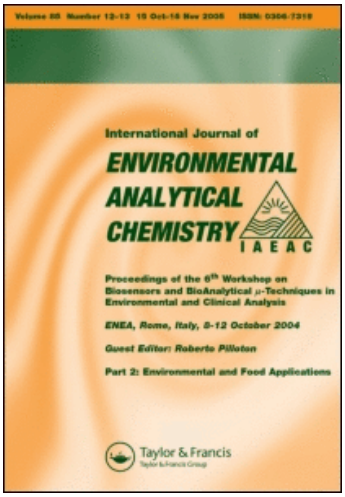

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Trace Determination of Domoic Acid in Sea Water and Phytoplankton by High-Performance Liquid Chromatography of the Fluorenylmethoxycarbonyl (FMOC) Derivative

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TRACE DETERMINATION OF DOMOIC ACID IN SEAWATER AND PHYTOPLANKTON BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE FLUORENYLMETHOXYCARBONYL (FMOC) DERIVATIVE*

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A method is presented for the trace determination of domoic acid, a neurotoxic amino acid responsible for cases of Amnesic Shellfish Poisoning resulting from the consumption of contaminated shellfish. The method involves pre-column derivatization with 9-fluorenylmethylchloroformate to form the FMOC derivative followed by reversed-phase HPLC with fluorescence detection. The detection limit for domoic acid in seawater and aqueous extracts is 15 pg/mL (50 pM) using gradient elution, a 20 μL injection volume, and a 2.1 mm I.D. microbore column. Use of dihydrokainic acid as an internal standard improved quantitation. The method was applied to the detection of domoic acid in seawater, in phytoplankton cultures (*Nitzschia pungens* forma *multiseries*), and in natural mixed phytoplankton assemblages in estuarine waters.

KEY WORDS: Domoic acid, neurotoxin, FMOC derivatization, reversed-phase HPLC, fluorescence detection, phytoplankton, *Nitzschia pungens*.

CA REGISTRY NUMBERS: Domoic acid 14277-97-5; Kainic acid 487-79-6; Dihydrokainic acid 52497-36-6; 9-Fluorenylmethylchloroformate 28920-43-6.

INTRODUCTION

Domoic acid (**1**, Figure 1) is a naturally-occurring secondary amino-acid that until

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recently was known to be present only in two species of red algae (*Chondria armata* and *Alsidium corallinum*) of the family Rhodomelaceae.[1,2] It is an excitatory amino-acid and neurotoxin, probably acting as an agonist to glutamate, a neurotransmitter in the central nervous system, in a manner analogous to the structurally related compound, kainic acid (**2**, Figure 1).[3] The potent neurological activities of **1** and **2** have attracted recent interest as neurobiological tools.[3–5]

During autumn 1987, domoic acid accumulated in cultivated blue mussels (*Mytilus edulis*) in a localized area of eastern Prince Edward Island (P. E. I.), Canada, in quantities sufficient to cause three fatalities and 153 cases of acute intoxication among persons who ate the shellfish.[6] The term Amnesic Shellfish Poisoning (ASP) has been proposed for this new toxic syndrome, as confusion, disorientation and memory loss are some of the characteristic symptoms observed in severely affected patients.[7] Analyses with HPLC using ultraviolet absorbance detection[8] showed that the toxin was concentrated in the mussels' engorged digestive glands, suggesting that phytoplankton or other particulate seston ingested by the mussels during their normal process of feeding was the source or at least the vector of the domoic acid.

To establish the role of phytoplankton in domoic acid production, it was necessary to have a method to measure domoic acid at trace levels in phytoplankton cultures, in plankton tows, and also in seawater itself, but no standard procedure existed. Because the ambient concentrations were much lower than in the toxic mussels, a method with both high selectivity and sensitivity was required. This was possible using fluorometric detection after a pre-column reaction to produce a fluorescent derivative separable by HPLC. The 9-fluorenylmethylchloroformate (FMOC-Cl) reagent (Figure 1) was chosen for investigation because of its rapid reaction with secondary amino acids at the pH of seawater,[9,10] the stability of its derivatives,[11,12] and the ease of automation.[13] These characteristics are not shared by some other widely-used amino acid reagents such as fluorescamine and *o*-phthalaldehyde.[14]

An early version of this FMOC-HPLC method was used recently to establish that the pennate diatom *Nitzschia pungens* forma *multiseries*, the dominant phytoplankter in the affected area of P.E.I. in late 1987, produces domoic acid in culture[15] and was the major source of toxin in the mussels.[16] The present paper reports on the optimization of the FMOC derivatization and HPLC of domoic acid, and on the application of the method to analyses of ambient seawater, phytoplankton cultures, and natural mixed phytoplankton assemblages in estuarine waters.

EXPERIMENTAL

Reagents and Materials

9-Fluorenylmethylchloroformate (FMOC-Cl) was purchased from Aldrich Chemical Company (Milwaukee, WI) and made up as a 15-mM solution in acetonitrile. The reagent was stored in 2-mL glass vials with teflon-lined caps in a desiccator at

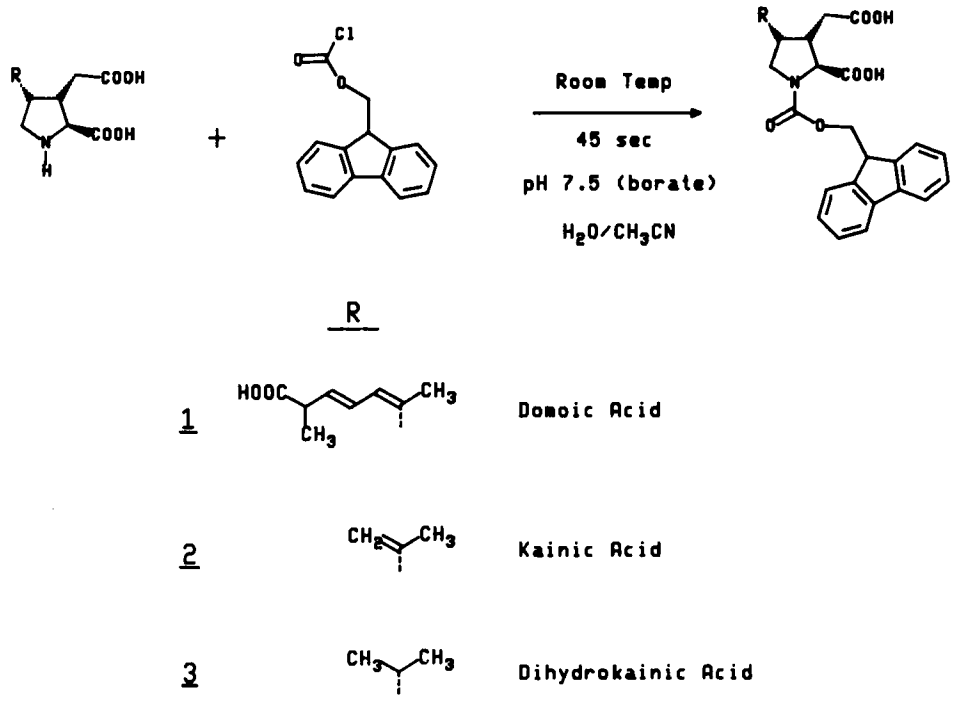

Figure 1 Schematic of the reaction of domoic (**1**), kainic (**2**) and dihydrokainic acids (**3**) with the FMOC-Cl reagent.

−20 °C. Aliquots were taken as needed and the excess was discarded. Borate buffer (1 M) was prepared from ortho-boric acid (Anachemia Canada Inc., Lachine, PQ) dissolved in deionized water and adjusted to pH 6.2 with 2 N sodium hydroxide.

Pure domoic acid (DA) was prepared from contaminated mussels as reported previously.[6] It can also be purchased from Diagnostic Chemicals (Charlottetown, P.E.I.) or from Sigma Chemical Company (St. Louis, MO). The primary domoic acid solution used for calibration of the HPLC when using the diode array detector (DAD) was 70 μg/mL domoic acid dissolved in acetonitrile/water (10:90 v/v). A calibration solution for the FMOC method (280 ng/mL; 0.90 μM domoic acid) was prepared by diluting the primary calibration solution into seawater (or into distilled water if the sample is in fresh water). An instrument calibration solution (DACS-1, 89 μg/mL) is now commercially available from the Marine Analytical Chemistry Standards Program of the National Research Council (Halifax, NS).

Dihydrokainic acid (DHKA), the internal standard, was purchased from Sigma. A 2.3-μg/mL solution (10.7 μM) was prepared by diluting a stock solution (113 μg/mL in acetonitrile/water, 10:90 v/v) by 50-fold into seawater.

Ethyl acetate used in the extraction was HPLC-grade (Fisher Scientific Ltd., Toronto, Ont.). Trifluoroacetic acid (99.5% pure) was purchased from BDH Inc. (Toronto). All solvents were HPLC-grade from Anachemia. Distilled water was

further purified to HPLC-grade by passage through a Millipore (Bedford, MA) Milli-Q water purification system equipped with ion-exchange and carbon filters. Seawater was filtered through a glass fiber filter (Type A/E, Gelman Sciences Inc., Ann Arbor, MI) prior to use.

Samples and Sampling

All samples of plankton and seawater were obtained from the Cardigan River (P.E.I.) estuary, the most severely affected site in 1987, and one that experienced a recurrence of the toxic diatom bloom in 1988.[16] Phytoplankton samples were collected by pumping a known volume of water (100 to 250 L) through a 28-μm net. The retained plankton were washed from the net with seawater, made up to 500 mL in a polycarbonate container, and transported to the laboratory on ice. A 500-mL sample of ambient surface seawater was collected at the same time. Sub-samples were taken for analyses after thorough mixing. Densities of *Nitzschia pungens* and other principal plankton species in these samples were assessed by counts of 5 to 10 replicate 20-μL aliquots.

Cultures of *N. pungens* forma *multiseries* were intitiated from a sample collected in March 1988[16] and maintained in f/2 medium[17] made with filtered seawater. Cultures were grown in triplicate at 10 °C under two irradiance levels (45 and 145 $\mu E \cdot m^{-2} \cdot s^{-1}$) provided by a bank of fluorescence tubes with a 10:14 hr light:dark cycle. The culture flasks were placed on a rotary shaker table (150 rpm agitation) and the position of the flasks was rotated daily. The cell density of the culture was obtained by a visual count in 2 to 4 replicate 5-μL aliquots.

Prior to derivatization, a homogeneous sub-sample (10 mL) of seawater, net-collected phytoplankton or whole culture (cells plus culture medium), was sonicated for 1 min at 100W using a 1-cm-diameter probe (Braun-sonic 1510) to disrupt the cells. Cell debris was then removed by filtration through a Millex-GS 0.22 μm disposable filter (Millipore Ltd.).

Derivatization

Sample (200 μL) and 1 M borate buffer (50 μL), plus DHKA internal standard solution (10 μL) were mixed in a glass test tube (10 × 75 mm) on a Vortex mixer for 10 sec. For seawater samples, the pH was approximately 7.5 at this point. The FMOC reagent (250 μL of 15 mM FMOC-Cl in acetonitrile) was added and the contents mixed. After exactly 45 seconds, the excess reagent was extracted into ethyl acetate (500 μL × 3) by rapid mixing. The organic layers were removed with a disposable glass pipette and discarded. The aqueous bottom layer was transferred to an autosampler vial for HPLC analysis. All reagents were delivered using Hamilton glass syringes, and the sample was delivered with an adjustable air-displacement pipette with disposable tips.

The control of contamination was important when working with samples having low domoic acid concentrations. All glassware, syringes and vials were rigorously washed (water/methanol/acetone). Disposable devices (pipette tips, syringe filters,

etc) were used whenever possible. Several control seawater samples (blanks), low level spikes and calibration solutions were run randomly within each set of samples for quality assurance.

HPLC Analysis

The chromatographic system consisted of a Hewlett-Packard HP1090M high-performance liquid chromatograph equipped with a DR5 solvent delivery system, variable volume (1 to 25 μL) injector and autosampler, heated column compartment, built-in HP1040 diode array detector (DAD), HP1046A fluorescence detector, and HP79994A data system. Settings for the fluorescence detector were: 264 nm excitation, 313 nm emission protected by a 280 nm cut-off filter, a photomultiplier gain of 10 to 17, and a xenon lamp pulse frequency of 55 Hz. For determinations with ultraviolet absorbance detection, the DAD was connected after the fluorescence detector and set to acquire at 242 nm with a bandwidth of 10 nm.[8]

Separations were performed on columns (25 cm × 4.6 mm I.D. or 2.1 mm I.D.) packed with either 5 μm Vydac 201TP (Separations Group, Hesperia, CA) or 5 μm LC-PAH (Supelco, Bellefonte, PA). The mobile phase was aqueous acetonitrile with 0.1% (v/v) trifluoroacetic acid (TFA) pumped at 1.0 mL/min for the wide-bore column and 0.2 mL/min for the narrow-bore column. Isocratic elution was performed with 40% acetonitrile and with the column at room temperature. Gradient elution was programmed linearly from 30% to 50% acetonitrile over 15 min, followed by an increase to 100% acetonitrile over 2 min which was maintained for 5 min before programming back to initial conditions over 2 min. Initial conditions were maintained for a further 12 min, resulting in a total cycle time of 36 min. The column temperature was 55 °C. Injection volumes of 10 and 25 μL were used with the wide-bore column for isocratic and gradient analyses, respectively. The corresponding injection volumes for the 2.1 mm I.D. column were 5 and 20 μL.

RESULTS AND DISCUSSION

Derivatization

The conditions used for the FMOC derivatization reaction with domoic acid (Figure 1) are very similar to those for other amino-acids.[9] Derivatization of amino acids with FMOC-Cl is usually conducted in basic media.[9-12] Since we were concerned primarily with the analysis of domoic acid dissolved in seawater, the pH of which is approximately 8, a slightly-acidic borate buffer (pH 6.2) could be used in preference to a phosphate buffer which, though of higher buffering capacity, causes peak-broadening in HPLC.[9] Experiments at different pH values showed that the pH of the buffered sample prior to addition of the FMOC-Cl reagent should be 7.5 ± 0.2 in order to achieve maximum yield of domoic acid derivative.

Acetonitrile was the most effective solvent for the FMOC-Cl reagent. Acetone, a recommended solvent in some published accounts,[12] was found to be incompatible with seawater solutions, causing clouding of the reaction mixture. A 15-mM solution of FMOC-Cl was used to ensure an excess of reagent for the derivatization of complex mixtures. Analyses showed complete reaction of domoic acid in concentrated standard solutions (tested up to 70 μg/mL) as well as phytoplankton extracts spiked with known levels of domoic acid (*vide infra*).

The reaction time was varied from 30 to 60 sec and as found in other studies,[12] the reaction was complete in 30 sec or less. An intermediate period of 45 sec was selected to give sufficient time for manipulation.

The FMOC derivative of domoic acid elutes from the HPLC column after 9-hydroxymethylfluorene (FMOC-OH), the hydrolysis product of FMOC-Cl. To avoid interference at trace levels, it is important to eliminate excess reagent soon after the derivatization is complete to prevent the formation of significant levels of the FMOC-OH side-product. Several solvents were evaluated for extraction of excess reagent. Ethyl acetate had the best combination of physical and chemical characteristics for this purpose. It has a greater extraction efficiency for FMOC-Cl and FMOC-OH than ethyl ether and is less volatile and potentially less explosive. One possible disadvantage of ethyl acetate is that it is slowly converted to acetic acid.[18] One batch of ethyl acetate that was quite acidic caused partial decomposition of FMOC-DA, as well as appreciable partitioning of FMOC-DA into the organic phase. Fresh solvent from small-volume bottles kept air tight must therefore be used. Another disadvantage of ethyl acetate is that several amino acids exhibit poor derivatization yields due to a higher organic solubility. An alternative to extraction is to quench excess reagent with a hydrophobic amine[13] which results in a derivative that elutes much later than amino acid derivatives. We have found that diethylamine is a suitable amine and that the reactions and analyses can be fully automated with an autosampler that can mix reagents. However, we have been able to achieve better detection limits for domoic acid with manual reaction using ethyl acetate extraction which results in the lowest levels of FMOC-OH and related side-products. In addition, gradient elution must be used with the quenching method to elute the side-product.

The stability of a derivative is an important consideration with automated analyses of large numbers of samples, many of which may remain at room temperature for several hours before analysis. The stability of FMOC-DA was tested by leaving 9 replicate derivatized calibration solutions (280 ng/mL) over 7 days, with 3 in the dark at 5 °C, 3 in the dark at room temperature and 3 on a window sill. Only slight differences were observed between the analytical results for the three groups and for a control group of 3 samples analyzed immediately. The relative standard deviation of 2.6% observed for the 12 samples is close to the overall method reproducibility. Derivatized solutions thus appear stable for up to one week in the light at room temperature, though normally they were analyzed within 24 hr or stored in a refrigerator.

Spectroscopy

Figure 2a presents the ultraviolet absorption spectra of domoic acid and its

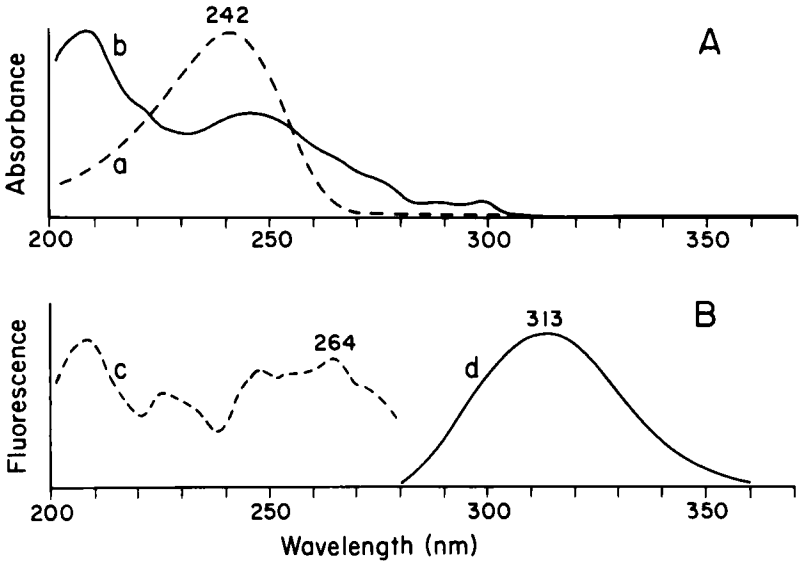

Figure 2 (A) Ultraviolet absorption spectra of: (a) domoic acid (DA); (b) its FMOC derivative (FMOC-DA). (B) Fluorescence spectra of FMOC-DA: (c) excitation scan; (d) emission scan.

FMOC derivative acquired with the diode array detector (DAD) during HPLC analyses. Domoic acid has a spectrum with a strong absorption band at 242 nm, while the spectrum of the derivative exhibits several additional bands due to the fluorenyl group. These spectra are useful for the confirmation of peak identity in concentrated solutions ($>0.5\,\mu$g/mL). The high molar absorptivity of domoic acid ($\varepsilon_{max}=26{,}100$ at pH 7)[8,19] allows for its detection at concentrations as low as 60 ng/mL by microbore HPLC with UV detection.[8]

The fluorescence excitation and emission spectra of the FMOC derivative are presented in Figure 2b. These spectra were acquired by a stopped-flow HPLC experiment conducted to determine optimum wavelengths for fluorescence detection. From this, 264 nm was selected as the excitation wavelength (λ_{ex}) and 313 nm was selected as the emission wavelength (λ_{em}).

Fast atom bombardment mass spectrometry (FAB-MS) of domoic acid[20] and its FMOC derivative in a glycerol-water matrix gave spectra with protonated molecular ions at m/z 312 and m/z 534, respectively. Such spectra are useful for confirmation of identity, but only for quantities greater than 1 ng. The new technique of ion spray mass spectrometry may be useful as it provides high sensitivity LC/MS detection of polar compounds such as domoic acid.[21]

HPLC Analysis

In the early stages of the study, HPLC analyses were conducted with isocratic elution, a conventional wide-bore (4.6 mm I.D.) column, and simultaneous detection by UV and fluorescence detectors. The UV detector was useful for establish-

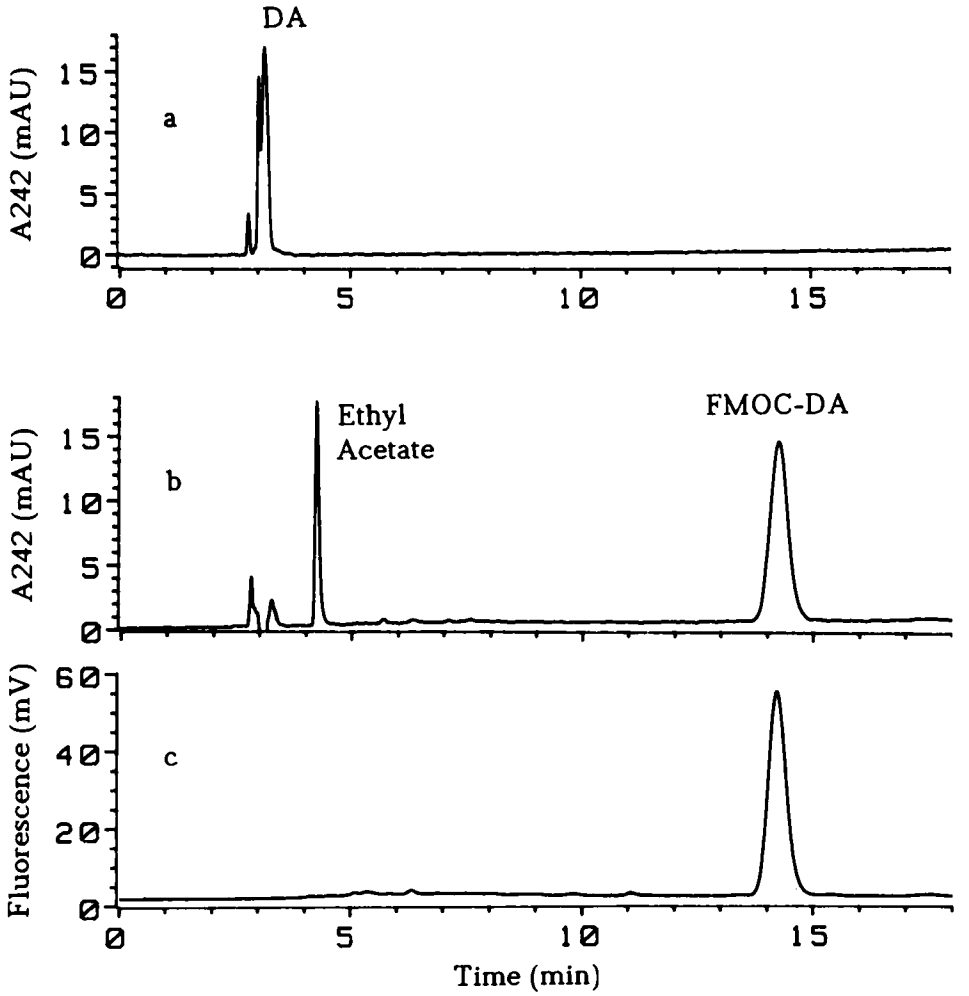

Figure 3 Isocratic reversed-phase HPLC of domoic acid in seawater at a relatively high concentration (8 μg/mL) before (a) and after (b, c) FMOC derivatization. Sequential detectors provided both the 242 nm UV absorbance (a, b) and fluorescence (λ_{ex} = 264 nm; λ_{em} = 313 nm) (c) chromatograms. Conditions: 25 cm × 4.6 mm I.D. column packed with 5 μm LC-PAH; column at room temperature; 1 mL/min aqueous 40% acetonitrile with 0.1% trifluoroacetic acid; 10 μL injection volume; fluorescence detector gain setting of 11.

ing that derivatization was complete for higher concentrations of analyte (Figure 3, 8 μg/mL). Underivatized domoic acid elutes very early in the chromatogram, whereas the derivative elutes much later. Figure 3 also illustrates the relative sensitivities of the UV and fluorescence detectors for the determination of domoic acid. The fluorescence detector had to be adjusted to a low gain setting to prevent saturation of the signal with the 8 μg/mL DA solution.

Investigation of various columns revealed that the retention time of FMOC-DA relative to that of the interfering FMOC-OH varies considerably between different manufacturers' stationary phases. The two compounds even co-elute on some columns. The best separations were observed with cross-linked stationary phases,

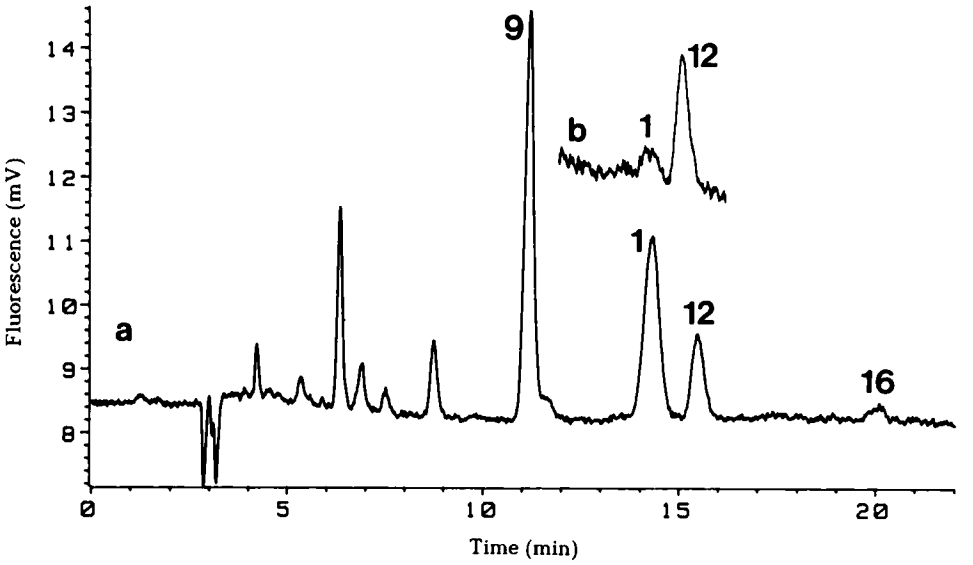

Figure 4 Isocratic HPLC analyses of FMOC derivatized samples of control seawater spiked with domoic acid (**1**) at (a) 8 ng/mL and (b) 0.8 ng/mL. Peaks **9** (FMOC-OH), **12** and **16** are side-products of the reaction. Conditions: same as in Figure 3, except that fluorescence detector gain settings were (a) 14 and (b) 15.

such as Vydac 201TP or Supelco LC-PAH, on which FMOC-DA elutes well after the FMOC-OH with an acidic mobile phase.

FMOC-DA is quite acidic due to its 3 carboxyl groups, the pKa values of which are 2.10, 3.72 and 4.97.[22] A mobile phase pH of 2 to 3 is required to effectively suppress ionization in order to prevent tailing due to adsorption on column active sites and to maximize the capacity factor (k') for increased separation from FMOC-OH. Trifluoroacetic acid (TFA) at 0.1% v/v concentration was found to be an excellent ion suppression agent and is convenient to use. Hexafluorobutyric acid was tried as an alternative to TFA, but to no obvious advantage. Attempts to decrease the k' of FMOC-DA to make it elute before FMOC-OH by using a pH greater than 3 resulted in severe peak tailing, especially at low concentrations.

The type and percentage of organic modifier in the mobile phase was examined. Acetonitrile gave better separations than methanol. The mobile phase finally selected for isocratic analyses was 40% acetonitrile in water plus 0.1% TFA. The k' for FMOC-DA was approximately 4 with the column at room temperature (Figure 3). The column temperature also affects the k' of FMOC-DA and its separation from the other components (*vide infra*).

The application of isocratic HPLC analysis to trace levels of DA in seawater is illustrated in Figure 4. At the 8 ng/mL level, the signals for FMOC-OH and two other side-products of the reagent (FMOC-X and FMOC-Y) are comparable to that of FMOC-DA. These peaks are well separated, however. The detection limit (signal/noise = 2) with the 4.6 mm I.D. column and a 10 μL injection volume was approximately 0.5 ng/mL DA in seawater (see inset in Figure 4).

Although analysis of DA by isocratic HPLC is clearly feasible, a better detection limit was required for environmental monitoring work. In addition, problems were encountered with plankton samples which gave late-eluting peaks that interfered with subsequent analyses. Gradient elution with microbore columns was therefore used to resolve these problems and also to profile other amino acids in the samples that elute together in an isocratic analysis. Gradient elution allows the injection of larger volumes of sample, provides better separation of complex mixtures, and flushes long eluting components from the column. Microbore columns provide a greater mass sensitivity with the concentration-dependent fluorescence detector. The disadvantages of this gradient method include longer analysis times due to the time required to equilibrate the column to initial conditions after a gradient run and the necessity to use smaller detector cell volumes because of the lower flow rates. With our present equipment, we were limited to 2.1 mm I.D. columns with a flow rate of 0.2 mL/min.

Figure 5 presents the optimized gradient, microbore HPLC analysis of an FMOC-derivatized sample of a culture of the diatom *N. pungens* grown in seawater-based growth medium (the application of this method to monitoring domoic acid in cultures is discussed further below). The peaks for DA and several amino acids are well resolved and sharp (Figure 5). Domoic acid elutes after proline in the sequence of natural amino acids (Table 1). None of the other amino acids frequently encountered in phytoplankton (Ser, Asp, Glu, Gly, Ala, Pro, Val)[23,24] have retention times close to that of domoic acid under these chromatographic conditions. Some additional peaks correspond to isomeric forms of DA previously observed in toxic mussel extracts.[8] A major advantage is the improved detection limit of 15 pg/mL (50 pM) DA in seawater, using a 20 μL injection volume and a higher gain setting on the detector (see inset in Figure 5). Only minor band-broadening was observed with a 20 μL injection in the gradient mode. An equivalent result could be obtained with a conventional 4.6 mm I.D. column using a 100 μL injection volume, but this would require a reaction scale-up if replicate analyses were required.

An internal standard was used for the analysis in Figure 5 in order to improve quantitation (*vide infra*). Kainic acid (**2**) was first considered as a possible internal standard but eluted too close to DA (Table 1); also, it is a natural product found in some marine organisms. Its saturated derivative, dihydrokainic acid (DHKA, **3**), was therefore chosen because it is a commercially available compound not known to exist in marine samples, it is an acidic secondary amino-acid like DA, and its derivative elutes after FMOC-DA.

Column temperature had a significant effect on the separation selectivity for the different components of the sample (Figure 6). As the temperature is increased, retention times of FMOC-derivatives decrease linearly, but not all at the same rate. From this data, a temperature of 55 °C was chosen for the optimal separation of DA and DHKA from the unknown reagent impurities (FMOC-X and FMOC-Y). This is not necessarily the best temperature for the separation and detection of other amino acids. For example, kainic acid (**2**) is not well resolved from DA under these conditions. Although it was not detected in any of the samples that we analyzed, it is possible that kainic acid may be present along with DA in some

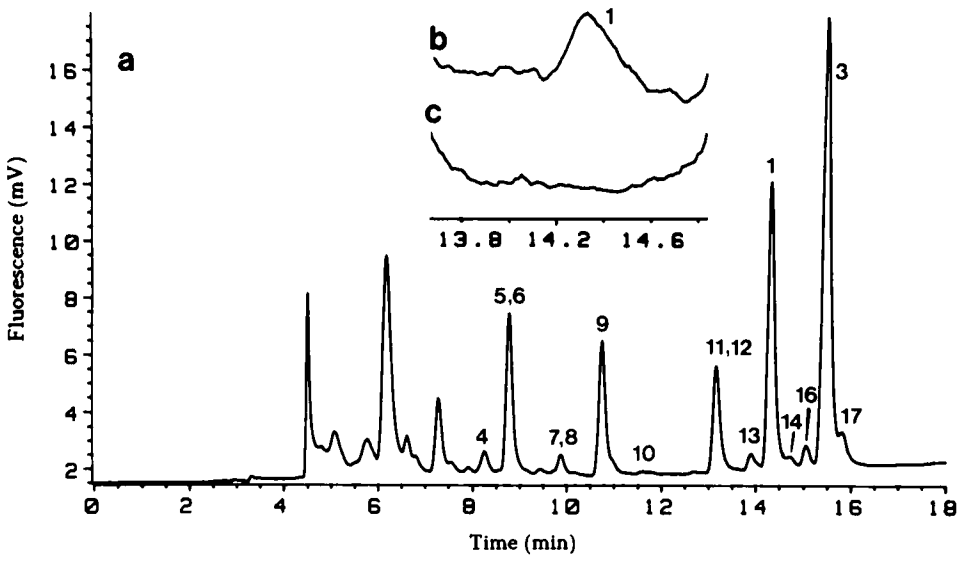

Figure 5 Gradient microbore HPLC analysis of FMOC-derivatized samples of: (a) *N. pungens* f. *multiseries* whole culture (cells plus medium) after sonication, filtration and addition of internal standard (**3**); (b) filtered control seawater spiked with 0.05 ng/mL domoic acid (**1**); and (c) filtered control seawater. See Table 1 for peak identities. Conditions: 25 cm × 2.1 mm I.D. column packed with 5 μm Vydac 201 TP; column at 55 °C; 0.2 mL/min aqueous acetonitrile with 0.1 % trifluoracetic acid (see Experimental for gradient conditions); injection volume: (a) 5 μL, (b, c) 20 μL; fluorescence detector gain settings: (a) 14, (b, c) 17.

Table 1 Gradient elution relative retention times (RRT) and relative molar responses (RMR) for FMOC derivatives of amino acids, relative to domoic acid (peak numbers used in Figures 4 and 5; conditions as in Figure 5).

Peak No.	*Compound*	*RRT*	*RMR*
4	Serine	0.58	0.31
5	Aspartic acid	0.60	0.29
6	Glutamic acid	0.61	0.26
7	Threonine	0.67	0.21
8	Glycine	0.69	0.15
9	FMOC-OH	0.76	–
10	Alanine	0.81	0.10
11	Proline	0.92	0.16
12	FMOC-X	0.92	–
13	Isomer of DA	0.97	–
1	Domoic acid (DA)	1.00	1.00
2	Kainic acid (KA)	1.01	0.98
14	Isomer of DA	1.03	–
15	Methionine	1.05	0.04
16	FMOC-Y	1.06	–
3	Dihydrokainic acid (DHKA)	1.09	0.99
17	DHKA impurity	1.11	–
18	Valine	1.11	0.014

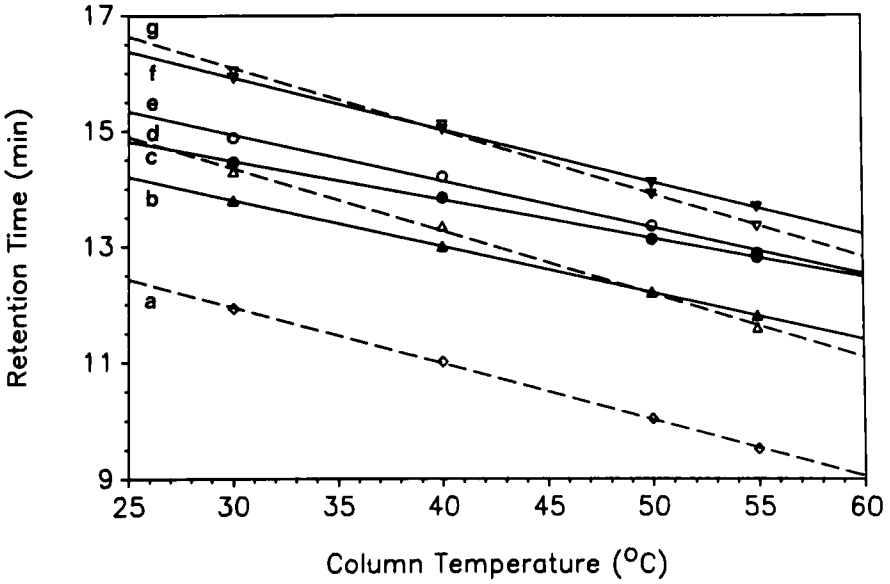

Figure 6 Effect of column temperature on the retention times of FMOC-derivatives in the gradient microbore HPLC analysis. Legend: (a) open diamond = FMOC-OH; (b) filled triangle = FMOC-Pro; (c) open triangle = FMOC-X (unknown side-product of reaction); (d) filled circle = FMOC-DA; (e) open circle = FMOC-KA; (f) filled inverted triangle = FMOC-DHKA; (g) open inverted triangle = FMOC-Y (second unknown side-product of reaction). Conditions: as in Figure 5, except that column temperature was varied.

marine samples. In such a situation, a different column temperature (e.g., 40 °C, see Figure 6) could be used to achieve good separation.

Quantitation

A calibration curve over the range 1 ng/mL to 1 μg/mL for the isocratic FMOC method showed sufficient linearity (correlation coefficient 0.99991) that a single-point external calibration was used initially. The reproducibility of peak areas for repeat injections of one derivatized calibration solution (280 μg/mL) made over one day was of the order of 1% relative standard deviation (RSD). Repeat derivatizations and analyses of calibration solutions by a single operator gave a reproducibility of approximately 2 to 3% RSD.

Figure 7 shows a plot of "normalized relative response" (the ratio of peak area to concentration normalized to the mean) versus concentration of DA in seawater for both isocratic FMOC and DAD methods. This presentation of the data demonstrates clearly that the response is linear to low concentrations and that there is no appreciable loss at trace levels in the derivatization or on the column. The calibration data for the two methods overlap in the 300 ng/mL to 1 μg/mL range. Thus, when concentrations are sufficient, both methods can be used to provide confirmation of identity and concentration. Note that the spread around the mean increases for each method as the limit of detection is approached (0.5 ng/mL FMOC; 60 ng/mL DAD). The FMOC method is preferable to the DAD

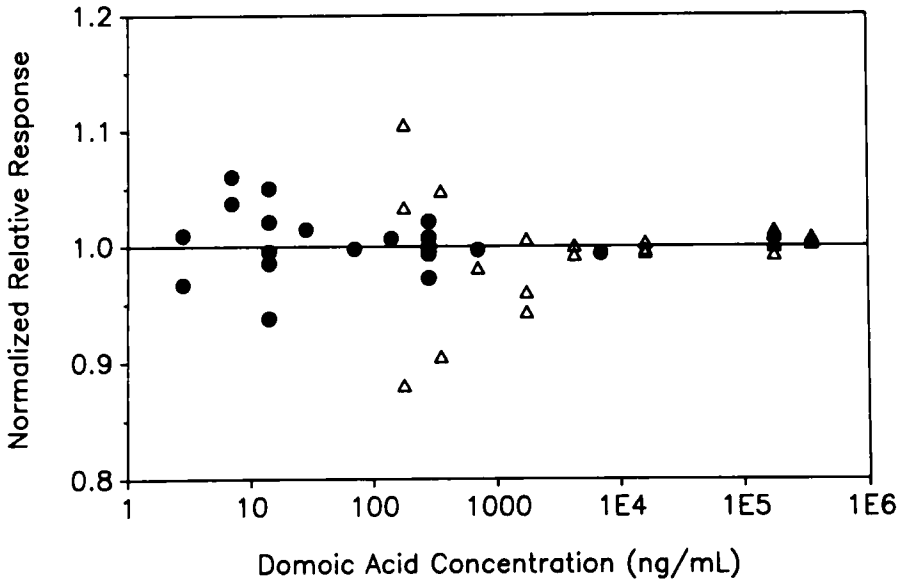

Figure 7 Comparison of the normalized relative response of domoic acid (ratio of peak area to concentration, normalized to the mean) for the FMOC/HPLC-fluorescence analysis (filled circles) with that for the HPLC-DAD analysis (open triangles).

method below a concentration of 1 μg/mL when a higher precision is required. Application of the FMOC method to concentrations greater than 1 μg/mL requires a decrease in detector gain and/or injection volume, or accurate dilutions of the sample. Concentrations lower than 1 ng/mL require the use of the microbore gradient method discussed above (15 pg/mL detection limit).

Although the precision of determination with the external calibration method was quite good with one operator, the precision was not nearly as good between operators, probably due to variations in the ethyl acetate extraction step. An even more serious problem can arise if there is drift in detector sensitivity between standard and sample analyses. Indeed, this was a problem with one fluorescence detector, which gradually decreased in sensitivity over a period of a few hours, thought to be due to a build-up of UV-absorbing ozone in the detector housing. In addition, it was important to have a method that would verify that the derivatization reaction was completely successful. An internal standard method was therefore investigated. As discussed earlier, dihydrokainic acid was found to be suitable for this purpose. In several instances, the internal standard has proved very useful in detecting faulty derivatizations due to matrix interferences or volumetric errors.

The overall reproducibility of the gradient elution method using internal standardization was excellent. For example, determinations on 6 replicate subsamples of a sonicated *N. pungens* culture with approximately 40 ng/mL DA gave an RSD of 2.5%. Replicate measurements over the 500 to 10 ng/mL range generally gave 2 to 4% RSD. Trace level determinations at the 1 ng/mL level gave RSD's on the order of 10%. It should also be noted that although retention times were very

Table 2 Intra- and inter-laboratory precision of analysis of natural phytoplankton samples taken from the Cardigan River.

	Experiment A[a]				*Experiment B*[b]		
Sample	*(DA) ng/mL*	*n*	*SD*	*% RSD*	*(DA) ng/mL*	*n*	*Overall % RSD*
Seawater[c]	3.6	3	0.4	11	3.5	1	9
100 L[d]	174.5	3	1.2	1	134.9	1	12
250 L[d]	168.2	3	4.3	3	175.7	1	3

[a]Subsampled, sonicated and filtered in one lab; derivatized and analyzed by two operators in one lab and by a third operator in a second lab.
[b]Subsampled, sonicated, filtered, derivatized and analyzed in the second lab by one operator.
[c]Ambient surface water sample with natural concentration of *N. pungens*.
[d]Plankton tow samples: plankton recovered from 100 L and 250 L of seawater and transferred to 0.5 L seawater.
(DA) = concentration of domoic acid in sample.
SD = standard deviation.
% RSD = percent relative standard deviation.

reproducible with the gradient system, the RSD usually being less than 0.4%, the internal standard served as a reference peak for the data system to recognize any unusual retention time variations.

Since this analytical method will likely be implemented in several regulatory laboratories, the interlaboratory reproducibility of the method was of particular concern. Table 2 shows some typical results for the analysis of field samples taken from Cardigan River, P.E.I., in the fall of 1988 during a recurrence of the *N. pungens* bloom (*vide infra*). Analyses by three different operators in two laboratories (with identical instrumentation) of three aliquots from a filtered subsample of surface estuarine water gave a concentration of 3.6 ± 0.4 ng/mL. Analysis of a second subsample by the third operator gave a value of 3.5 ng/mL. This represents an overall interlaboratory precision of 9% RSD at the 4 ng/mL level. Two phytoplankton concentrates, produced by filtrations of 100 and 250 L of estuarine water through a 28-μm net and washing the cells into 0.5 L, were analyzed in a similar manner. The only significant difference noticed, that between Experiments A and B for the 100 L net sample, is consistent with the heterogeneity of such natural samples.

Three experiments were conducted to assess the accuracy of the analytical procedures, *viz.*: spike recovery, standard addition, and comparison with a second method. Experiments in which known amounts of domoic acid were spiked into control seawater and control diatom (*Nitzschia seriata*) culture samples showed recoveries consistently better than 90% at levels as low as 1 ng/mL. The standard addition experiment was conducted by spiking known amounts of DA into aliquots of an *N. pungens* culture sample, adjusting each to equal volume with seawater, and analyzing. A plot of the ratio of peak areas of DA and internal standard versus the concentration of the spike showed good linearity of response (correlation coefficient = 0.9993). More importantly, the x-intercept established that the actual concentration in the unspiked sample was 46 ng/mL. Comparing this with an internal standard method result of 45 ng/mL showed that the accuracy of the method was acceptable. Finally, 15 samples were analyzed by both the

FMOC/HPLC-fluorescence and HPLC-DAD methods. For some samples, either a dilution or concentration step was required to get the concentration in the appropriate range. The results showed a good correlation between the two methods (slope = 0.982, correlation coefficient = 0.9986). An important observation from this experiment was that calibration of the FMOC method should be performed with a solution of DA in a medium as similar as possible to that of the sample because there are slight differences in the final yields of FMOC-DA and FMOC-DHKA after ethyl acetate extraction for seawater versus distilled water.

Applications

The application of the FMOC method to free domoic acid dissolved in seawater required only filtration for sample preparation (to eliminate particulate material which could block the column). However, an examination of sampling and extraction requirements was in order for samples containing phytoplankton suspended in seawater. Two questions were addressed: (a) what is the best method for separating cells and seawater for determination of extra- and intra-cellular levels of DA; and (b) what is the best method of extracting intracellular DA into aqueous solution for FMOC derivatization?

Three methods were considered for separation of cells from seawater: (a) filtration using glass fiber filters; (b) filtration using polycarbonate (3-μm pore size Nuclepore) filters; and (c) centrifugation. The glass fiber filters presented two problems. The first was erratic results for trace levels of DA in the filtrate. Adsorption on the filter was suspected and demonstrated by one experiment in which a seawater sample was passed through a series of filters and analyzed for DA at each stage. It was observed that the concentration of DA diminished at each stage. The second problem was associated with the extraction of DA from cells which could not be easily resuspended in seawater. Sonication of the glass fiber filter plus cells in water followed by centrifugation to isolate the aqueous extract consistently gave lower concentration values for intracellular DA than did the other methods. Centrifugation or Nuclepore filtration allowed the resuspension of cells in seawater, and both gave comparable results for intra- and extra-cellular concentrations of DA. In addition, a good mass balance was achieved for both of these methods in a comparison of the level determined for a total sample versus the levels in the separated phases.

The extraction of DA from cells suspended in seawater was best achieved by a brief (1 min) sonication to disrupt the cells. Cell debris was then removed by filtration through a 0.22-μm disposable filter. Boiling the sample released domoic acid from the cells, but irreproducible quantitative results occurred with some samples, especially macroalgae (Rhodophyta)[8] where the domoic acid appears to be lost by binding to other materials (probably carbohydrates in the well-known "Maillard" reaction).

Figure 8 illustrates the successful application of these analytical procedures to monitoring the production of DA by cultures of *N. pungens*. The curves for cell density and DA concentration in the whole culture grown at two irradiance levels showed that negligible amounts of DA were produced during the log phase of

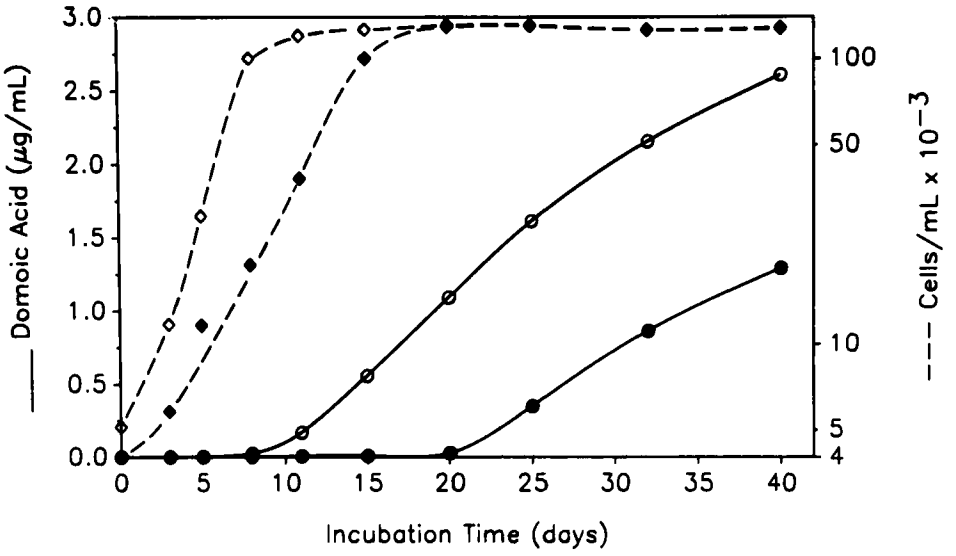

Figure 8 Domoic acid production (circles) and cell densities (diamonds) in *N. pungens* f. *multiseries* cultures maintained at two irradiance levels: 145 (open symbols) and 45 (filled symbols) $\mu E \cdot m^{-2} \cdot s^{-1}$.

growth. Production only during the stationary phase indicated clearly that domoic acid is a secondary metabolite. The overall production level of DA in the whole culture amounts to 20 to 30 pg/cell after 40 days. Analyses of the separated phases using the Nuclepore filtration method showed that intracellular levels of DA rose to a plateau of approximately 5 pg/cell and then declined slightly after 15 days; dissolved DA continued to increase in the medium (manuscript in preparation). The smooth curves in Figure 8 for the increase in DA concentration attest to the effectiveness of the analytical method.

An excellent example of the application of the FMOC method is an investigation of a phytoplankton bloom dominated by *N. pungens* that occurred in late 1988 in the Cardigan River, the principal site of the November 1987 mussel contamination incident. Figure 9 shows the results of measurements of *N. pungens* and domoic acid concentration in ambient estuarine water samples at a single site during November and December. The increase in cell numbers of *N. pungens* was accompanied by an increase in DA concentration. Conversely, the decline in the bloom was followed by a decrease in DA. These two measurements provided a timely warning of a potential mussel toxicity problem (which did occur 5–10 days later), allowing mussel-growers to make commercial decisions.

The FMOC method has been applied to a variety of phytoplankton species and macroalgae.[16] The only taxa in which we have detected DA are *N. pungens* f. *multiseries* and *Chondria baileyana*. Even *Nitzschia seriata*, a species very closely related to the former, produced no detectable levels of DA when grown in culture.[16]

Application of the method to shellfish tissue samples has been achieved, but requires a clean-up of the extract before derivatization (manuscript in preparation).

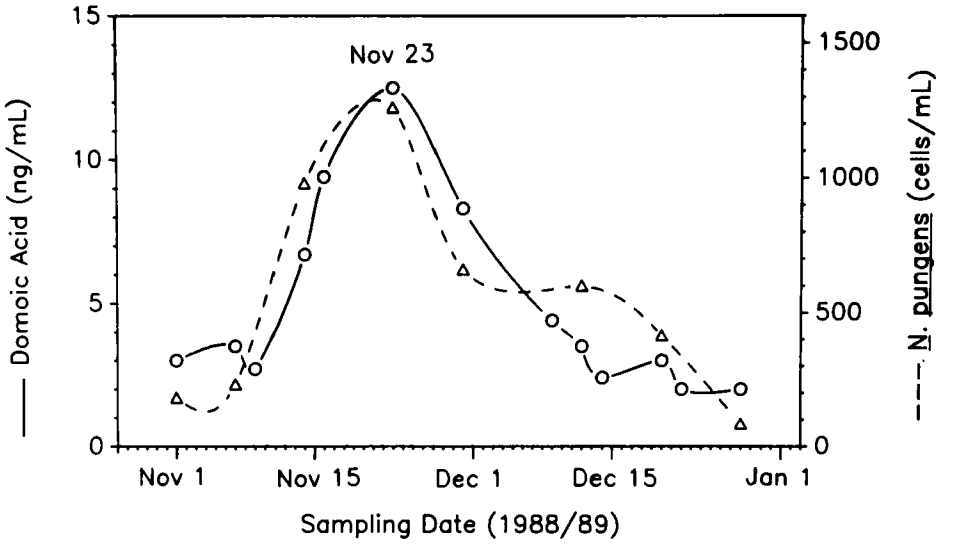

Figure 9 Domoic acid (circles) and cells of *N. pungens* f. *multiseries* (triangles) in surface water samples from the Cardigan River (P.E.I.) estuary (autumn 1988).

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