Microbial utilization of the neurotoxin domoic acid: blue mussels (*Mytilus edulis*) and soft shell clams (*Mya arenaria*) as sources of the microorganisms

James E. Stewart, L.J. Marks, M.W. Gilgan, E. Pfeiffer, and B.M. Zwicker

Abstract: The neurotoxin domoic acid is produced in quantity by the diatom *Pseudo-nitzschia multiseries* and is released to the environment directly and indirectly via food chains. Presumably there is a mechanism for the biodegradation and disposal of domoic acid and as bacteria are logical candidates for such an activity, a search for bacteria competent to carry out biodegradation of domoic acid was initiated. Extensive trials with a wide variety of bacteria isolated mainly from muds and waters taken from the marine environment showed that the ability to grow on or degrade domoic acid was rare; in fact, domoic acid was inhibitory to resting cells or growing cultures of most of these bacteria. In contrast, using enrichment techniques, it was possible to isolate from molluscan species that eliminate domoic acid readily, i.e., blue mussels (*Mytilus edulis*) and soft-shell clams (*Mya arenaria*), bacteria that exhibited growth with and biodegradation of domoic acid when supplemented with low concentrations of growth factors. The species that retain domoic acid for lengthy periods, such as sea scallops (*Placopecten magellanicus)* and red mussels *(Modiolus modiolus)*, only occasionally yielded bacteria with this capability. The differences may be a result of the mechanisms used by the different shellfish in dealing with domoic acid, i.e., freely available in the blue mussels and soft shell clams but likely sequestered in the digestive glands of sea scallops and red mussels and thus, largely unavailable for bacterial utilization. The results show that *Mytilus edulis* and *Mya arenaria,* almost uniquely, are prime and reliable sources of domoic acid utilizing bacteria. These findings suggest a strong possibility that autochthonous bacteria may be significant factors in the elimination of the neurotoxin in these two species of shellfish.

Key words: bacteria, neurotoxin, domoic acid, elimination, bivalve molluscs.

Résumé : L'acide domoïque est une neurotoxine produite en quantité par la diatomée *Pseudo-nitzchia multiseries* et elle est directement ou indirectement libérée dans l'environnement par les biais des chaînes alimentaires. Comme il doit exister un mécanisme de biodégradation et d'élimination de l'acide domoïque et comme les bactéries sont des candidates logiques à ce type d'activité, nous avons cherché des bactéries qui auraient la compétence de dégrader l'acide domoïque. De nombreux essais avec une grande variété de bactéries isolées de boues ou d'eaux provenant d'environnements marins ont révélé que la capacité de croître en présence d'acide domoïque ou de dégrader cet acide était un phénomène rare. En fait l'acide domoïque inhibait les cellules au repos ou en culture de la plupart de ces espèces bactériennes. Par contre, en utilisant des techniques d'enrichissement, il a été possible d'isoler chez des mollusques qui éliminent facilement l'acide domoïque, c'est-à-dire, les moules bleues (*Mytilus edulis*) et les palourdes à coquille molle (*Mya arenaria*), des bactéries capables de croître en présence d'acide domoïque et de le biodégader lorsque le milieu de culture est enrichi de facteurs de croissance en faible concentration. Les espèces qui retenaient longtemps l'acide domoïque comme les pétoncles (*Placopecten magellanicus*) et les moules rouges (*Modiolus modiolus*) contenaient rarement ce type de bactérie. Ces différences pourraient être reliées aux différents mécanismes avec lesquels les mollusques vivent en présence d'acide domoïque, c'est-à-dire, accès facile chez les moules bleues et les palourdes à coquille molle mais séquestration possible dans les glandes digestives chez les pétoncles et les moules rouges, donc non-disponibilité particulière pour les bactéries. Les résultats ont confirmé que *Mytilus edulis* et *Mya arenaria* sont à peu près les seuls hôtes fiables de bactéries utilisant l'acide domoïque. Ces résultats laissent croire fortement que les bactéries autochtones pourraient être des facteurs significatifs dans l'élimination de cette neurotoxine chez ces deux espèces de mollusque.

Mots clés : bactéries, neurotoxine, acide domoïque, élimination, mollusques bivalves.
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Introduction

Many species of marine microalgae produce substances that are highly toxic; because the algae are widespread and periodically form large concentrations in the form of population blooms, the amounts of toxins produced are considerable (Hallegraeff 1995; Smayda 1990). These toxins constitute a significant environmental hazard as they can accumulate in molluscan shellfish, which rely on phytoplankton as food; when the algal toxin concentrations are high enough they can cause major adverse physiological effects and the death of molluscan shellfish (Gainey and Shumway 1988) and other species feeding directly upon the algae or in contact with the algae. At lesser concentrations the shellfish live (Shumway 1990), but the accumulated toxins are poisonous to those that consume the shellfish, including fish, marine mammals, and people (Taylor 1990). Although these toxins are widespread, enter the environment in large quantities, and cause considerable damage (Sundström et al. 1990; Hallegraeff 1995), there is no information on their fates and eventual dispositions. As this information is important to the overall problem of marine toxins as health problems and environmental hazards, an investigation was begun to acquire information on the fates of marine toxins. Domoic acid appeared to be an appropriate initial target because of its availability and its importance locally.

The neurotoxin domoic acid was the cause of the 1987 human intoxication episode that arose from consumption of cultivated blue mussels (*Mytilus edulis)* containing large concentrations of the toxin (Perl et al. 1990; Subba Rao et al. 1988; Bates et al. 1989; Bird et al. 1988; Wright et al. 1989; Todd 1990, 1993). Because of its impact on memory, among other ill effects, domoic acid intoxication was named amnesic shellfish poisoning (Perl et al. 1990). The mussels causing the initial amnesic shellfish poisoning episode had been grown in the estuary of the tidal Cardigan River, Prince Edward Island, Canada and possessed levels of domoic acid ranging up to 900 μg/g (almost 3 μmol/g) of soft tissue (Quilliam et al. 1989; Wright et al. 1989). Current Canadian regulations prohibit the sale of shellfish tissues containing 20 μg/g or more of domoic acid.

The toxin is produced by a diatom (Subba Rao et al. 1988; Bates et al. 1989), now called *Pseudo-nitzschia multiseries* (Hasle 1995), a bloom of which was serving at that time as food for the mussels (Subba Rao et al. 1988; Bates et al. 1989). Subsequently domoic acid, as reviewed by Villac et al. (1993), has been shown to occur widely in the marine environment.

Recent local examples indicate that substantial amounts of domoic acid are found routinely in the digestive glands but not in the adductor muscles of offshore sea scallops (*Placopecten magellanicus)* from Georges Bank or Browns Bank (10–200 μg/g) and frequently in Bay of Fundy sea scallops (Gilgan 1996). Offshore scallops are tested for domoic acid only in April and May as their digestive glands are usually toxic with paralytic shellfish poisons during the rest of the year and thus, only the adductor muscles are available for sale. This chronic level of domoic acid in the sea scallops can be enhanced markedly and episodically. In the April–May period of 1995, sea scallops on Georges Bank had concentrations of domoic acid in their digestive glands in excess of 1300 μg/g (>4 μmol/g) and up to 150 μg/g in the roe, while Browns Bank scallops had >2500 μg/g (8 μmol/g) in their digestive glands. The affected scallops were distributed relatively evenly over the banks (Burns 1996). The single highest individual value recorded for Browns Bank was 4300 μg/g (>13 μmol/g) of scallop digestive gland. The source of domoic acid in this 1995 episode was not discovered.

Thus domoic acid, a water soluble, chemically stable, and highly toxic substance produced in quantity in nature, is a good candidate for investigations on toxin biodegradation and disposal. As bacteria are logical candidates to mediate such an activity, a search for bacteria competent to carry out biodegradation of domoic acid was instituted.

Materials and methods

Medium for domoic acid enrichments

The basal medium for the domoic acid enrichments consisted of half strength artificial sea water (MacLeod 1968) plus 0.1% yeast extract (Difco, Detroit, Mich.) adjusted to pH 7.6 and steam sterilized. Domoic acid (Diagnostic Chemicals Ltd., Charlottetown, P.E.I.) in distilled water, sterilized by filtration, was added where required to give a final concentration of 0.5 mg/mL (1.6 μmol/mL).

Bacterial groups

Initially available (group A)

Bacteria used in the initial experiments on the degradation of or growth at the expense of domoic acid were taken from bacterial collections made earlier from the Bedford Basin (Halifax Harbour) and Digby Bay (Bay of Fundy) waters, lobster intestinal tracts, and a variety of other marine sources. In addition, a number of bacteria were isolated (using Bacto marine agar and broth No. 2216; Difco) from Cardigan River estuarine sediments collected the previous day and maintained at 5°C until the isolations were completed.

Seawater, sediments, and garden soil (group B)

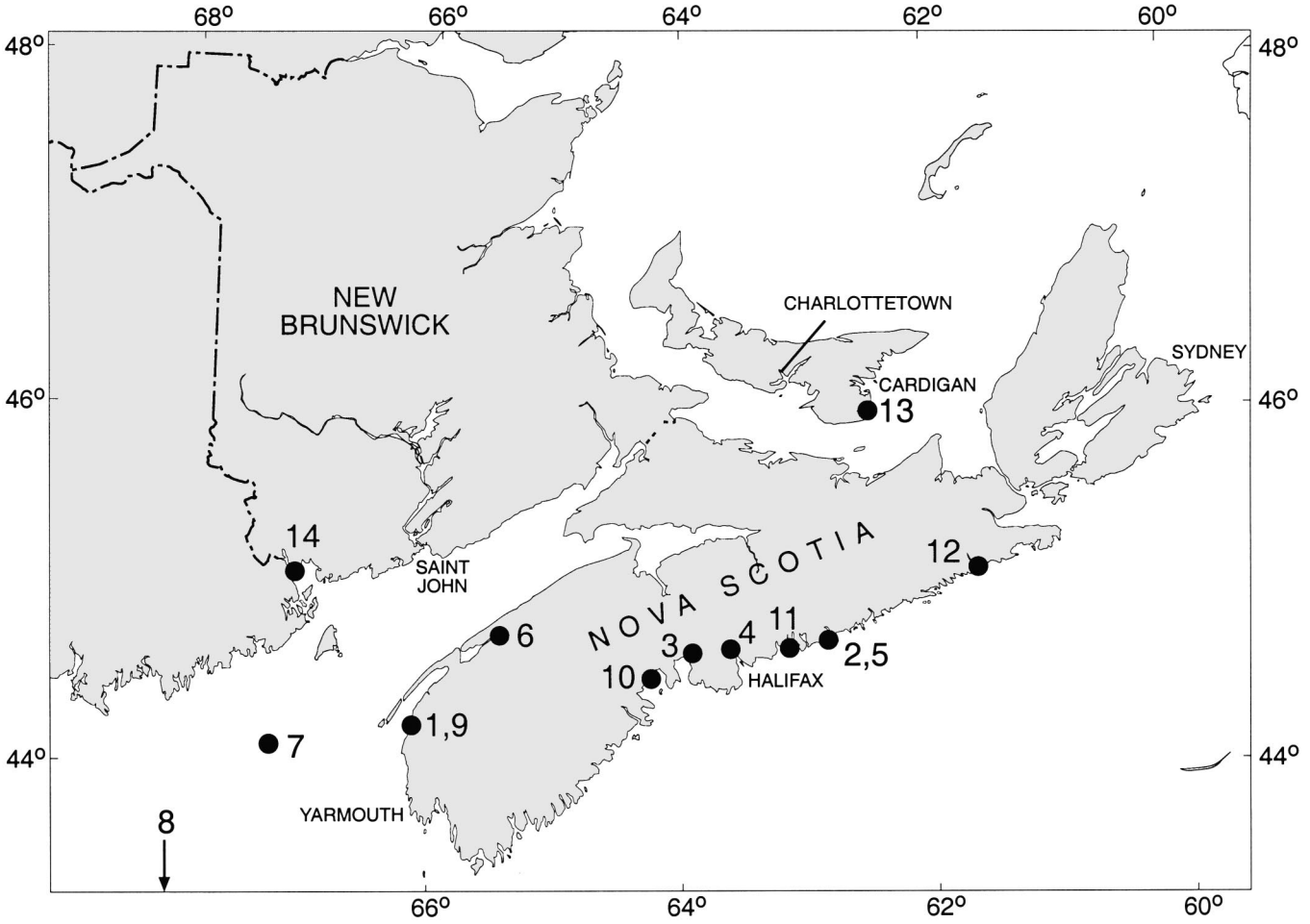
Domoic acid (0.5 mg/mL) enrichment procedures were applied to seawater taken from Bedford Basin, muds from the Cardigan River estuary, and local garden soil. These enrichments were also run with and without yeast extract in the basal medium as past experience had shown that some bacteria isolated from these environments would not grow without added growth factors.

From molluscs (group C)

As it was possible that bacteria could be involved in the clearance of domoic acid in molluscan shellfish, the search for bacteria competent to grow at the expense of domoic acid focused on several common molluscan shellfish species in which domoic acid had been observed. These were blue mussels, sea scallops, red mussels (*Modiolus modiolus*), and soft-shell clams (*Mya arenaria*). The widespread inshore and offshore locations from which the various samples were taken are shown in Fig. 1. Supernatant fluid from five homogenates of mussels (each made from 10 mussels) from southeast Nova Scotia were also used as source material.

Those bacteria found associated with molluscs and apparently capable of growing at the expense of domoic acid were isolated by aseptically removing small sections (approximately 0.5 g) of the internal food transport – gill system and the digestive tract – gland tissue from each of the various molluscs and homogenizing these in 5 mL of 3% sterile NaCl. Supernatant fluid (50 μL) from each homogenate was transferred to a single well of a 96-microwell plate (TC Microwell 96 FSI; Nunc, Roskilde, Denmark) containing 200 μL of basal medium supplemented with domoic acid and to a parallel well containing basal medium without domoic acid. This procedure was carried out in duplicate. The covered plates were then incubated at

Fig. 1. Map showing the locations of domoic acid occurrences and the origin of the samples listed in Table 1.



20°C for 24–48 h and increases in absorbance were read at 590 nm with a Thermomax microplate reader (Molecular Devices, Menlo Park, Calif.). Aliquots (10 µL) were transferred aseptically from each well of the first plate to corresponding new wells containing the same two media and incubated for an additional 48 h. This was repeated and the readings after 48 h of incubation of the third transfer were used to gauge bacterial growth stimulated by the presence of domoic acid.

Wells containing domoic acid that exhibited bacterial growth enhanced over that in parallel wells not supplemented with domoic acid were declared positive. Enhanced growth was defined as a difference ≥0.1 optical density (OD) units in both of the replicates containing domoic acid compared to the OD recorded for growth in wells in which domoic acid was absent. Most positive samples exhibited differences of between 0.1 and 0.6 OD units.

A loopful of fluid from each of the wells declared positive and each of the parallel wells not supplied with domoic acid was streaked on the Bacto marine agar and incubated at 20°C; all individual colonies with different appearances were picked into marine broth, restreaked to assure purity, and then maintained on marine agar. The approximately 260 bacterial isolates were identified to the genus level following the scheme of Austin (1982) as modified by Austin (1988), supplemented as required by Hansen and Sørheim (1991), Holt (1984), and Bauman et al. (1972) and using data acquired as described in Stewart et al. (1997).

Manometric procedures

Oxygen uptake in the presence of various substrates was measured at 28°C using a Warburg respirometer (Braun, Model UV85) and the procedures outlined by Umbreit et al. (1949). Whole cells grown at 20°C for 24–72 h, depending on the bacterium, in the yeast extract broth of MacLeod (1968) plus 0.5% glucose and 0.2% peptone on a platform shaker (100 rpm) (Psycrotherm; New Brunswick Scientific Co., Edison, N.J.) were used. The cells used in respiration studies were harvested by centrifugation (10 000 × *g* for 15 min at 5°C), washed twice with 0.1 M phosphate buffer (pH 7.7) containing 1.5% NaCl, and resuspended in the same buffer to give approximately 8 mg (dry weight) cells/mL.

Each Warburg vessel contained 1 mL of 0.1 M potassium phosphate buffer (pH 7.7) in 1.5% NaCl, 0.5 mL of resting cells, 1.3 mL of distilled water, 0.2 mL containing the substrate, and 0.2 mL of 15% KOH plus a filter paper strip in the centre well for the absorption of carbon dioxide. One vessel with the substrate replaced by 0.2 mL of distilled water was used to measure the endogenous respiration.

Domoic acid inhibition tests

The basal medium of MacLeod (1968) modified to contain 0.05% glucose, 0.05% yeast extract, and 1.5% agar (10 mL/100 mm diameter petri plate) was used to provide the growth test surfaces. The inoculum was grown in the same medium but without agar. An aliquot (0.05 mL) of a 24- to 48-h culture was spread evenly over the agar surface to form a bacterial lawn. Sterile Bacto concentration disks (0.25-in. diameter (1 in. = 25.4 mm); Difco) in which a range of domoic acid concentrations (or its analogue, kainic acid) had been absorbed were then pressed onto the agar surface. The plates were

incubated at 20°C and the full diameters of the clear zones of inhibition were recorded in millimetres.

Domoic acid

Domoic acid was determined quantitatively on (*i*) the clarified bacterial culture filtrates, (*ii*) the extracts of scallop digestive gland tissue individually, or (*iii*) pooled separate samples of each of the following scallop tissues: adductor muscles, gonads, or remaining tissue. The scallop tissue extracts were prepared by the standard paralytic shellfish poisons extraction procedure (Helrich 1990). The determinations were made on the culture filtrates or scallop tissue extracts (after they had been mixed 1:1 with methanol and passed through 0.22-μm-pore size cellulose acetate or nylon filters); those filtrates made from animal tissues were diluted further, i.e., 1:1 with distilled water. This was followed by high performance liquid chromatography (HPLC) using the procedure of Bird et al. (1988) as modified by Gilgan et al. (1990). The column effluent was monitored at 242 nm. Performance of the system was evaluated before and after each run using the certified reference standard, NRC/IMB DACS-IB, and two in-house reference materials that also allowed quantitation of the results.

Growth with domoic acid

Selected bacterial isolates were grown in MacLeod's (1968) yeast extract broth medium to which varying concentrations of domoic acid, sterilized by filtration, had been added. The inocula were standardized to 0.3 OD units at 590 nm; the inoculum (70 μL) was added to 1.36 mL of the yeast extract broth medium containing various additives at different concentrations. The inoculated medium was then incubated at 20°C for the periods specified. The cultures were clarified by centrifugation (10 000 × *g* for 15 min at 5°C) and the supernatant fluids were then mixed 1:1 with methanol and passed through 0.22-μm-pore-size membrane filters in preparation for HPLC determination of domoic acid.

***Pseudo-nitzschia multiseries* NPH extract**

Diatom cells grown axenically (20 mL) were harvested by centrifugation (1500 × *g* for 10 min), resuspended in 2 mL of distilled water, and then ruptured by treatment for 3 min with a Sonic Dismembrator 300 (Artek System Corp.) equipped with a titanium microtip and operated at 30% maximum power (ca. 100 W). The resulting mixture was heated at 100°C for 5 min, cooled, and then sterilized by filtration through a 0.22-μm-pore-size membrane. The sterile supernatant fluid was stored at –10°C.

Results

Initial manometric trials were conducted with 16 different bacterial isolates from bacterial group A (as designated in Materials and methods) in which the oxygen consumption of resting cells was measured in the presence of domoic acid, kainic acid, and various other more usual substrates such as glucose, glutamic acid, or proline. The results for two of these trials, representative of work with all 16 bacterial isolates, are presented in Fig. 2. Although the resting cells were metabolically active, as demonstrated by their ready oxidation of other substrates (Fig. 2), the results were uniformly negative with regard to the utilization of domoic acid or kainic acid despite several repetitions of each trial with a number of variations and regardless of the original sources of the bacteria. The 16 bacterial isolates used included *Escherichia coli* (for reference purposes), a *Pseudomonas perolens* strain isolated earlier from a lobster intestinal tract, *Moraxella* and *Alteromonas* strains isolated from close association with the diatom *Pseudo-nitzschia multiseries* (Stewart et al. 1997), and others from our marine collection or isolated from Cardigan estuary sediments (categorized as *Pseudomonas, Alteromonas*, and *Acinetobacter* strains). The data show that, in fact, domoic acid and kainic acid actually inhibit bacterial endogenous (basal) metabolism in resting cells over a broad range of concentrations.

As the results with the manometric trials exhibited domoic acid inhibition of the metabolism of resting cells, a series of exposures of bacteria to domoic acid and kainic acid during growth were undertaken with 14 of the bacterial cultures from bacterial group A. We employed the approach used in determining antibiotic sensitivities, i.e., bacterial lawns grown on the surface of agar plates with disks containing known concentrations of domoic acid or kainic acid pressed on the surface at the outset.

The results showed that in all cases bacterial growth was inhibited. The inhibition zones for all bacteria were virtually the same, i.e., approximately 9 mm for 5 μmol of domoic acid, 8 mm for 2.5 μmol, 7 mm for 0.625 μmol, and a trace or none for 0.25 μmol; the domoic acid inhibition of growth was concentration dependent. Varying the concentration of the glucose or yeast extract had no effect on the inhibition, but, as expected, it did affect the quality of the bacterial lawns. Without yeast extract the lawns were thinner. Kainic acid at 2.5 μmol/disk produced inhibition zones of about 7 mm in diameter.

These findings were confirmed by attempting to grow these same 14 bacterial isolates in microwell plates containing the liquid medium used in the domoic acid enrichment approach, i.e., with and without 0.5 mg domoic acid/mL of medium. Despite repeated trials, enhanced growth in the presence of domoic acid was not observed with these organisms.

A series of domoic acid enrichments performed subsequently using Bedford Basin seawater and Cardigan River estuarine muds (bacterial group B) did not yield bacteria that grew at the expense of domoic acid. Enrichments using local garden soil, however, did yield two isolates that were stimulated by the presence of domoic acid in the basal medium, i.e., minus yeast extract. This capacity to use domoic acid, however, did not persist after these isolates had been transferred several times on marine agar. It was concluded from all the foregoing results that the capacity to degrade domoic acid was rare and the search needed to be broadened.

Other workers had shown that domoic acid disappeared readily from mussels and clams (Scarratt et al. 1991); on the other hand, sea scallops were believed to retain toxins. The experiment illustrated in Fig. 3 confirms this for domoic acid clearance from scallops. Scallops taken from the Bay of Fundy on April 28, 1988 proved to have significant levels of domoic acid in the digestive glands. The domoic acid concentration varied widely from one scallop to another over a range of 60-165 μg/g of digestive gland tissue. Upon storage, unfed at ambient temperatures in flowing filtered seawater, the average level of domoic acid in the digestive glands fluctuated on an individual basis, but as the measurement made on the 568th day of storage showed, a substantial part of the initial domoic acid in the digestive glands was still retained by the scallops. As can be seen by the regression line drawn in Fig. 3, the domoic acid decline over the storage period was modest. Of the other tissues measured, no domoic acid was detected in the adductor muscle on days 0, 2, or 10. The gonads had 2.3–2.5 μg domoic acid/g on days 0 and 2, but domoic acid was not detectable in the 10-day gonadal sample.

Fig. 2. Manometric measurements with resting cells. (*a*) *Alteromonas* sp. from Bedford Basin. (*b*) *Pseudomonas* sp. from the Bay of Fundy.

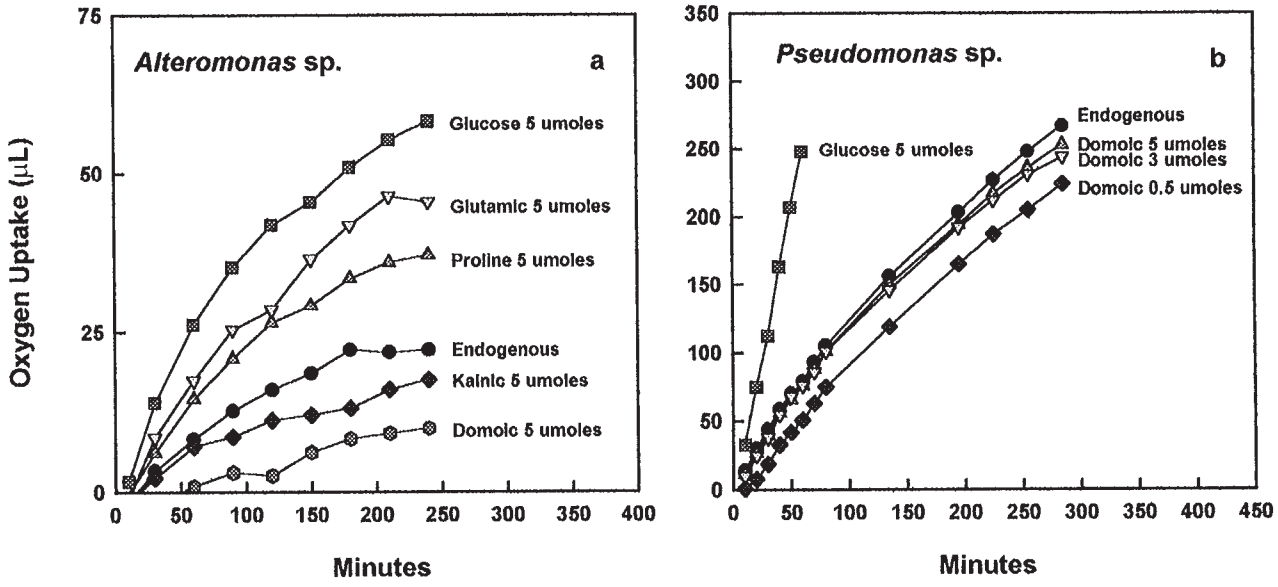
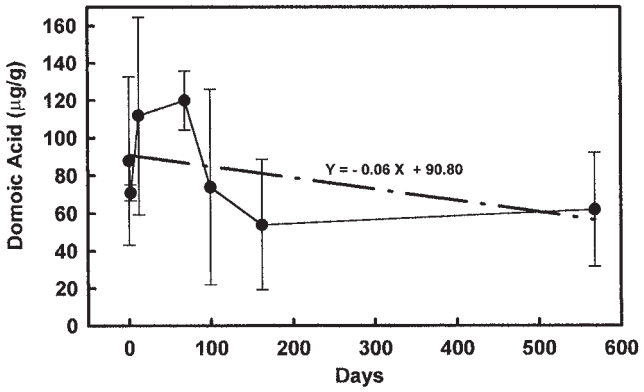


Fig. 3. Domoic acid levels ± SD in unfed sea scallops plus the regression line calculated using these values. Sixty scallops (10–12 cm diameter) collected April 28, 1988 from the Bay of Fundy were placed on the floor of a 90 × 90 cm square tank with aerated filtered flowing sea water (flow rate of 2 L/min) at ambient temperature. On day 0, eight scallops were taken to establish baseline values; all subsequent samples consisted of five scallops each selected at random. Temperatures ranged from ca. 1 to 15°C over the storage period.



The search for bacteria competent to utilize domoic acid focused on several common shellfish species in which domoic acid had been observed. These were blue mussels, sea scallops, red mussels, and soft shell clams. Results obtained in the molluscan shellfish (bacterial group C) survey are reported in Table 1 and show that bacteria whose growth was stimulated by domoic acid were routinely present in blue mussels and soft shell clams. The 260 bacterial isolates from the molluscan sources from various locations (Fig. 1) were identified to the genus level as shown in Table 2, illustrating the range and types of bacteria isolated from the various shellfish.

The growth of 60 of the isolates from bacterial group C was compared using microwell plates and the enrichment medium with domoic acid incorporated. In pure culture not all grew well at the expense of domoic acid and some did not grow at all. Apparently not all of the isolates from the enrichments had been growing at the direct expense of the domoic acid or they were unable to utilize domoic acid while growing in pure culture. The five bacterial isolates exhibiting the greatest growth enhancement with domoic acid in this trial were selected and used, along with *Moraxella* strain N-7 and *Alteromonas* strain N-9 (isolated from *Pseudo-nitzschia multiseries* cultures), to assess quantitatively their capacity to utilize domoic acid present at 40 and 80 nmol/mL. Depending upon the particular bacterium and trial, the domoic acid utilization ranged up to 57% of the substrate (Table 3); the additives did not affect the results greatly.

Discussion

As discussed in Stanier et al. (1963) and Koch (1985), microbial metabolic capacities for the utilization of different compounds are products of the long-term availability of these compounds and the direct association of the microorganisms with the specific substrates. This is used to advantage in enrichment cultures to select for microorganisms with specific and often unusual metabolic capacities. The niches in which otherwise refractory compounds are available come to be populated by microorganisms that have specific constitutive or adaptive capacities for metabolizing them. In effect, these niches in which unique compounds are metabolically available constitute natural enrichment cultures for such organisms. These principles are illustrated in the results obtained in this study.

The conclusion drawn from work with a wide variety of nonmolluscan bacterial isolates from seawater and marine muds from the location in which the toxic episode originated and garden soil is that domoic acid and, to the extent that it was tested, its near relative kainic acid are highly refractory. In fact, domoic acid exhibited the capacity to inhibit the endogenous metabolism of resting cells of many of the bacteria tested and generally inhibited growth even at low concentra-

Table 1. Results of the survey carried out in 1993 for the presence of bacteria whose growth was promoted by domoic acid.

Molluscan samples	Origin of samples*	Month	No. of molluscs yielding bacteria positive for growth with domoic acid/Total
Blue mussels (C) (5 homogenates)	Southwest N.S. (1)	Sept.	All
Blue mussels (C)	Ship Harbour, N.S. (2)	Sept.	10/11
Blue mussels (W)	St. Margaret's Bay, N.S. (3)	Oct.	10/10
Blue mussels (W)	Bedford Basin, N.S. (4)	Oct.	7/7
Blue mussels (W)	Ship Harbour, N.S. (5)	Oct.	8/8
Sea scallops (W)	Annapolis Basin, N.S. (6)	Oct.	2/10
Sea scallops (W)	Lurcher Shoal (7)	Oct.	1/10
Sea scallops (W)	Georges Bank (8)	Oct.	0/10
Red mussels (W)	Centreville, N.S. (9)	Nov.	2/10
Sea scallops (C)	Mahone Bay, N.S. (10)	Nov.	1/10
Soft-shell clams (W)	Jeddore, N.S. (11)	Nov.	1/10
Sea scallops (C)	Country Harbour, N.S. (12)	Nov.	0/10
Blue mussels (C)	St. Mary's Bay, P.E.I. (13)	Nov.	10/10
Soft-shell clams (W)	St. Andrew's, N.B. (14)	Dec.	8/10

Notes: The concentration of domoic acid was 1.6 μmol/mL of medium. C, cultivated; W, wild.
*Numbers in parentheses refer to those on Fig. 1.

Table 2. Identity of bacterial isolates from the molluscs.

Molluscs	Genera isolated from domoic acid enriched cultures	Genera isolated from parallel cultures without domoic acid
Blue mussels	*Alteromonas*, *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Photobacterium*	*Alteromonas*, *Micrococcus*, *Vibrio*, *Brevibacterium*, *Pseudomonas*, *Coryneform*, *Acinetobacter*, *Streptococcus*, *Aeromonas*
Sea scallops	*Alteromonas*, *Vibrio*, *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter*, *Photobacterium*, *Nocardia*	*Alteromonas*, *Coryneform*, *Vibrio*, *Micrococcus*, *Pseudomonas*, *Paracoccus*, *Aeromonas*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter*
Red mussels	*Alteromonas*, *Vibrio*, *Flavobacterium*, *Alcaligenes*	*Alteromonas*, *Pseudomonas*, *Vibrio*, *Coryneform*, *Flavobacterium*
Soft-shell clams	*Alteromonas*, *Vibrio*, *Acinetobacter*, *Aeromonas*, *Pseudomonas*, *Flavobacterium*	*Alteromonas*, *Vibrio*, *Acinetobacter*, *Aeromonas*, *Pseudomonas*

tions, as shown by exposure to domoic acid using bacterial lawns and disks on agar plates; the inhibition of growth was concentration dependent at levels consistent with, or far less than, the amounts recorded in nature.

In contrast, microorganisms whose growth was enhanced by the presence of domoic acid were found routinely in the two species of shellfish shown to eliminate domoic acid readily, the blue mussel and the soft shell clam, but less frequently or seldom in the two shellfish species that did not, i.e., the sea scallop and the red mussel. All four species are suspension type filter feeders and all four have been shown to accumulate and retain domoic acid varyingly.

The blue mussel has been shown to accumulate high levels of domoic acid (Quilliam et al. 1989; Wright et al. 1989) but also to eliminate this toxin readily in a matter of a few days or weeks depending upon environmental conditions and circumstances (Scarratt et al. 1991). Interestingly, Silvert and Subba Rao (1992), in developing a model of the clearance of domoic acid from blue mussels, concluded that at very high levels of domoic acid the depuration rate was depressed and the domoic acid was retained for lengthier periods. Novaczek et al. (1992) concluded that in the blue mussel, the bulk of the domoic acid probably resided in the gut lumen as it was cleared readily; prolonged retention of domoic acid in the mussel coincided only with massive concentrations of the neurotoxin. This occurs during intensive feeding upon the diatom *Pseudo-nitzschia multiseries,* as noted by Bates et al. (1989), resulting in mussels gorged with the diatom (Scarratt 1996). The studies of Osada and Stewart (1997) and Stewart et al. (1997) suggest that this massive buildup of domoic acid in the shellfish, coincident with gorging on the diatom, probably occurs in situ under such circumstances.

In contrast, the short-term studies of Wohlgeschaffen et al. (1992) and Douglas et al. (1997) showed that scallops accumulated domoic acid more slowly than mussels fed diets of the toxic diatom *Pseudo-nitzschia multiseries* but retained it with far higher efficiencies. Douglas et al. (1997) found that domoic acid accumulation was concentrated largely in the digestive gland when the shellfish were fed toxic *Pseudo-nitzschia multiseries*. The peak value of domoic acid in the digestive gland fell rapidly when the toxic diatom diet was replaced by a diet of toxin-free *Chaetoceros muelleri* and *Thalassiosira*

Table 3. Bacterial utilization of domoic acid.

	Initial domoic acid concentrations in culture medium					
	40 nmol/mL			80 nmol/mL		
Bacteria	Trial 1	Trial 2	+ 0.2 mM Na_2SiO	No additives	+ 0.2% glucose	+ 0.2% glucose + 1% *Pseudo-nitzschia multiseries* extract*
Moraxella strain N-7	14.4	10.4	12.4	0	5.6	11.2
Alteromonas strain N-9	19.6	4.4	6.8	0	7.2	8.0
Alteromonas strain 38	18.0	15.6	15.6	0	7.2	8.8
Alteromonas strain 64	8.4	15.6	14.0	6.4	6.4	16.0
Pseudomonas strain 104	11.2	2.8	6.8	2.4	2.4	6.4
Alteromonas strain 242	13.6	22.8	18.0	13.6	12.0	13.6
Alteromonas strain 257	8.0	22.8	17.6	17.6	12.8	18.4
Average	13.3	13.5	13.0	5.7	7.7	11.8

Notes: Amounts used are in nanomoles. Cultures were incubated for 14 days at 20°C. All cultures were run in duplicate; values given are the means of the duplicate cultures.

*from *Pseudo-nitzschia multiseries* NPH.

pseudonana. Douglas et al. (1997), however, emphasize that even after 14 days on a non-domoic acid diet, the domoic acid concentration in the digestive gland remained relatively high. Unfortunately the experiments of Wohlgeschaffen et al. (1992) and Douglas et al. (1997) with scallops were terminated before domoic acid was eliminated.

As already noted in the Introduction, sea scallops have a reputation for retaining toxins. This reputation is deserved (Fig. 3); the level of domoic acid in the digestive gland was only slightly lower at the end of the 19-month depuration period than it was at the beginning. Additional limited confirmation of this can be found in the 1996 reports from the Fish Inspection Services (Burns 1996), which report that sea scallops from the Georges Bank and Browns Bank fishing areas have domoic acid levels in their digestive glands that, while less than those of the previous year, were still impressively high (i.e., about 75% of what they had been in 1995).

It seems likely that the high levels of domoic acid measured in 1996 in the sea scallops from Georges Bank and Browns Bank were residues from the unusually high levels of the previous year, as massive domoic acid accumulations in scallops generally appear to be episodic and the depuration trial indicated a prolonged clearance time. The fisheries harvest is based primarily upon the young rapidly growing 4-, 5-, and 6-year classes where up to 60% of the available year classes are taken annually; recruitment through growth to replace those fished gives the misleading impression of an unchanging population (Black et al. 1993). The growth rate of the year classes predominantly fished (Black et al. 1993) is sufficient to account for the apparent approximate 25% decline in domoic acid concentration by dilution through growth of individual specimens following a single massive exposure to domoic acid in the spring of 1995.

In blue mussels and sea scallops, the presence or absence of bacteria whose growth was enhanced by the presence of domoic acid appeared to parallel the capacity of these shellfish to eliminate the toxin. That these bacteria were actually able to utilize domoic acid in relatively low concentrations is demonstrated in Table 3, where selected bacterial isolates were shown to cause the disappearance of up to 57% of the substrate. It is quite likely that in a more suitable medium with critical ingredients constantly replenished and with mixed rather than pure cultures, greater amounts of the domoic acid would be metabolized within a relatively short time.

The array of microorganisms displayed in Table 2 cannot, of course, be described as the native microflora of the various shellfish species, as it is the product of isolations made from media three transfers removed from the original shellfish. It is interesting, however, to compare the genera isolated from cultures containing domoic acid with those isolated from the cultures without it. It appears that fewer genera appear in the domoic acid enrichment cultures than in the parallel growth medium. Aside from this, it is clear that the internal populations of bacteria in the shellfish are varied and exhibit considerable commonality; no one molluscan species appears to have a unique or definitive microflora based upon bacterial identity alone. Metabolic capacity, judging from the differences in Table 1, however, appears to reflect a degree of uniqueness that suggests marked differences in the utilization of domoic acid in the different shellfish. The virtual absence in the sea scallop of bacterial capacities for domoic acid utilization coupled with its long-term retention suggest that the stored domoic acid is made generally unavailable in its digestive gland almost immediately following its entry into the digestive system.

The wide range of bacteria resident in the shellfish internal food transport system of the gills and the digestive system raise questions as to their possible roles. Various authors have suggested that gut and gill flora of invertebrates play a role in supplying the nutritional requirements of the host. Fong and Mann (1980) described the bacterial transfer of amino acids in the sea urchin, while Dando and Spiro (1993) proved the capability of autotrophic microorganisms living under anaerobic conditions on the gills of thyasirid bivalves, *Thyasira sarsi* and *Thyasira equalis*, to supply all of the nonmineral nutritional needs of these two shellfish species. As a demonstration of the basis for the involvement of bacteria in these transfers and transformations, McHenery et al. (1979) showed the presence of powerful lysozymes associated with the crystalline style and digestive glands of *Mytilus edulis, Volsella modiolus*, *Chlamys operculatus*, and *Tellina tenuis* and the gills and digestive

glands of *Mya arenaria* and that a wide variety of molluscan shellfish were capable of metabolizing bacteria (McHenery and Birkbeck 1985). They concluded that the primary role of the lysozyme-like enzymes was not host protection as previously suggested but rather was nutritional through the digestion of bacteria.

A collateral and incidental mutual advantage to such a nutritional strategy would be the acquisition and maintenance within the bivalves of bacterial populations with diverse metabolic capacities based upon long-term associations, i.e., enrichment. Among the attributes of the selected species and strains of bacteria in certain shellfish could be the capacity to biodegrade various unique algal products such as domoic acid. It would seem unlikely and illogical to assume that the relationships between the presence or absence of domoic acid utilizing bacteria and the respective positive and negative capacities for the clearance of domoic acid by the different shellfish are explained simply by coincidence.

In conclusion, we suggest that bacteria could play a significant role in domoic acid elimination in certain molluscan species, e.g., *Mytilus edulis* and possibly *Mya arenaria*, but apparently not in *Placopecten magellanicus*. Further studies are required to show precisely whether and how much of the toxin elimination from the shellfish is attributable to bacterial action. If the necessary proof is forthcoming, possible practical applications point to the opportunities for detoxifying certain shellfish by favouring relevant autochthonous microorganisms rather than by eliminating or inhibiting the resident bacteria as occurs in current shellfish depuration procedures.

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