish are experienced commonly when no algal blooms have been noted, e.g. much of the chronic

Paralytic Shellfish Poisoning in the Bay of Fundy, the recorded instances of Diarrhetic Shellfish Poisoning on the Nova Scotian south shore, and the domoic acid in scallops from Georges Bank in 1995 to name a few. The problems caused by phycotoxins in freshwater are often the result of blooms of cyanobacteria (formerly known as blue-green algae) which make the waters toxic, resulting in large losses among wildlife and domestic animals. In the marine environment the most newsworthy occurrences have been major intoxications among peo-

ple who have eaten filter-feeding shellfish (molluscs) which have fed on toxigenic algae, thereby accumulating large amounts of the toxins. Many of these toxic episodes have resulted in fatalities and others in temporary and permanent disabilities. In addition, a number of studies have implicated marine toxins in large fish kills in the wild (adult and larval fish), major kills of marine mammals (whales, porpoises, and seals), as well as threats to cage cultured finfish, e.g. the microcystin-like toxin causing Netpen Liver Disease resulting in serious mortalities among farmed salmon.

In addition to the toxigenic aspects, an arresting and important element, which has not previously received attention, is the role plankton play as vectors for disease agent propagation and transmission. A recent review (Patz *et al.* 1996) examining the question of climate change and infectious diseases looked at the data on the seasonality of cholera outbreaks. The causative agent the bacterium, *Vibrio cholerae*, adheres to the surfaces of both phytoplankton and zooplankton and is transported and transmitted along with the plankton to shellfish and thence to humans;

the bacterial numbers parallel the plankton numbers and the bacterium lives on contributions of nutrients exuded by the plankton. Thus the cholera outbreaks, which are a function of the numbers of *V. cholerae*, increase with the plankton numbers in the spring and fall. Other bacteria have been observed as resident on plankton, and the evidence is increasing showing that plankton are an important vector for spread of diseases in water. This is of major consequence to public health generally and to aquaculture operations in particular, e.g. as indicated by Nese and Enger (1993) who found the salmon pathogen *Aeromonas salmonicida* (the causative agent of furunculosis) carried on marine plankton in the vicinity of the salmon net pens.

Following the 1987 domoic acid/mussel crisis the decision was taken to incorporate a national perspective in dealing with phycotoxins. Accordingly, the Phycotoxins Working Group (PWG), composed of representative toxin investigators from all Department of Fisheries & Oceans (DFO) regions, was formed. It is a national advisory and program management body which reports to the National Science Directors' Committee (NSDC) and is concerned with research projects whose objectives include:

- identification of algae and microorganisms that produce harmful blooms or toxins
- investigation of the distribution of these organisms and the environmental factors that control this distribution
- investigation of the nature and magnitude of the impact of phycotoxins on aquaculture and harvest fisheries
- identification and quantification of toxins by chemical or bioassay techniques and developing innovative, simple analytical methodologies that address both research and product certification requirements
- establishment of the role of toxins in nature and competitive impacts on other species;
- elucidation of the dynamics of blooms and toxin production (nutritional, physiological, biochemical and microbial)
- investigation of the fate of toxins in nature (foodweb transfers, biotransformations) development of warning systems, predictive models and countermeasures; and
- investigation of the effects of toxins on aquatic organisms.

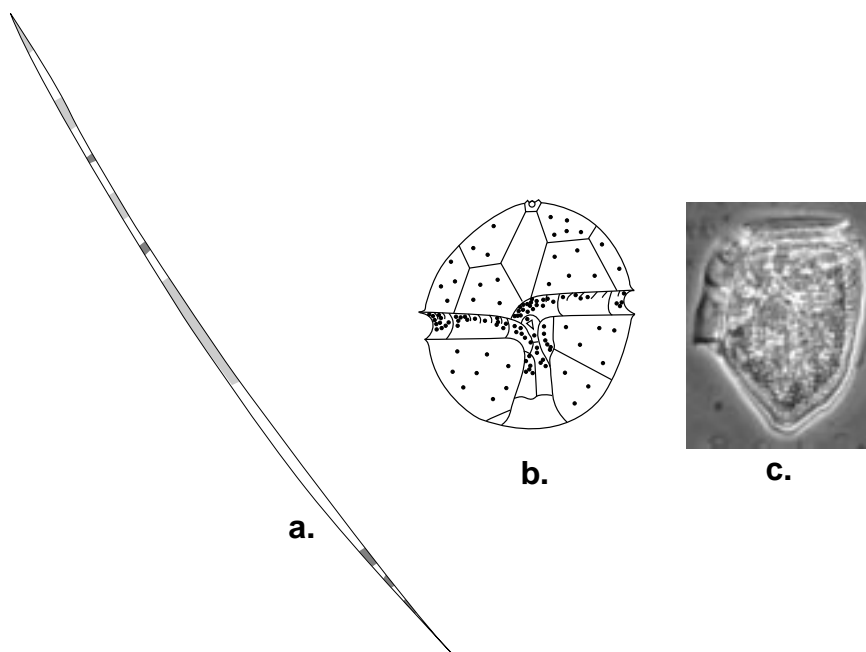


Figure 1. Toxicogenic taxa: a. *Pseudonitzschia multiseries*, b. *Alexandrium tamarense*, c. *Dinophysis norvegica*

Atlantic Canada

In Atlantic Canada, marine toxins pose three major threats. These are Paralytic Shellfish Poisoning (PSP) caused by members of the saxitoxin group, Amnesic Shellfish Poisoning (ASP) caused by domoic acid, and Diarrhetic Shellfish Poisoning (DSP) caused by a family of toxins which include okadaic acid and related dinophysistoxins, pectenotoxins and yessotoxins (Fig. 1 and 2).

Paralytic Shellfish Poisoning resulting from toxins produced by dinoflagellates occurs on both Canadian coasts. In the east it has occurred chronically in the Bay of Fundy and upper Gulf of St. Lawrence with periodic outbreaks elsewhere such as the lower portion of the Gulf of St. Lawrence and Newfoundland. In 1987, the problem of shellfish poisonings broadened dramatically with the addition of a new neurotoxin, domoic acid, which caused a condition subsequently named Amnesic Shellfish Poisoning. This toxin was produced by a diatom, the name of which evolved from *Nitzschia pungens* through several intermediates to its current sobriquet, *Pseudonitzschia multiseries*. The mussels cultured in Cardigan Bay, Prince Edward Island, fed on a bloom of this diatom and accumulated massive levels of the toxin, i.e. up to 900 mg/g of soft tissue which is about 45 times

the current legally permitted maximum in Canada. Consumers of the mussels suffered an intoxication which resulted in about 150 people being hospitalized; around a dozen were seriously and apparently permanently disabled, and ultimately 3 definitely and possibly a total of 5 died as a direct result of the intoxication.

Diarrhetic Shellfish Poisoning has been confirmed recently in the Maritimes; anecdotal evidence, however, suggests that it has been present for a long time. Elsewhere it is believed to be produced by marine algae (*Dinophysis* sp. and others), but its actual source in Atlantic Canada has not been identified yet. To date it has not been considered as serious a problem locally as the other two toxins.

In discussing the problems arising from toxins a fatalistic attitude is commonly adopted in which it is stated that the toxins will always be with us and that the simplest and most successful course would be to learn how to manage around the toxins. This course may, in fact, be valid for domoic acid in mussels which rid themselves of it readily, but it does not apply to domoic acid or PSP in scallops or other species which retain these toxins. It should be remembered that the toxin problems are completely analogous to the problems

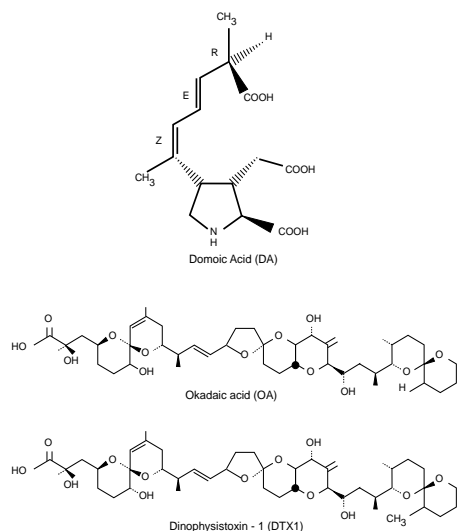


Figure: 2a Toxins: Domoic acid (DA), Okadaic acid (OA), Dinophysistoxin-1 (DTX 1)

posed 150 years ago by infectious diseases. These proved to be amenable to investigation; the basic understanding derived resulted in diagnostic procedures, prediction, protection, and cures. Exactly the same possibilities exist within the phycotoxins field as results gained over the past several years demonstrate, portions of which are presented below.

Domoic Acid: Physiological Aspects of Production

After the identification of the neurotoxin, domoic acid, as the cause of the 1987 mussel crisis by scientists at the National Research Council (NRC) Laboratories in Halifax (Bird *et al.* 1988; Wright *et al.* 1989), the source was discovered to be the diatom *Nitzschia pungens* (Subba Rao *et al.* 1988); the toxigenic strains are now named *Pseudo-nitzschia multiseriis*. Subsequently, both the diatom and the neurotoxin have been shown to be distributed widely and, in fact, have been particularly troublesome in California where mass die-offs of seabirds have been attributed to the consumption of domoic acid-contaminated anchovies. Major disruptive episodes have occurred also in the shellfish industries of the U.S. Pacific Northwest.

Experimental studies have shown that various strains of *P. multiseriis* yield varying amounts of toxin; all have one element in common, they tend to produce domoic

acid mainly after growth has entered the stationary phase. This phase occurs after the nutrients sustaining exponential growth are depleted or culture conditions have deteriorated. In either or both events the culture has entered a period of physiological stress (Pan *et al.* 1996a,b), as shown in Figure 3. When the culture was grown in media in which the concentrations of phosphorous or silica were below the required optimum, domoic acid production was enhanced; this enhancement of domoic acid was eliminated proportionately by adding to the growth medium graded amounts of phosphorous or silica, thereby overcoming the nutrient stress (Fig. 4 and 5). Lithium was found in relatively high concentrations at Cardigan in 1987 and through growth studies was shown to enhance significantly the production of domoic acid by *P. multiseriis*, as shown in Figure 6 (Subba Rao *et al.*, in preparation). Another influence is the ammonium concentration; Bates *et al.* (1993) showed that very high concentrations of this ion tended to inhibit growth of *P. multiseriis* compared to growth with the same nitrogen levels in the form of nitrate. The cells that did grow, however, produced higher concentrations of domoic acid than those growing in the presence of nitrate alone.

Parallel to these findings, work by McLachlan *et al.* (1993) showed that a marker compound, gluconolactone, appeared only in fluids from mussels shown by high-performance liquid chromatography (HPLC) to be contaminated with domoic acid. Gluconolactone was found also in a methanolic extract of a bacterium isolated from close association with the diatom, *P. multiseriis*, but not from extracts made from the diatom.

Exposure of the diatom to varying levels of gluconolactone (more properly an equilibrium mixture of gluconic acid/gluconolactone; the acid is a powerful sequestering agent) showed domoic acid production was enhanced in its presence and that the effect was concentration dependent (Fig. 7). As the concentration of domoic acid increased in relation to increasing concentrations of gluconic acid/gluconolactone, the proportion of domoic acid released by the diatom to the culture filtrate increased (Osada and Stewart, in press).

Studies by Stewart *et al.* (in press) with bacteria isolated from close associations with *P. multiseriis* revealed that, of the four *P. multiseriis* strains examined, each had at least one strain of associated bacteria capable of producing, from glucose, large amounts of gluconic acid/gluconolactone. Each diatom strain also had other bacterial strains which grew best with amino acids. It was concluded that these bacteria lived in a symbiotic relationship with the diatom. Further studies showed the *P. multiseriis* grown under standard conditions and in different salinities had substantial amounts of glucose free in the cell, and at the highest salinity accumulated a substantial quantity of sorbitol, a presumed osmolyte.

Thus, ingestion, by mussels or other molluscan shellfish, of masses of the *P. multiseriis* containing varying levels of domoic acid, as occurred during the 1987 domoic acid/mussel crisis, would bring together the ingredients required to give rise to the circumstances detected within the mussels by McLachlan *et al.* (1993). These were *P. multiseriis* cells containing domoic acid and substantial quantities of glucose which could be released through injury to or rupture of the diatom, bacteria capable of converting the glucose to gluconic acid/gluconolactone and intact *P. multiseriis* cells (observed by Scarratt, personal communication) which would still be metabolically active. The gluconic acid/gluconolactone acting upon these cells would be expected to stimulate and further enhance the production of domoic acid. Thus, it is probable that a significant por-

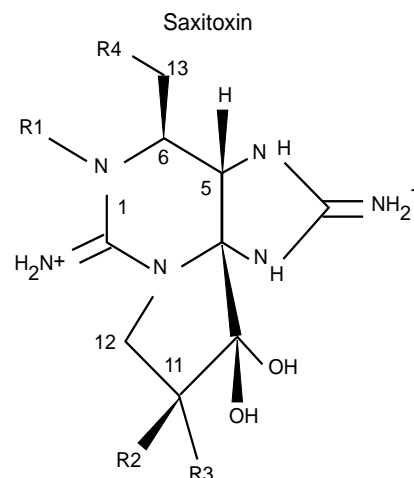


Figure: 2b Saxitoxin

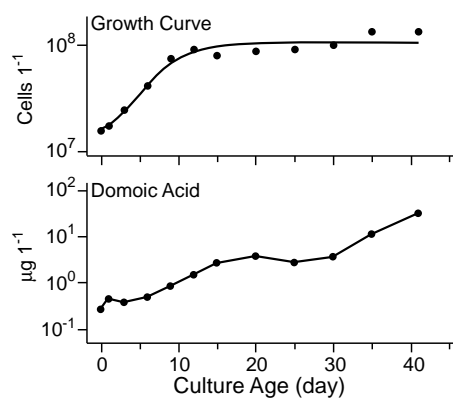


Figure 3. Growth curve of *P. multiseriis* and variations in domoic acid levels in a batch culture.

tion of the domoic acid present is actually synthesized within the mussel after ingestion of *P. multiseriis*. We believe the reason for this lies in the chemical nature of the two agents gluconic acid/gluconolactone and domoic acid.

Gluconic acid is a powerful sequestering agent and for that reason is produced commercially for inclusion in cleaning compounds. Domoic acid, as well, has the structure of a powerful chelating agent. The chemical nature of the antagonism suggests the role of domoic acid is that of a chemical scavenger and control agent for the diatom. The proof for this consists of the following: when nutrients become scarce at the end of the exponential period of growth, domoic acid production is enhanced; when nutrients such as phosphorous or silica are present in the media in limiting concentrations, domoic acid production again is enhanced. It is, however, reduced when the nutrients previously in limited supply are returned to normal levels. When a sequestering agent such as gluconic acid is present to tie up various nutrients, domoic acid production is enhanced in proportion to the concentration of gluconic acid present and is released to the surrounding medium to counteract the effect of the antagonistic agent. In addition, when high concentrations of various materials, i.e. lithium or excess silicates, are present major amounts of domoic acid are synthesized and released presumably in attempts to sequester these materials. Much of this would be expected to occur within the shellfish following their ingestion of the diatoms thereby giving rise

to the high levels of domoic acid found in the shellfish.

Domoic Acid: Possible Clearance Mechanism

Domoic acid has been shown to be produced widely in nature and in quantity; as it does not appear to accumulate beyond a certain point, mechanisms must exist for its degradation and disposal. Bacteria in the marine environment are prime choices to mediate this activity. Bacteria from the mussel culture area of Cardigan Bay, Prince Edward Island, Bedford Basin, Nova Scotia, the Bay of Fundy, and other marine sources were examined for growth at the expense of domoic acid and the capacity of resting cells to oxidize it using manometric procedures. Despite extensive and intensive trials, the results were uniformly negative. Clearly, the capacity to grow on and utilize domoic acid is not a common microbial attribute.

Published studies have shown that blue mussels (*Mytilus edulis*) routinely are capable of reducing the concentrations of accumulated domoic acid relatively rapidly; in contrast, the results from trials as well as anecdotal evidence indicate that the sea scallop (*Placopecten magellanicus*) eliminates domoic acid very slowly. Through application of enrichment techniques, using gill and digestive gland tissue, we showed that 45 of 46 individual mussels possessed bacteria, the growth of which was enhanced to a limited, but significant extent by domoic acid; in addition, 5 pooled soft-tissue homogenates (each representing 10 mussels) also yielded similar bacteria. Nine of 20 softshell clams (*Mya arenaria*) and 2 of 10 red mussels (*Modiolus modiolus*) had bacteria whose growth was stimulated by domoic acid, while only four of 60 scallops taken from six different locations were positive for such bacteria.

The dominant bacterial genus appeared to be *Alteromonas* followed by *Pseudomonas* sp. Substrate utilization trials were carried out with five of the bacterial isolates which had shown the greatest growth in the presence of domoic acid. A significant portion of the substrates presented, domoic acid or saxitoxin, (depending upon the isolate), disappeared after incubation at 20°C.

It was concluded that the blue mussel

virtually always possessed microflora which could utilize domoic acid, while the softshell clam was more variable. The sea scallop and red mussels only occasionally had such organisms. Domoic acid clearance from molluscan shellfish species, as judged from limited trials and anecdotal evidence appear to parallel these microbial findings.

To account for the different microbial capacities evident in the various molluscan species, it is necessary to postulate a selection mechanism; this might involve selection of bacterial types by molluscan lysozymes such as those described in the literature on *M. edulis*. As these bacteria have the potential to play a significant role in toxin elimination in certain molluscan species, it could be profitable to explore these leads. If confirmed as a significant

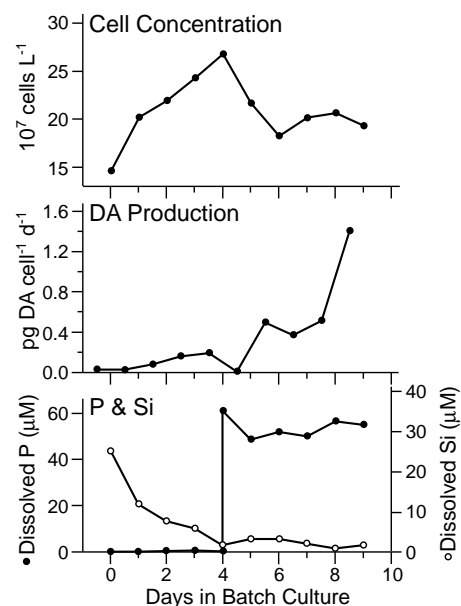


Figure 4 Variations in intracellular domoic acid and dissolved phosphorus in relation to growth rate in *P. multiseriis* culture. Note at low phosphorus levels the division rates were low which coincided with high levels of domoic acid.

toxin clearance mechanism in molluscs, practical applications of bacterial clearance could include detoxification procedures based upon favouring autochthonous domoic acid and saxitoxin utilizing bacteria and possibly implanting relevant bacteria (or transferring their capacities to autochthonous bacteria) in those molluscan species which appear to select against the toxin utilizing bacteria. This approach would be

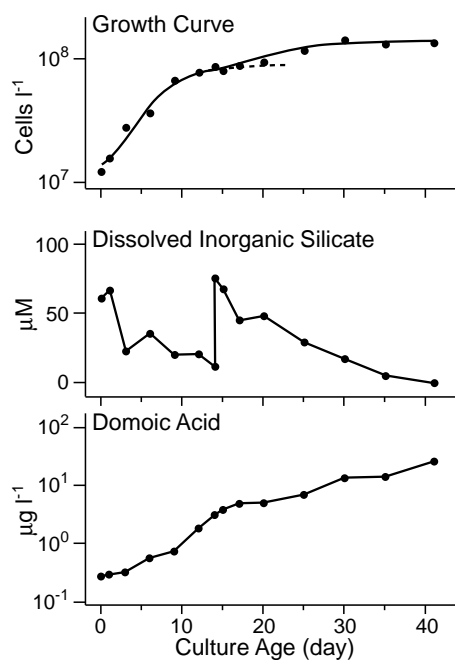


Figure 5 Variations in intracellular domoic acid and dissolved silica in relation to growth rate in *P. multiseriis* culture. Note at low silica levels the division rates were low which coincided with high levels of domoic acid.

in distinct contrast to current shellfish depuration methods which are aimed at eliminating bacteria from the shellfish.

Thus, we now have a much clearer picture of the physiology of the diatom, the probable and important role of domoic acid in the diatom's survival and dominance, many of the factors affecting domoic acid production including the role of excess nutrients and pollutants, the probability that much of the domoic acid is formed within the mussel and other affected shellfish and that bacteria possessed by certain shellfish species are capable of degrading domoic acid and at least one of the major PSP toxins.

We also found that *P. multiseriis* growth was stimulated by the presence of an amino acid through the intervention of the bacteria associated with the diatom, raising the possibility that the presence of organic wastes from land run-offs, sewage and other sources, including the aquaculture units themselves, can contribute to blooms of this as well as other diatoms and dinoflagellates. As nitrification and pollution are important elements the process could be qualitative as well as quantitative; i.e. the precise

algal species involved and its eventual abundance could be determined largely by materials contributed by man.

Unfortunately space does not permit similar coverage of the work being done at Bedford Institute of Oceanography (BIO) on other toxins. Extensive studies have been carried out with the dinoflagellate, *Alexandrium*, a producer of toxins of the saxitoxin family (PSP). The detailed results and methods developed provide insights comparable in certain ways to those provided by the studies with the diatom, *P. multiseriis*, and its production of domoic acid. In addition, attempts have been made to culture in the laboratory algae believed responsible for producing the toxins causing Diarrhetic Shellfish Poisoning; this is a feat that has not been accomplished anywhere to date.

General Remarks

A partial listing of the advances made within Atlantic Canada since the domoic acid crisis of 1987 is impressive. The major obstacles to analytical work with ASP and PSP have been overcome by the provision of reference material for the critical qualitative and quantitative analyses for ASP and PSP. Domoic acid, produced on Prince Edward Island, now is available commercially in quantity; saxitoxin, neosaxitoxin, and gonyautoxins II and III prepared as certified standards can be purchased. Analytical methods for domoic

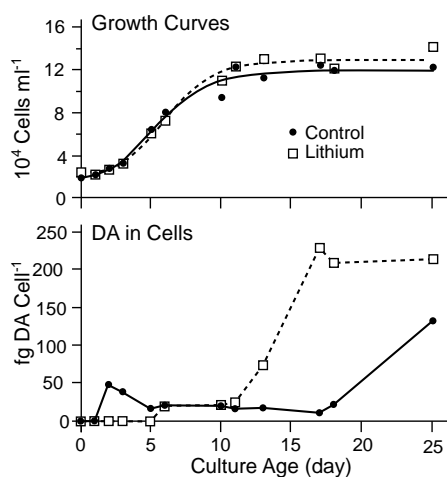


Figure 6 Effect of 385.6 mmol lithium enrichment on cell concentration and intracellular domoic acid in cultures of *P. multiseriis*.

acid, the saxitoxins and DSP toxins have been developed or improved; these include more convenient chemical, serological and bioassay techniques, improved growth techniques, biochemical approaches and surveys with area-wide analyses made of some of the data collected over the past seven years. These activities have provided insights which have made the Canadian Atlantic area a focal point for marine toxins work. With the methodology and techniques now available, the analytical instruments in place, the chemical reference materials and the benefits of an extensive and intensive learning experience, the opportunities for major advances in this field locally have never been better. This position, if capitalized upon, comes at a good time as prospects are very real for an escalation of molluscan shellfish production, especially for some sea scallop culture ventures.

If the Japanese experience (from zero to 250,000 tonnes/year in a 14-year period) with sea scallop culture is repeated here to any degree, it is quite possible that the yield from culture could form a substantial portion of the total regional scallop production within the foreseeable future. The sea scallop industry, which is already the biggest fisheries money earner in the region, is now grossing over \$200 million/year; this income is based on the utilization of the adductor muscle only, the only part of the scallop reliably free of PSP. The remainder of the soft parts (65%) are discarded in landfills. There are, however, large markets looking for reliable, continuing supplies of substantially greater portions of the now discarded scallop material, i.e. rims and roe if it could be provided toxin free. It is possible that knowledge gained through the toxin studies could provide the means to overcome the toxins obstacles.

To date, sea scallop culture areas have not been hit by PSP, and while this fortunate state of affairs continues small operators have been able to take advantage of it to break even by selling the whole scallop and thus making substantially more than they could by selling only the adductor muscle. The opportunity to safeguard the integrity and extend the profitability of a developing industry through the further development and application of technology by exploiting the overall advances made in the Atlantic area to date is good and very

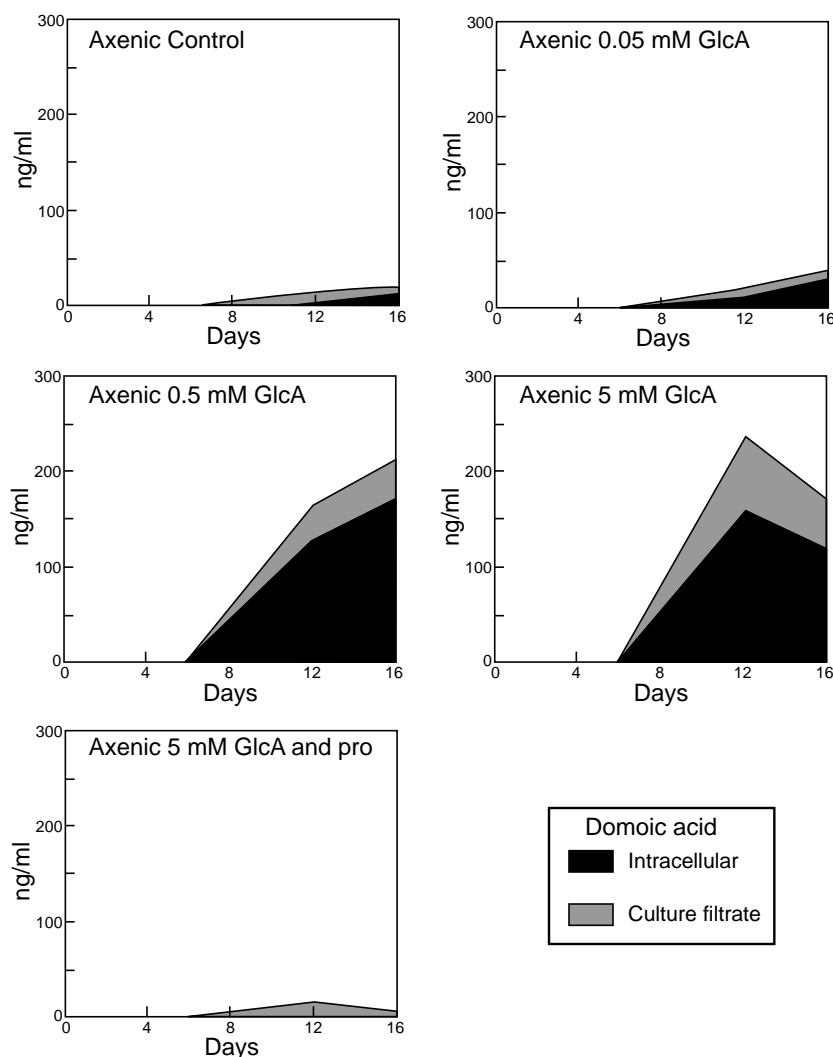


Figure 7 Concentration of domoic acid produced by axenic *P. multiseriis* cultured with various concentrations of gluconic acid/gluconolactone in the presence and absence of proline (5 mM).

definitely would be consistent with the departmental interest in Coastal Zone Management. The phycotoxin problems fit comfortably within this context as they are quite broad and actually encompass issues of habitat and environmental concerns as well as fisheries, aquaculture and recreational aspects.

Conclusions

Conclusions derived from these and other studies include the very real possibility that an important part of the problems stemming from toxins, in this case domoic acid, could be a direct consequence of man's own activities. By acquisition of the information outlined above and logical extensions of it we are in a better position to understand how and where the problems

are likely to arise. Steps can be taken then to avoid the worst consequences and to exploit the possibility that the bacterial populations of the shellfish can biodegrade the toxins and thus either eliminate the toxins before their concentrations in the shellfish become prohibitive or aid in clearance afterward. With these and similar kinds of data for other toxins, we will also have the basis for selecting different and more effective approaches to monitoring than we have employed in the past.

In closing, it is worth reiterating the essence of the statement made earlier in this essay. The basic understandings derived from the phycotoxins studies are permitting a partial realization of the aims for improved diagnostic procedures, prediction, protection and cures with promise, as

more work is done, to permit their full realization. The potential benefits for the fishing industry, and furthering our understanding of phytoplankton dynamics in the Coastal Zone are considerable.

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