ELSEVIER

Deep-Sea Research II 52 (2005) 2501–2521

DEEP-SEA RESEARCH
PART II

www.elsevier.com/locate/dsr2

Toxin variability in natural populations of *Alexandrium fundyense* in Casco Bay, Maine—evidence of nitrogen limitation

N.J. Poulton[a,*], B.A. Keafer[b], D.M. Anderson[b]

[a]*Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, 180 McKown Point Road, P.O. Box 475, ME 04575, USA*
[b]*Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02453, USA*

Accepted 28 June 2005
Available online 10 October 2005

Abstract

The dinoflagellate *Alexandrium fundyense* is a common, recurring harmful algal bloom (HAB) species in the Gulf of Maine. To date, most physiological measurements of phytoplankton in the field provide data on the entire community, yet efforts to obtain species-specific data are particularly important for understanding the ecological and physiological dynamics of HAB species, such as, *Alexandrium. Alexandrium* spp., do not usually dominate the planktonic community in the Gulf of Maine, but are of great interest due to the potent toxins produced. In order to determine the nutritional status of *Alexandrium* spp. in natural populations, indicators of nutrient deprivation need to be identified that are specific to that one species. To date, the saxitoxin content of *A. fundyense* is known to vary under different environmental conditions such as nitrogen and phosphorous limitation. However, in batch culture the composition of the toxin (the relative amounts of each saxitoxin derivative per cell) appears to be a stable quantity and thus is sometimes viewed as a biochemical marker of individual strains. In more recent studies, toxin composition has been shown to vary during progressive N- and P-limitation, once the cells are given time to achieve steady state in semi-continuous, nutrient-limited cultures. Using both the absolute toxin concentrations and relative proportion (mole % total toxin) of each toxin derivative, N- and P-limitation can be distinguished based on the observed trends in the different saxitoxin derivatives. In this study, we examine the toxin content and composition in natural *A. fundyense* populations during a spring bloom in Casco Bay, ME from April–June of 1998. This allows us to examine whether *A. fundyense* populations in the western Gulf of Maine are sufficiently homogenous to permit the detection of toxin composition and toxin content differences through time and space, and if so, to determine whether those changes are indicative of a particular nutritional state (e.g., N-limitation). Using both toxin composition and toxin ratios determined from field samples during an *A. fundyense* bloom, the ratios generally correlated with N-limitation in the Casco Bay region.

Keywords: *Alexandrium*; Environmental factors; Saxitoxin; Gulf of Maine; Paralytic shellfish poisoning; Nitrogen limitation

1. Introduction

In phytoplankton ecology the ability to measure a single species' physiological responses to changes in environmental conditions is challenging. The development and succession of phytoplankton species in

*Corresponding author. Tel.: +1 207 633 9600; fax: +1 207 633 9641.
E-mail address: npoulton@alum.mit.edu (N.J. Poulton).

0967-0645/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dsr2.2005.06.029

the marine coastal environment can depend on the nitrogen and phosphorous (N and P) requirements of individual organisms (Rhee, 1978). The ability to determine and monitor the nutritional status of a single species is difficult due to the heterogeneity of the plankton population. Currently, many measurements of phytoplankton in the field provide data on the entire community (i.e. chlorophyll, CHN, productivity), yet efforts to obtain species-specific data are particularly important for harmful algal bloom (HAB) species such as the toxic dinoflagellate *Alexandrium fundyense*. Dinoflagellates, such as *Alexandrium* spp., do not usually dominate the planktonic community in the Gulf of Maine, but are of great interest due to the potent toxins they produce. In order to determine the nutritional status and the conditions under which *Alexandrium* spp. grow in natural populations, diagnostic indicators or responses to nutrients need to be identified that are specific to that one species or species complex.

A unique feature of many *Alexandrium* spp. that can be exploited is that they are toxic, and that toxicity varies with different nutritional conditions (Anderson et al., 1990a, b; Cembella, 1998). Many *Alexandrium* spp. produce saxitoxin (STX), a neurotoxin that causes paralytic shellfish poisoning (PSP) in many regions of the world (Hallegraeff, 1993). More than 21 known STX derivatives have been identified (Cembella, 1998). Different species or strains of *Alexandrium* can produce different quantities and combinations of these toxins. Toxicity of *Alexandrium* spp. can be quantified in terms of: (1) toxin content (the overall toxicity of the cell calculated as the sum of the molar content of each derivative, expressed as pmol cell^{-1}, or as the integrated potency of all STX derivatives present, expressed as STX equivalents cell^{-1}); or (2) toxin composition (the number and relative proportions of the STX derivatives in the cell (Hall, 1982; Cembella et al., 1987; Anderson, 1990).

Numerous studies have examined changes in toxin content and composition using batch cultures. The effects of varying a single environmental parameter such as irradiance, temperature, salinity or inorganic nutrients (nitrogen and phosphorous) have been shown to affect toxin content for several different *Alexandrium* strains (Etheridge and Roesler, 2005). In particular, depletion of nitrogen (N) causes toxin content to decrease in parallel with other general physiological changes that occur during N-stress (i.e. chlorosis, increased C:N, and decreased protein content; White, 1978; Boyer et al., 1987; Ogata et al., 1987; Anderson et al., 1990b; Flynn et al., 1994, 1996; Matsuda et al., 1996; Parkhill and Cembella, 1999). Two exceptions to the decrease in toxin content during stress are growth under P-limited conditions and growth at sub-optimal temperatures. In both cases, toxin content increases dramatically (Hall, 1982; Boyer et al., 1987; Anderson et al., 1990a, b; Flynn et al., 1994, 1996). Since phosphate stress can affect amino acid and protein metabolism, it has been proposed (Anderson, 1990; Flynn et al., 1994) that P-stressed cells may allocate the nitrogen that cannot be used for proteins toward STX production (a nitrogen-rich compound).

Based on results from batch culture studies under fixed conditions, toxin composition has been considered a stable feature—a fixed genetic trait that can be used to distinguish strains or species, as a biochemical fingerprint (Boyer et al., 1987; Anderson, 1990; Sako et al., 1995; Matsuda et al., 1996; Cembella, 1998). Indeed, during nutrient replete conditions in batch culture, toxin composition does appear to be invariant and can serve as a chemotaxonomic marker for different *Alexandrium* strains (Cembella, 1998). However, recent studies have shown that once cells are given time to achieve steady state in semi-continuous, nutrient-limited cultures, toxin composition does vary during progressive N- and P-limitation (Anderson et al., 1990a; MacIntyre et al., 1997). Using either the absolute toxin abundance or relative proportion (mol% total toxin) of each toxin derivative, N- and P-limitation can be distinguished based on the observed trends in the different STX derivatives. For example, during N- and P-limitation the absolute toxin abundance decreases and increases, respectively (Anderson et al., 1990a, b). Other environmental conditions such as salinity and temperature have limited affects on toxin composition (Taroncher-Oldenburg et al., 1999; Etheridge and Roesler, 2005).

This suggests that toxin composition could be used to assess the nutritional status of *Alexandrium* spp. in field populations, as long as the changes in toxin composition due to nutrient stress have been characterized from isolates of a particular region. To date, modifications in toxin composition using nutrient-limited semi-continuous culture have been characterized for *A. fundyense* strains from the Gulf of Maine and *A. tamarense* from the Gulf of St. Lawrence, Canada (Anderson et al., 1990a;

MacIntyre et al., 1997). Variability in toxin content and toxin composition of *Alexandrium* spp. has not been examined in detail in field populations. In this study, we examine the toxin content and composition in natural *A. fundyense*[1] populations during a spring bloom in Casco Bay, ME from April to June of 1998. This allows us to examine whether *A. fundyense* populations in the western Gulf of Maine are sufficiently homogenous to permit the detection of toxin composition and content differences through time and space, and if so, to determine whether those changes are indicative of a particular nutritional state (e.g., N-limitation).

2. Material and methods

This investigation was carried out during a portion of a 5-year study (ECOHAB-GOM) of the ecology and oceanography of toxic *A. fundyense* blooms in the Gulf of Maine. This multidisciplinary study is exploring cell distributions and dynamics of *A. fundyense* blooms from the Bay of Fundy, Canada, to Cape Ann, Massachusetts. The research presented here was part of a series of small-scale cruises conducted to understand the environmental regulation of toxic blooms and to characterize the environmental factors that govern their initiation, growth, maintenance, and termination.

2.1. Sampling

During the first year of the ECOHAB-GOM study, a series of 11 cruises within Casco Bay in the western Gulf of Maine sampled between April and June 1998 along two transects that extended from Casco Bay to about 20 km offshore (Fig. 1). Depth profiles for temperature and salinity were acquired using a Falmouth Scientifics CTD/rosette system. Data were post-processed into 0.5-m depth bins using MatLab software.

Samples for *A. fundyense* abundance and nutrients were collected from rosetted-Niskin bottles at

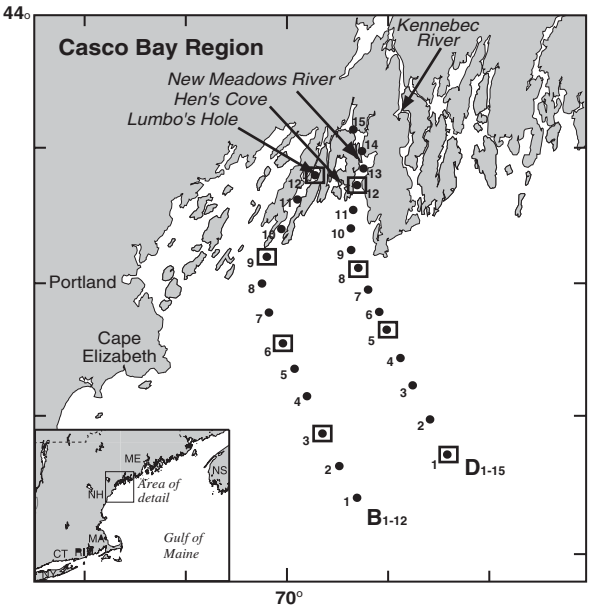

Fig. 1. Chart of the Casco Bay Region along the Maine coast within the Gulf of Maine, showing the location of the shore and cruise sampling sites. Boxed sites represent locations where toxin samples were collected along cruise Transect B (Stations: B3, B6, B9 and B12) and Transect D (Stations: D1, D5, D8 and D12).

the surface (1 m), 3.5, 7, 10 and 20 m at all stations (see Fig. 1). The top three samples were combined to give one surface layer abundance estimate, designated as a "pooled sample". For each *A. fundyense* abundance measurement, 2 l from each depth were sieved onto a 20-μm mesh Nitex screen, and the phytoplankton retained on the screen was backwashed and preserved in 5% borate-buffered formalin (v/v) in 15-ml centrifuge tubes and stored at 4 °C until analysis. In addition, samples were collected to determine dissolved organic and inorganic nitrogen at 1, 3.5, 7, 10 and 20 m, respectively. Nutrient samples were collected during Cruises 1, 2, 4, 6, 8, 10 and 11 and frozen until analysis.

Samples for *A. fundyense* toxin content and composition were collected at four stations along each transect, evenly distributed from inshore to offshore (Fig. 1). Seawater was collected near the surface using a combination of the ship's seawater deck hose (intake at ~1 m) and a submersible pump that was set just below the surface (intake at ~0.5 m). At these stations, seawater was passed through a 20-μm plankton net for approximately 30–45 min. The flow rate was measured at the beginning of each cruise using a calibrated carboy and remained constant during the each survey. It

[1]Both *A. tamarense* and *A. fundyense* occur in the Gulf of Maine (Anderson et al., 1994). We consider these to be varieties of the same species (Anderson et al., 1994; Scholin et al., 1995). Neither antibody nor oligonucleotide probes can distinguish between them, and only detailed analysis of the thecal plates on individual cells can provide this resolution. This is not practical for large numbers of field samples. Accordingly, for the purpose of this study, the name *A. fundyense* is used to refer to both forms.

ranged from 26 to 52 l min^{-1} with an estimated 700–1600 l of seawater passing through the net depending on whether one or two hoses (deck and/or submersible pump) were used for the collection. The collected material was then back-washed onto a 20-μm Nitex sieve and re-suspended into a 50-ml conical centrifuge tube. From the 50-ml sample, 2 ml were removed for cell counts and preserved using buffered formalin, 5% final concentration (v/v). The remaining 48 ml were split into two fractions (24 ml each). The first fraction was sieved through a series of sequential meshes of 100, 64 and 20 μm, to obtain the plankton fractions 64–100 and 20–64 μm. The 64–100 and 20–64 μm fractions were re-suspended with filtered seawater and filtered onto 47-mm Whatman (934-AH) glass fiber filters. The 64–100 and 20–64 μm filter fractions were placed into pre-weighed 15-ml centrifuge tubes containing 2 ml 0.1 M hydrochloric acid (HCl) and 2 ml 0.5 M acetic acid (HOAc), respectively. The other 24 ml of the original plankton sample were filtered directly onto a Whatman 47-mm filter and placed into a pre-weighed 15-ml centrifuge tube containing 2 ml 0.1 M HCL. All 15-ml centrifuge tubes containing filters were frozen at −20 °C until analysis.

In addition to the transect stations, samples were collected from three shore stations during the same time frame—Lumbo's Hole, Cundy's Harbor (D12) and Hen's Cove (Fig. 1). Sampling from the shore stations took place from April 27 through June 17, 1998. Each shore station was sampled approximately every 2–5 days.

For each shore station, samples for toxin analysis, specifically toxin composition, were collected using the same method used for the shipboard sampling, but with a few modifications. Briefly, between 700 and 1550 l of water were filtered through a 20-μm plankton net at each station. Afterwards, the 20-μm plankton sample was re-suspended into 500 ml and sieved sequentially through 100-, 64- and 20-μm mesh sieves. The 64–100 and 20–64 μm fractions were re-suspended with filtered seawater and filtered onto a Whatman 47-mm filter (934-AH) and placed into pre-weighed 15 ml centrifuge tubes containing 2 ml 0.5 M HOAc. All toxin samples were frozen at −20 °C until analysis. To determine the ambient *A. fundyense* cell concentration at each station, 8 l of surface seawater were collected and filtered onto a 20-μm sieve, back-washed into a 15-ml centrifuge tube and preserved with borate-buffered formalin (5% final concentration; v/v).

2.2. *Alexandrium fundyense* quantification

Cell abundance was determined using an immunofluorescence labeling protocol with a monoclonal antibody (MAb) probe. The antibody labeling uses an *Alexandrium* genus-specific probe, MAb M-8751-1, which has been shown to cross-react with *A. fundyense* isolates from the Gulf of Maine (Adachi et al., 1993; Sako et al., 1993). Recently, it has been shown that antibody-labeling variability due to environmental conditions (physiological changes) is minimal and that accurate manual counts are possible despite expected changes in nutrition through time (Anderson et al., 1999).

Labeling protocols for *Alexandrium* sp. using immunofluorescence have been described previously (Anderson et al., 1999; Turner et al., 2000), but the method has recently been modified for direct labeling and visualization on a filter (Anderson et al., 2005; Keafer et al., 2005a). Briefly, a 0.5–7.4 ml aliquot of a preserved field sample (note each aliquot of concentrated field sample contained the equivalent of one or more liters of filtered seawater) was filtered onto 25-mm Whatman Cyclopore polycarbonate, membrane filters (5.0 μm). The cells on the filter were initially blocked with 1 ml of 5% normal goat serum (NGS; Sigma Chemical Co.) in 0.02 M phosphate-buffered saline (PBS) for 30 min at room temperature (RT). After gentle filtration, 315 μl of primary MAb M8751-1 (diluted 1:50 v/v with 5% NGS/PBS) were added, incubated for 30 min at RT, and rinsed 3 × with 5 ml 0.5% NGS in 0.02 M PBS. The secondary antibody [300 μl of a goat anti-mouse IgG secondary antibody conjugated to fluorecein isothiocyanate (GAM-FITC; Molecular Probes, Inc.) diluted 1:300 v/v with 5% NGS/PBS] was then applied to the filter and incubated for another 30 min at RT. After final washing (3 × with 5 ml 0.5% NGS in 0.02 M PBS), the filter was semi-permanently mounted on a glass slide with a coverslip using 80% glycerol in PBS as the mounting media and stored at 4 °C in the dark until microscopic analysis (within days). Positive control samples using preserved cultured *A. fundyense* cells were processed simultaneously to ensure that the cells were consistently stained.

The slides were examined using an epifluorescence Zeiss Axioskop equipped with a FITC filter set (excitation = BP 450–490 nm, emission = LP 520 nm) at 100 ×. The entire area of the filter was enumerated for the presence of *A. fundyense* cells (stained bright green around the periphery of the

cell). Cells were only enumerated if a cell was positively labeled with FITC green fluorescence, indicative of the *Alexandrium*-specific MAb, and contained natural red fluorescence, indicative of chlorophyll. Subsequent to the current study, antibody M8751-1 was found unable to discriminate between *A. fundyense* and the potentially co-occurring, *A. ostenfeldii* (Anderson et al., 2005; Gribble et al., 2005). The possible implications of this cross-reactivity are considered in detail in the Discussion section below.

2.3. Nutrient analysis

Frozen nutrient samples were analyzed for dissolved inorganic nutrients, specifically, nitrate and phosphate using a Lachat Autoanalyzer following standard methods (Love et al., 2005). Total dissolved N (TDN) was determined following Valderrama (1981) using a Lachat Analyzer. Dissolved organic N (DON) was determined by the difference of the total inorganic nitrogen (DIN) from the total dissolved N (TDN).

2.4. Culture controls

Prior to the field season, during the fall of 1997, *A. fundyense* strains from Casco Bay, Maine were isolated from germinated cysts and rendered clonal in the laboratory. For comparison to the toxin data from Casco Bay, the toxin content and composition of six different Casco Bay isolates was determined. Six cultures were grown in triplicate 25 ml volumes of nutrient-replete *f*/2 medium (Guillard, 1975), made with 0.2-μm filtered Vineyard Sound seawater (VSW). The *f*/2 medium was modified by adding H_2SeO_3 and reducing the concentration of $CuSO_4 \cdot 5H_2O$ to a final concentration of 10^{-8} M. The cultures were incubated at 15 °C using an irradiance of 200 μmol quanta $m^{-1} s^{-2}$ on a 14 h:10 h light:-dark cycle. One additional isolate (CB-301) also was grown under nitrogen-limited, semi-continuous conditions following the protocol of MacIntyre et al. (1997).

Once the six nutrient-replete cultures reached mid-exponential growth as monitored by cell abundance and fluorescence during nutrient replete conditions, the cultures were harvested for cell density and toxin determinations. The CB-301 N-limited semi-continuous culture was harvested once the culture stabilized and maintained a constant cell concentration. From each culture, three 1.5-ml cell count samples were removed and preserved with Utermohl's solution. Cell concentrations were determined by counting at least 400 cells per sample in a Sedgewick–Rafter counting chamber. A 15-ml sample for toxin extraction was removed and placed into a pre-weighed, polypropylene 15-ml centrifuge tube and centrifuged (5000*g*, 5 min, 23 °C), the supernatant aspirated, and 500 μl of 0.5 HOAc were added to each cell pellet.

2.5. Toxin extraction and analyses

Toxins were extracted by thawing the frozen HOAc field samples and sonifying both types of samples (field and cultured) with a Branson sonic cell disrupter for 20 s at a setting of 6 A while the samples were suspended in an ice-water bath. Afterwards, each sample was weighed and centrifuged (5000*g*, 5 min, 25 °C). The total weight of the toxin sample required to determine the toxin extraction volume (TEV) was calculated by subtracting the final sample weight from the initial 15 ml tube weight. For the field samples only, 1 ml of supernatant was passed through a Millipore, Sep-Pak C18 cartridge following the manufacturers' recommendations. The column eluent was then spun in a Millipore 10,000 MW cutoff filter at 4000*g* for 5 min. From the purified field samples and the centrifuged cultured cell extracts, approximately 200 μl from each were loaded into separate auto-sampler vials, and analyzed for STX derivatives by HPLC using the three-step isocratic elution method of Oshima et al. (1989) following the modifications of Anderson et al. (1994). The total toxin extraction weight (TEW) and total toxin extraction volume (TEV) for the field samples was calculated as follows:

$$\mathrm{TEW} = (\text{final sample wt.} - \text{tube tare wt.} - \text{filter wt.}),$$

$$\text{Vol. of SW \& algae} = \frac{(\mathrm{TEW} - \text{acid wt.})}{d},$$

$$\mathrm{TEV} = (\text{Vol. of SW} + \text{algae}) + \text{acid Vol.},$$

where the weights (wt.) are in kg and volumes (Vol.) represented in l, the filter weight is the dry weight in kg and d represents the density of seawater (SW). The ambient toxin concentration in the seawater surface layer at each station (Fig. 2) was calculated using the following equation:

$$\text{pmol toxin l}^{-1} = \frac{[T] \cdot \mathrm{TEV}}{V_{\mathrm{SW}}},$$

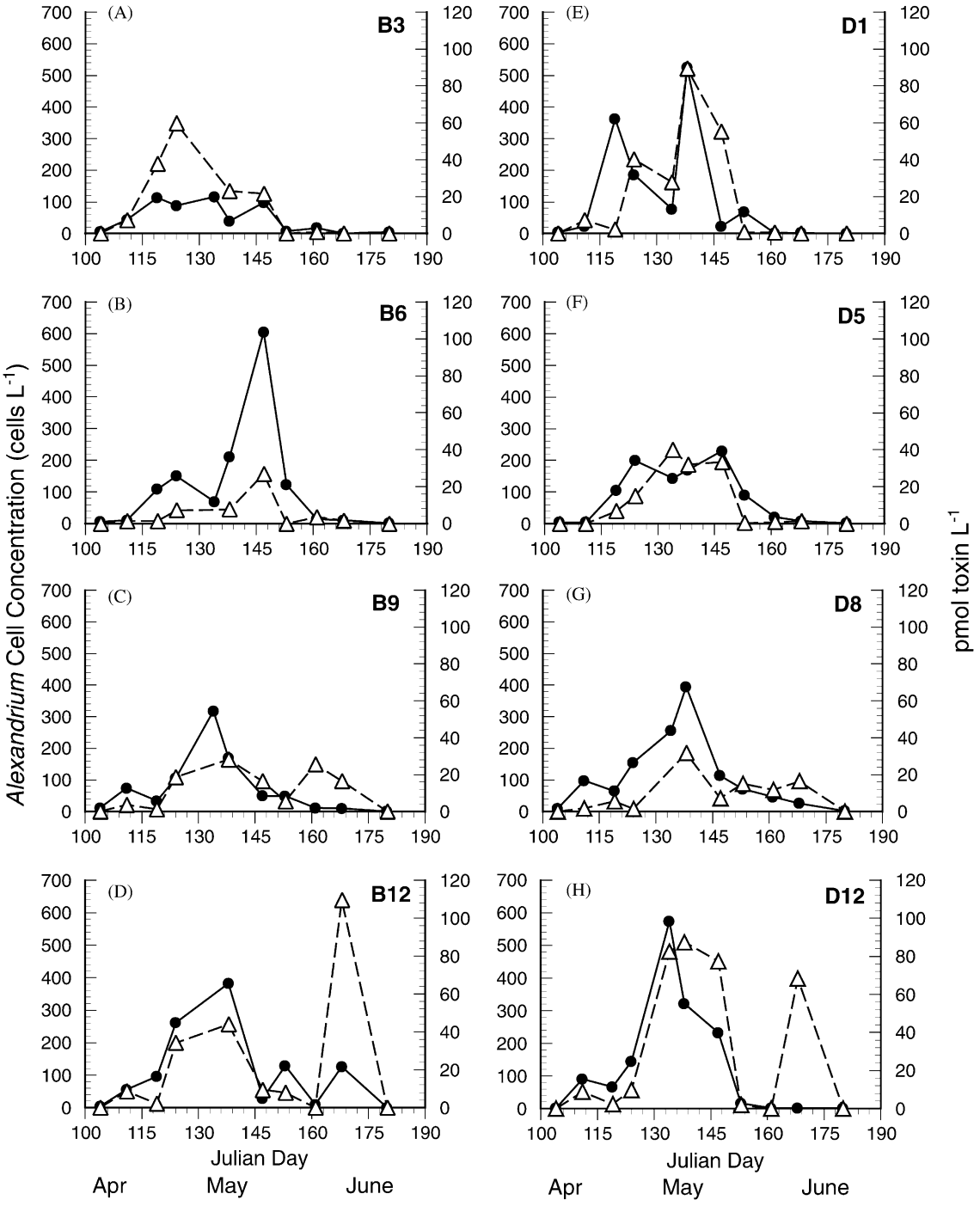

Fig. 2. Surface toxin concentrations and *Alexandrium fundyense* cell abundance at shipboard stations during the 1998 field season. The data from Transect line B are located in graphs (A)–(D) and the data from Transect line D are located in graphs (E)–(H). The black lines (●) represent the cell concentrations determined from seawater samples that were pooled from 1, 3.5 and 7 m. Using the secondary *y*-axis, the dashed lines (Δ) represent the calculated total toxin concentration (pmol toxin l^{-1}) in the surface seawater from each shipboard station. Both the cell and toxin cell concentrations in the surface seawater correlated well, demonstrating the strong association of *A. fundyense* with toxin distribution, as expected.

where [T] is the toxin concentration as determined by the HPLC in pM, TEV is the total toxin extraction volume as described previously, and V_{SW} is the volume of ambient surface seawater that was collected and filtered for toxin extraction. The toxin abbreviations used throughout this paper are: STX, saxitoxin; NEO, neosaxitoxin; GTX 1,4, gonyautoxins 1 and 4; GTX 2,3, gonyautoxins 2 and 3; GTX 5, gonyautoxin 5; GTX 6, gonyautoxin 6; C1,2, toxins C1 and C2. To determine whether toxin composition (mol% total toxin) changes significantly through time and distance from shore, a two-way ANOVA was performed for each derivative of the two different transects (B vs. D—considered replicates) as a factor of time (cruise) and distance (station). Tukey's multiple comparison test was used to determine differences among individual times and stations.

3. Results

3.1. *A. fundyense* distributions

A. fundyense was detected in the water column during all 11 cruises in the Casco Bay region from April to June of 1998 (Fig. 2). At only a few stations during the last cruise in late June was *A. fundyense* considered undetectable (detection limit = 1 cell l^{-1}). *A. fundyense* cell concentrations fluctuated through time, with considerable variability between stations. At the beginning of the field season in early April, cell abundance was low (between 1 and 50 cells l^{-1}). In late-April, the cell abundance increased rapidly at both the inshore and offshore stations. At all stations, the cell distribution was either bimodal, with peaks in early May and mid-June, or evidenced a single, broader peak between May and June, declining thereafter. For almost all shipboard and shore stations the bloom reached a maximum density in mid-May. At the cruise stations, the highest cell density within the top 7 m was ca. 600 cells l^{-1}, however, at the three shore stations (Fig. 3), the cell abundance attained much higher cell densities (ca. 2500–4000 cells l^{-1}). The large differences may reflect the difference in sampling methods; the shore counts were from a single surface sample as *Alexandrium* cells tend to concentrate at the surface in the Gulf of Maine, whereas the shipboard station counts were from a pooled sample representing three depths. Overall, the cell concentrations at the inshore stations appeared to have higher cell numbers at the peak of the bloom with an additional peak in cell abundance occurring in mid-June (Fig. 2D). Typically bloom densities in the Gulf of Maine do not reach higher than 10,000 cells l^{-1} (Anderson, 1997; Keafer et al., 2005a).

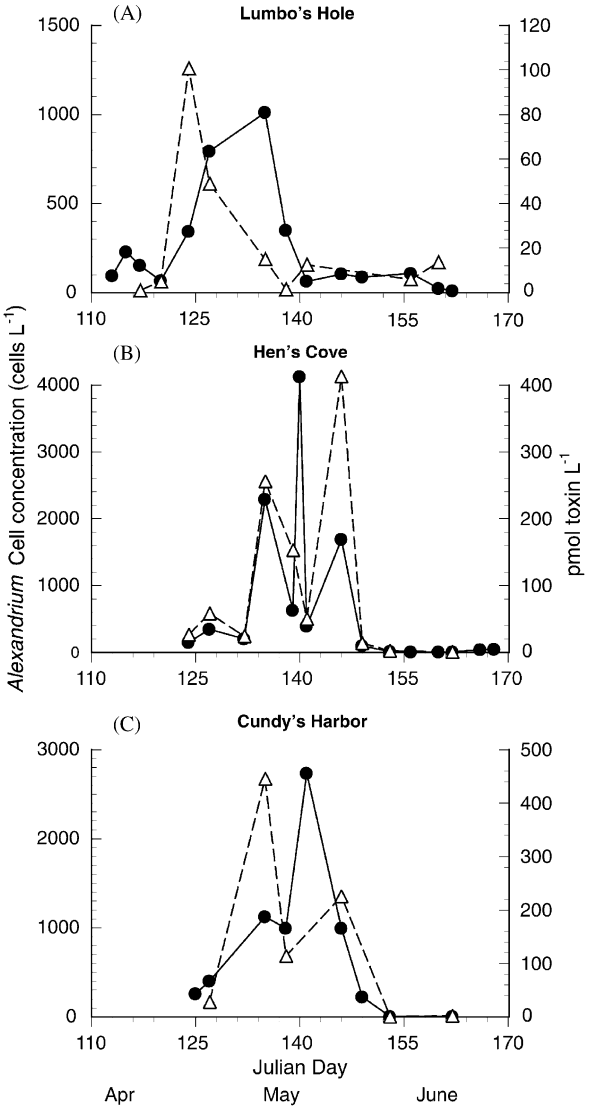

Fig. 3. Surface toxin concentrations and *Alexandrium fundyense* cell abundance at three shore stations, (A) Lumbo's Hole, (B) Hen's Cove and (C) Cundy's Harbor, during the 1998 field season. The black lines (●) represent cell concentrations determined from discrete surface (1 m) seawater samples. Using the secondary y-axis, the dashed lines (Δ) represent the calculated total toxin concentration (pmol toxin l^{-1}) in the surface seawater from each station.

3.2. Physical and chemical distributions

The influence of low-salinity waters (<32 psu) of the Kennebec River plume and the adjacent waters

of the western Maine Coastal Current is quite apparent in the study area and is described elsewhere (Churchill et al., 2005; Keafer et al., 2005b; Janzen et al., 2005). The seawater temperature changed significantly over the course of the Casco Bay field season. Sea-surface temperatures ranged from 4 to 14 °C from early April to late June. In mid-April, the water temperature was relatively uniform within the top 25 m, ranging between 4 and 6 °C. As stratification developed during May and into the beginning of June due to both runoff and vernal warming, a warm surface layer (12–14 °C) became apparent within the top 5 m, with a rapid temperature gradient beginning at 7–9 m with much cooler temperatures (6–10 °C) below 10 m.

Total dissolved inorganic nitrogen (DIN) concentrations changed significantly in the surface layer and vertically, with depth. Initially, high DIN values were reported throughout the water column (4.0–7.0 μM) during cruise 1 in early April (Fig. 4A). The DIN in the surface layer then declined rapidly over the next 2 weeks, while maintaining higher concentrations below 7 m. DIN values were low but measurable in surface waters in May (0.01–1.8 μM), but were below detection throughout most of the water column during Cruise 8 (June 1–4, 1998). However, during most cruises DIN values were higher (2–4 μM) at depths greater than 20 m. During Cruise 10 (June 15–19, 1998) DIN values increased briefly in the surface layer, especially at the inshore stations on the B transect (B9 and B12). This surface-layer nutrient increase (top 7 m) also was observed in the ambient phosphate concentrations. The reported increases correlate with a freshwater runoff event in the Kennebec River that occurred during a week-long period of precipitation at the beginning of June.

Phosphate concentrations ranged between 0.1 and 1.3 μM throughout the field season. In the surface layer, phosphate concentrations ranged from 0.1 to 0.84 μM (Fig. 4B), with the largest values reported during Cruise 1 in early April. At 20 m, phosphate concentrations were between 0.3 and 1.3 μM, and at no time during the field season was phosphate undetectable.

N:P atomic ratios were calculated for all stations throughout the field season (Fig. 4C) using the concentrations of DIN and phosphate within the top 7 m (pooled samples) at all stations. During Cruise 1 in early April, the highest N:P ratios ranged from 6 to 10, but as the field season progressed, N:P ratios declined rapidly, to between

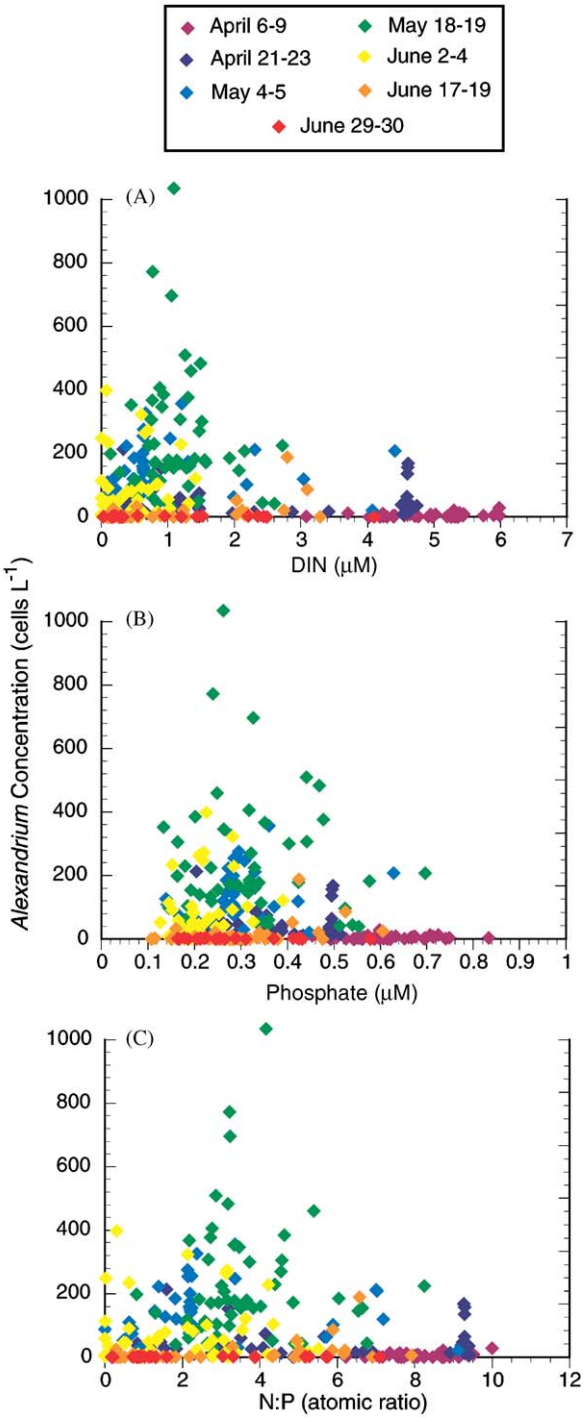

Fig. 4. Surface (top 7 m) nutrient concentrations and N:P ratios vs. *Alexandrium fundyense* abundance during the Casco Bay, Maine field season. (A) DIN, (B) Phosphate and (C) N:P (atomic ratio). Each color represents a different cruise time frame beginning in mid-April and ending in late June 1998.

0 and 6. As *A. fundyense* concentrations increased throughout the field season the largest cell concentrations were associated with very low DIN (0.5–1.5 μM; Fig 4A), phosphate (0.2–0.4 μM; Fig 4B) and very low N:P ratios (ranging from 2 to 5; Fig. 4C).

3.3. Variations in toxin concentration and content

The 20–64 μm size fraction samples that contained *A. fundyense* from eight shipboard transect stations and three shore stations were assessed for toxin content and composition using HPLC. During the 11 cruises, the D transect was occupied all 11 times yielding a total of 44 samples. The B transect was occupied 10 of 11 times (weather limited one sampling effort) yielding 40 samples. At Lumbo's Hole, Hen's Cove and Cundy's Harbor shore stations, 13, 12 and 8 toxin samples were collected, respectively, from April 17 to June 17, 1998. Of the 117 samples collected, 93 contained detectable levels of STX. Of the 24 samples that contained no toxins, 16 were from early April and late June when *A. fundyense* cell abundance was low or undetectable.

Total toxin concentrations in the surface of the water column (pmol toxin l^{-1}) increased and then decreased through time for both the shore and transect samples, mimicking the patterns of *A. fundyense* abundance in the water column as the field season progressed (Figs. 2 and 3). The cell abundance from each pumped toxin sample along the B and D transects correlated with the ambient cell concentrations determined from the pooled bottle samples (Fig. 2). The highest toxin concentrations (100–400 pmol STX l^{-1}) were reported between May 14 and 27 at both the shore and transect stations; however, for some shore stations (Lumbo's Hole and Cundy's Harbor) high levels of toxicity preceded times when high cell concentrations were observed (Figs. 2 and 3). The lowest toxin values (0–10 pmol STX l^{-1}) corresponded to the times at the beginning and end of the field season when *A. fundyense* concentrations were low, but toxin concentrations were still detectable.

Toxin content (fmol $cell^{-1}$) was calculated at transect stations (Fig. 5) using the cell abundance from each toxin sample and the total toxin concentration was measured by HPLC. These values were highly variable, ranging from 36 to 325 fmol $cell^{-1}$. Values for toxin content were not calculated for stations with low or undetectable cell abundance or toxin concentrations, as occurred for most samples during early April and late June.

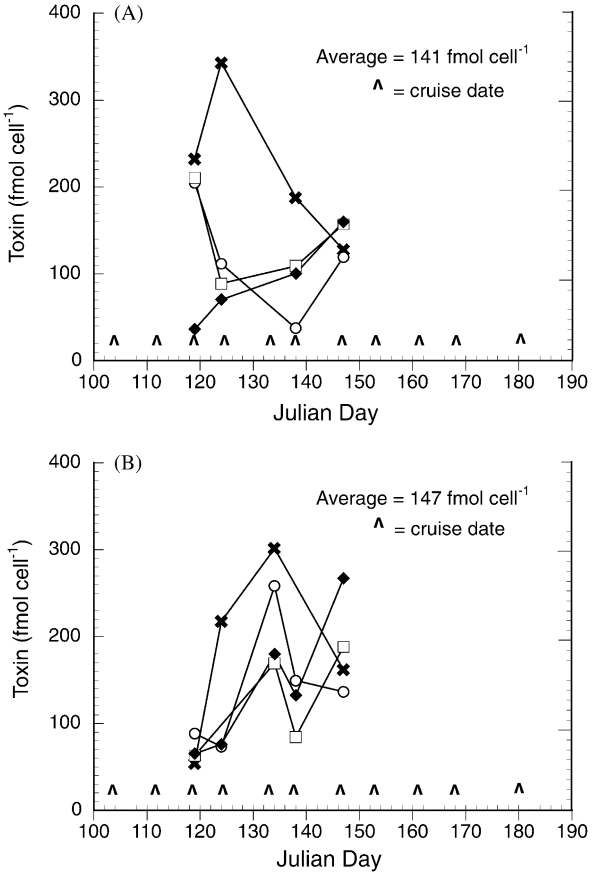

Fig. 5. Cellular toxin content (fmol $cell^{-1}$) determined at different stations over the course of the field season. (A) Transect B; Station B3 (×), Station B6 (○), Station B9 (□), Station B12 (♦), (B) Transect D; Station D1 (×), Station D5 (○), Station D8 (□), Station D12 (♦).

The toxin content (fmol $cell^{-1}$) of isolates from Casco Bay (CB-isolates) ranged from 113 to 155 fmol $cell^{-1}$ (mean 128), during nitrogen-replete conditions in laboratory cultures. One isolate (CB-301) also was grown under nitrogen-limited, semi-continuous conditions and analyzed for toxin composition using HPLC. At extreme levels of nitrogen-limitation, growth rate slowed to 0.05 d^{-1} and cellular toxin content decreased to 33 fmol $cell^{-1}$.

3.4. Variations in toxin composition

Changes in the relative proportions (mole percent of total toxin) of the main STX derivatives and toxin ratios (mol:mol) were used to examine trends

over the course of the field season. Of the more than 20 naturally occurring STX derivatives (Oshima, 1995), Casco Bay field samples were found to contain 10 different derivatives. The major toxins present (in order of relative abundance as mol% total toxin) were GTX 3, GTX 4, NEO, STX and C2. Toxins found in minor abundance were GTX 5, GTX 2, GTX 1, C1 and dcGTX3. For interpretation of the compositional analysis, GTX 1 & 4, GTX 2 & 3 and C1 & C2 were grouped together, since the epimeric pairs GTX 1,4, GTX 2,3 and C 1,2 are subject to rapid interconversions (Oshima et al., 1993).

Over the course of the field season, significant differences in the toxin composition profile were observed in the pumped plankton samples (Figs. 6 and 7). In most cases, the relative molar abundance of NEO and STX decreased through time for both transect and shore stations, whereas, GTX 1,4 increased. C 1,2 and GTX 2,3 remained relatively constant at the transect stations, but C 1,2 decreased through time at the three shore stations and GTX 2,3 increased.

For the transect data only, a two-way ANOVA with no interactions between transects B & D showed significant differences between the averaged means of the relative proportions (mol% of total toxin) of NEO, STX, GTX 1,4 and GTX 2,3 with respect to TIME ($P<0.001$) and STATION ($P<0.001-0.004$). The two transects were considered as replicates. Prior to performing the two-way ANOVA, the data were transformed using a square root function to fulfill the normality requirement of the statistical test. Afterwards, Tukey's multiple comparison test was used to determine whether significant differences were observed between specific Times (cruises) and Stations.

In addition to determining the relative abundances of the different toxins, toxin ratios (mol:mol) of different STX derivatives were calculated as follows:

$$\text{Toxin Ratio } [T_A : T_B] = \left[\frac{T_A}{T_B}\right],$$

where $[T_A]$ is the absolute toxin concentration of derivative A (μM) and $[T_B]$ is the absolute toxin concentration of derivative B (μM). Over the course of the field season, significant increases were observed in the following toxin ratios at most stations along transect B and D: GTX1,4:STX, GTX2,3:STX and NEO:STX (Figs. 8 and 9). Other ratios, such as GTX1,4:NEO, C1,2:STX and GTX2,3:STX increased but not with the same magnitude. Overall, along the two transects, some ratios differed by a factor of 5 or more, others only 2 or 3 fold. When comparing the inshore stations (B12 and D12) to the offshore stations (B3, B6 and D1, D5), the increase in the GTX1,4:STX and GTX2,3:STX ratios towards the end of June were generally larger for the offshore (between 20 and 50) than the onshore stations (between 10 and 15). At the shore stations (Lumbo's Hole, Hen's Cove and Cundy's Harbor—Fig. 10) lower magnitudes of the GTX1,4:STX and GTX2,3:STX ratios also were observed. Overall, the magnitudes of the ratios at the shore stations and the inshore stations along the B and D transects were similar.

4. Discussion

It has been demonstrated previously that the toxin content and composition of *Alexandrium* species vary in a systematic manner as a result of N- and P-limitation in semi-continuous culture (Anderson et al., 1990a), but these patterns have not been examined in detail during *Alexandrium* spp. blooms in the natural environment. This study demonstrates that changes in toxin composition did occur over the course of a spring bloom in the Casco Bay region of the Gulf of Maine, and that these changes are generally consistent with increasing levels of N-stress as the bloom season progressed. There is also a suggestion that the level of N-limitation was more severe in offshore compared to near-shore waters, again evidenced by toxin composition patterns and the relative amounts of the different STX derivatives. These results demonstrate the great potential of field toxin measurements, and in particular toxin composition variability and specific toxin ratios as indicators of N-limitation in natural *Alexandrium* spp. populations.

4.1. Nutrient availability

During the 1998 field season, a rapid reduction of available nutrients (specifically N) accompanied relatively large increases in *A. fundyense* concentrations in the water column (Figs. 2 and 4). During this period of bloom development (April 29–May 14), surface waters were essentially depleted of DIN (Townsend et al., 2001, 2005). Surface waters of the southwestern Gulf of Maine are predominately N-limited during the spring season, as demonstrated

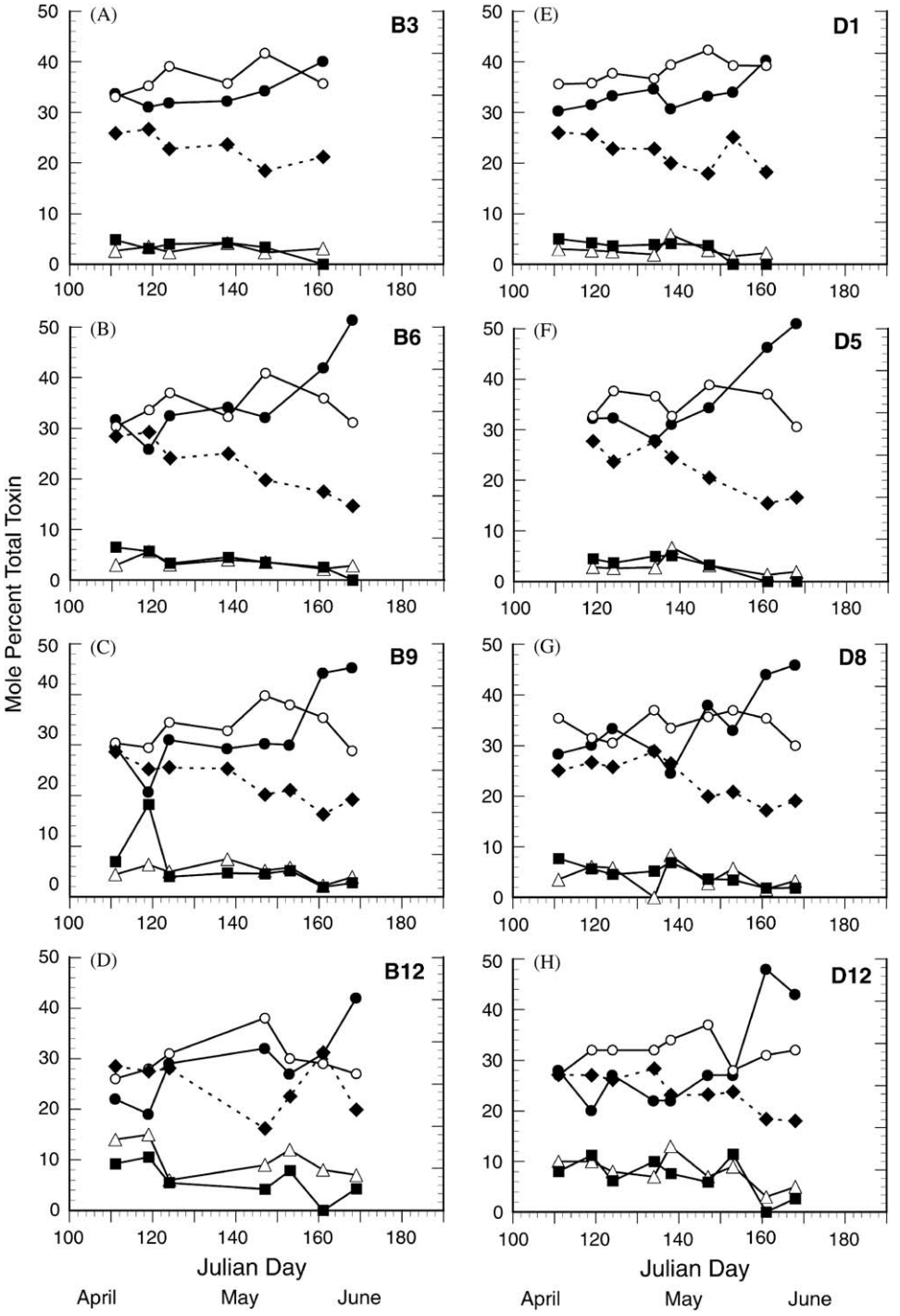

Fig. 6. Relative proportions (mol% total toxin) of the different STX derivatives, at stations along Transect B (A–D) and Transect D (E–H) during cruises (2–10); GTX2,3 (○), GTX1,4 (●), NEO (♦), STX (■) and C1,2 (Δ).

by Martorano (1997), Love et al. (2005) and by the low atomic N:P ratios observed in this study (Fig. 4C). N:P ratios at the beginning of the field season were relatively high (~8–10) but decreased to much lower values (0–5) during the latter part of the field season when *A. fundyense* abundance peaked. All N:P values were below the optimal Redfield ratio of 16 (Redfield, 1958), indicative of potentially

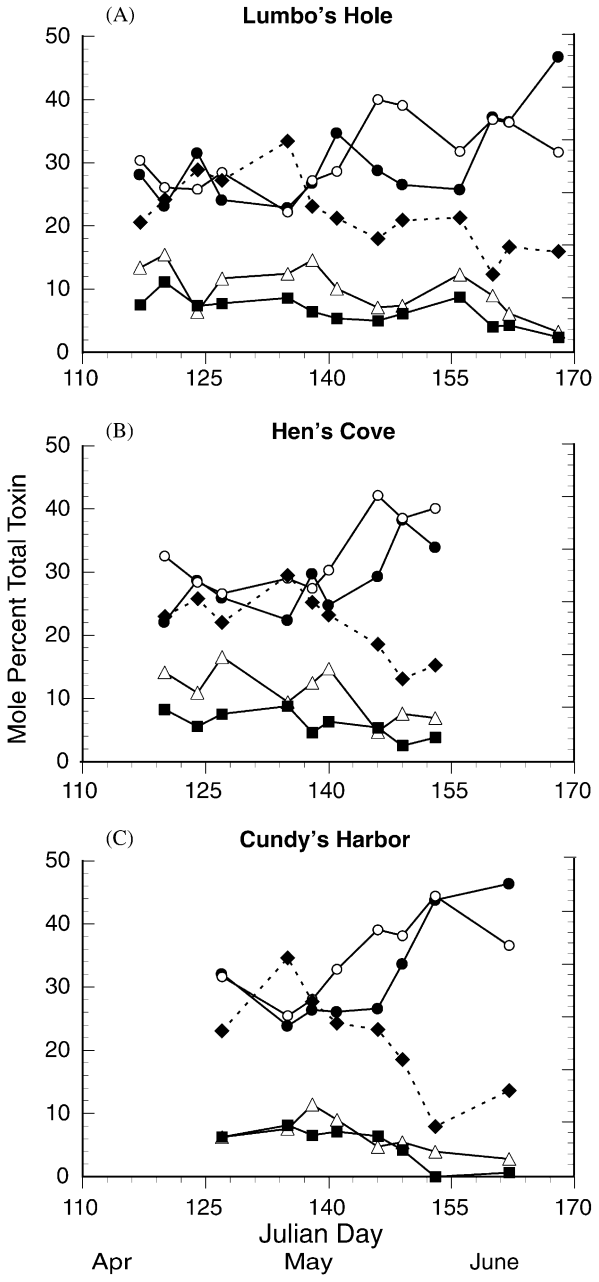

Fig. 7. Relative proportions (mol% total toxin) of different STX derivatives at three shore stations, throughout the field season. (A) Lumbo's Hole, (B) Hen's Cove, (C) Cundy's Harbor: GTX2,3 (○), GTX1,4 (●), NEO (♦), STX (■) and C1,2 (Δ).

N-limited conditions. Nutrient values at inshore stations were generally higher compared to offshore stations. This reflects freshwater inputs from the Kennebec and New Meadows rivers, which are known to contribute significantly to the surface nutrient pool in the southwestern Gulf of Maine (Martorano, 1997; Love et al., 2005). The spike in nutrients within the Casco Bay region in mid-June is a primary example of this influence (Love et al., 2005).

The peak of the *A. fundyense* bloom throughout the region was short in duration—approximately 2–3 weeks. The rapid decline in cell abundance observed at many stations may reflect decreased nutrient availability (specifically N) or another environmental factor such as wind forcing that would physically move the bloom away from the study area (Franks and Anderson, 1992). Keafer et al. (2005a) have suggested that the decline was partially related to strong upwelling favorable conditions that occurred in the late May–early June period after the mid-May abundance peak that limited further downcoast transport of coastal populations. The lack of downcoast transport during that time may have contributed to the lack of nutrient-rich waters coming from the eastern Gulf of Maine into the study area. The decrease in N availability should result in the loss of cells due to cyst formation (Anderson, 1998). During maximum bloom densities, *A. fundyense* cells are capable of sexual reproduction, where it is hypothesized that the formation of planozygotes and cysts can occur rapidly, decreasing the population of cells at the surface as cysts settle to bottom sediments. Sexuality is typically induced in laboratory cultures of *A. fundyense* by N- or P-limitation (Anderson et al., 1984; Anderson and Lindquist, 1985).

A decline in *A. fundyense* cell numbers was observed at all stations through time, and the changes in toxin composition described in Sections 4.2 and 4.3 suggest that the *A. fundyense* population in the Casco Bay region was N-limited even though there was significant N available below the pycnocline. This is consistent with mesocosm observations of Casco Bay isolates of *A. fundyense* (Poulton, 2001) which demonstrate that some strains do not exhibit diel vertical migration behavior (DVM) when N-limited. It is also consistent with field observations that indicate the lack of diel vertical migration in *A. fundyense* populations in the GOM (Townsend et al., 2005; Martin et al., 2005; D. Anderson, unpublished obs.).

Since toxicity parameters can be measured with no interference from co-occurring organisms, they have the potential to serve as biomarkers of N-limitation for this toxigenic species. Based on the observed changes in toxin composition, we

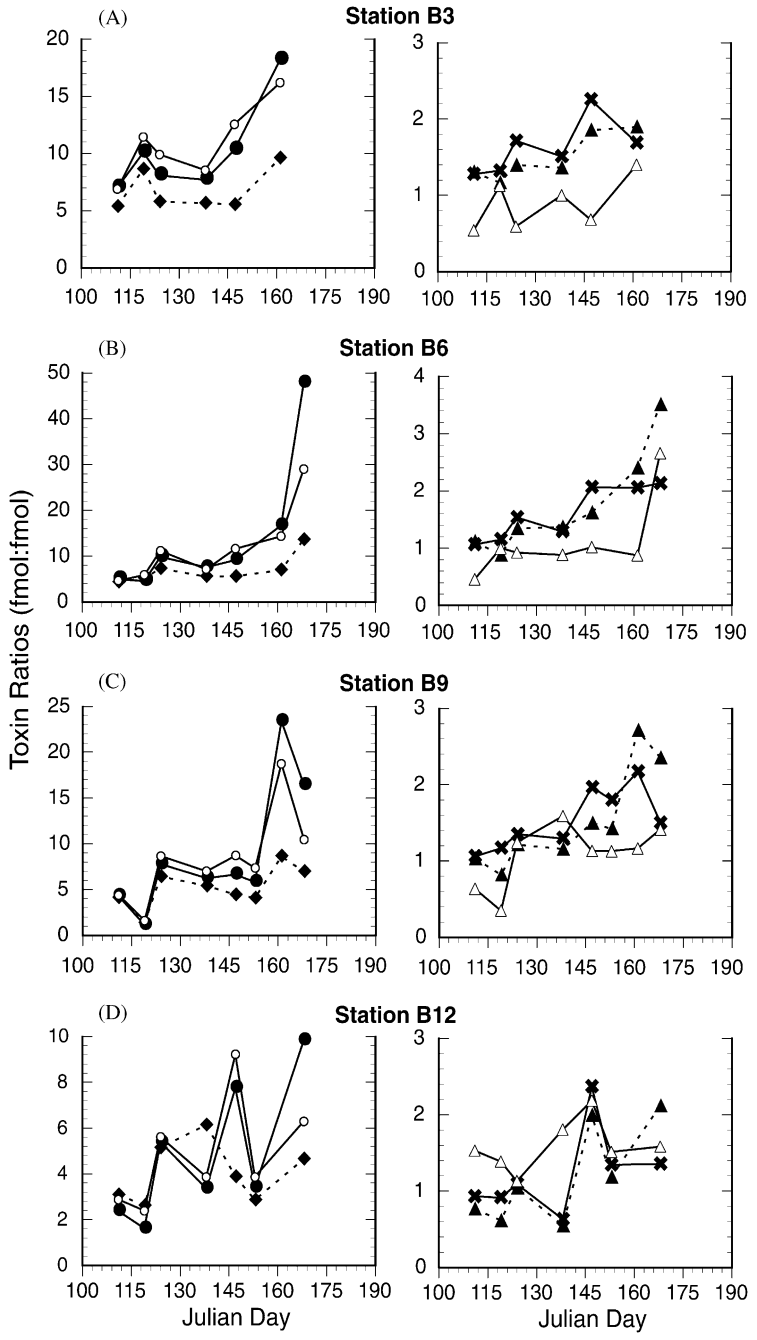

Fig. 8. Toxin ratios (mol:mol) of different STX derivatives at different shipboard stations along the Transect B during the 1998 Casco Bay field season: (A) Station B3, (B) Station B6, (C) Station B9, (D) Station B12; GTX1,4:STX (●), GTX2,3:STX (○), NEO:STX (♦), C1,2:STX (Δ), GTX2,3:NEO (×), GTX1,4:NEO (▲).

hypothesize that field populations of *A. fundyense* became progressively more N-limited over the course of the 1998 field season in the Casco Bay region, due to their inability to access deep nutrients. The combination of changes in internal physiology leading to lower growth rate and sexual induction leading to cyst formation caused cell abundance to decline rapidly by mid-June.

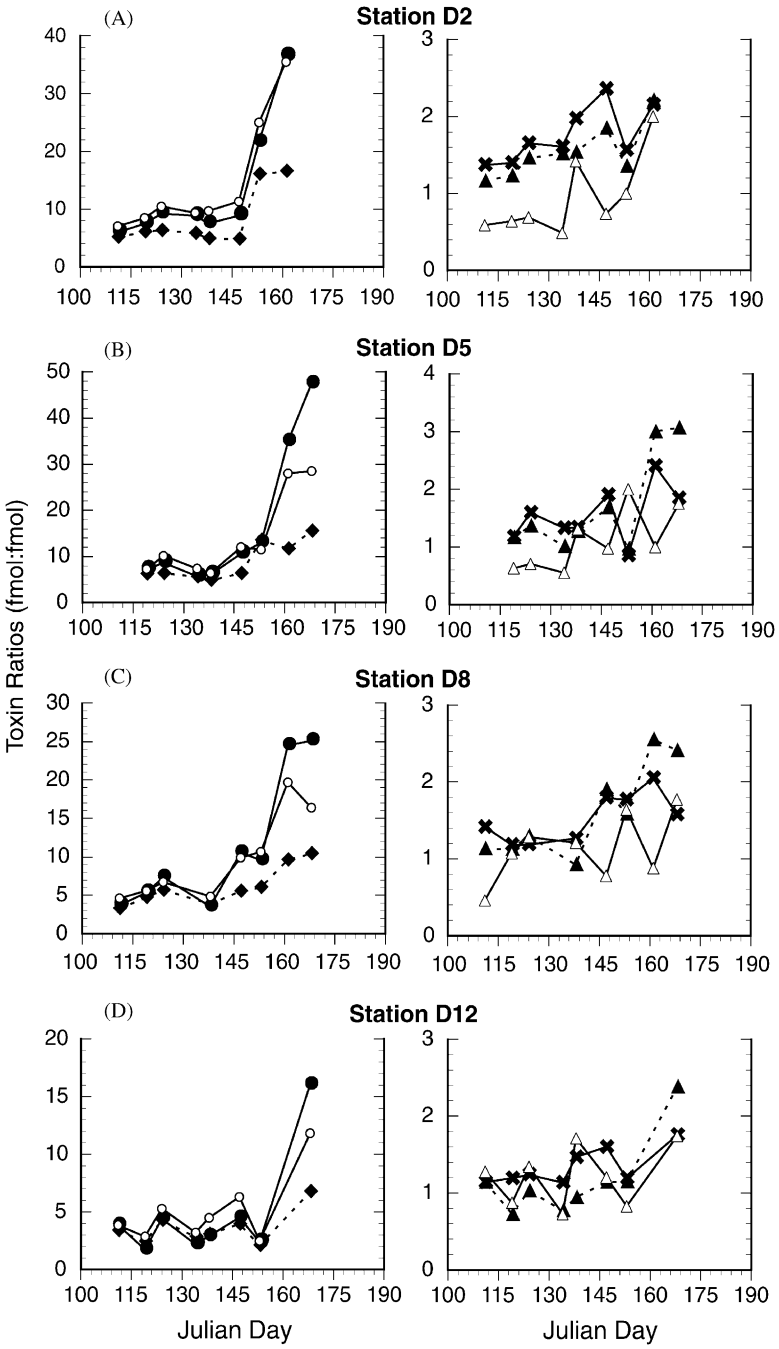

Fig. 9. Toxin ratios (mol:mol) of different STX derivatives at different shipboard stations along the Transect D during the1998 Casco Bay field season. (A) Station D3, (B) Station D6, (C) Station D9, (D) Station D12; GTX1,4:STX (●), GTX2,3:STX (○), NEO:STX (♦), C1,2:STX (Δ), GTX2,3:NEO (×), GTX1,4:NEO (▲).

4.2. Toxin concentrations and content

Based on previous laboratory studies of *A. fundyense* isolates, two of the many internal biochemical changes that occur during N-limitation are a reduction in toxin content (toxin cell quota) and changes in toxin composition (Anderson et al., 1990a; MacIntyre et al., 1997; Poulton, 2001). Here we describe changes in toxin concentration (pmol toxin l^{-1}) and content (fmol toxin $cell^{-1}$) in field

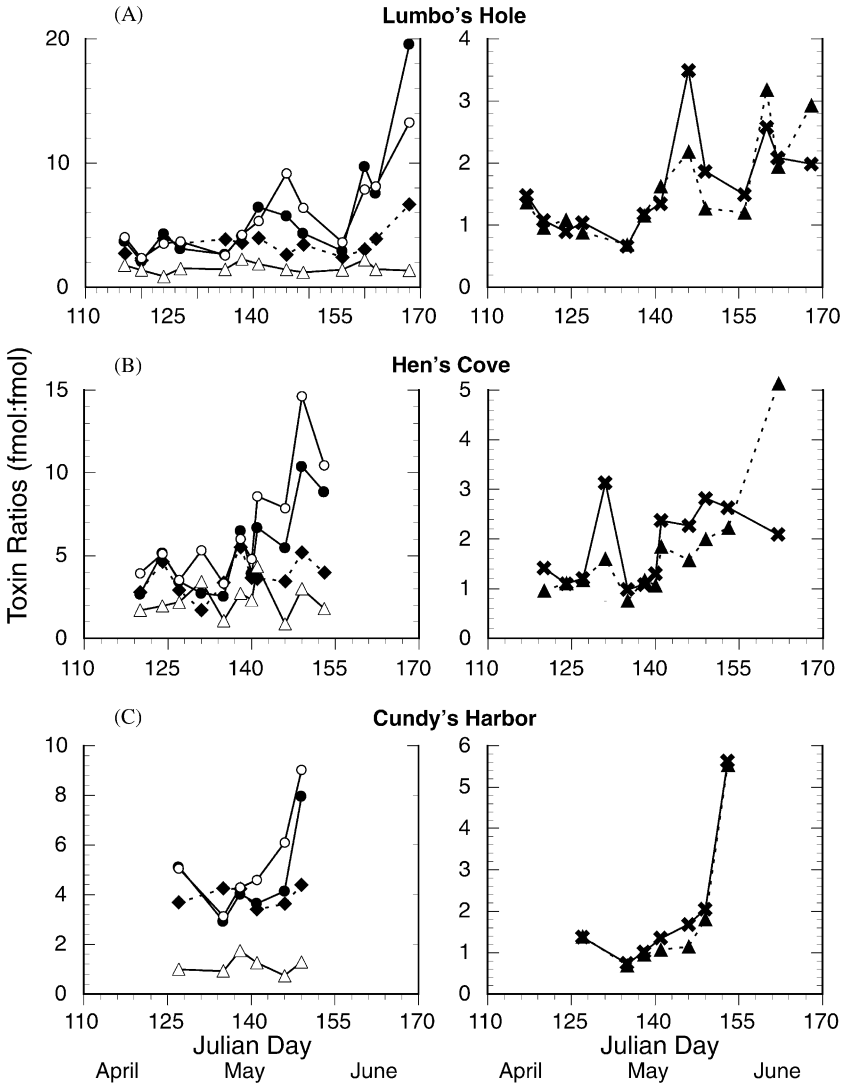

Fig. 10. Toxin ratios (mol:mol) of different STX derivatives at three shore stations, (A) Lumbo's Hole, (B) Hen's Cove and (C) Cundy's Harbor, during the 1998 Casco Bay field season; GTX1,4:STX (●), GTX2,3:STX (○), NEO:STX (♦), C1,2:STX (Δ), GTX2,3:NEO (×), GTX1,4:NEO (▲).

samples throughout an *A. fundyense* bloom. During the 1998 field season, changes in ambient toxin concentration in the smallest size class (20–64 μm) mimicked the increases and decreases in *A. fundyense* cell abundance (Figs. 2 and 3). This close association supports the claim that STX is a unique biochemical marker for *A. fundyense* in this region.

Toxin content represents an equilibrium between the rate of anabolism and catabolism, cell growth and division (Anderson et al., 1990b; Cembella, 1998). Therefore, changes in environmental factors, such as irradiance (Ogata et al., 1987; Parkhill and Cembella, 1999; Etheridge and Roesler, 2005), temperature (Hall, 1982; Anderson et al., 1990b; Etheridge and Roesler, 2005), salinity (White, 1978; Parkhill and Cembella, 1999; Etheridge and Roesler, 2005), macronutrients (N & P) (Hall, 1982; Boyer et al., 1987; Anderson et al., 1990a, b; Flynn et al., 1996) and turbulence (Estrada and Berdalet, 1998) can all affect toxin content. Fig. 5 shows the calculated toxin content of the *A. fundyense* cells collected in the field. The data are highly variable and incomplete, as it was not possible to accurately calculate the toxin content of many field samples due to low cell and toxin concentrations. Dividing one highly uncertain number by another

compounds the error. We conclude that toxin content is not a useful indicator of nutritional status in these particular field samples. We do note, however, that Doucette et al. (2005) also report highly variable toxin content values for this same *A. fundyense* bloom, determined by using highly sensitive receptor binding assay measurements of toxin in plankton size fractions. Those authors report toxin cell quotas that ranged widely from ca. 10 to 2000 fmol STX equiv. $cell^{-1}$.

A related parameter, the ambient toxin concentration (pmol toxin l^{-1}), is more accurate since the measured toxicity is expressed as a function of the volume of water pumped, which is readily measured. However, very low toxin concentrations measured at the beginning and end of the bloom make it difficult to interpret changes in toxin concentration that might have occurred as the bloom declined. There is, therefore, too much uncertainty in the field toxin content and ambient toxin concentration data to permit conclusions to be drawn about stresses such as nutrient limitation using these two parameters. However, a different trend was observed in the toxin composition as ambient nutrient concentrations declined.

4.3. Toxin composition and ratios

Toxin content varies with a broad range of physio-chemical conditions, but the toxin composition or toxin profile has been considered a stable chemo-taxonomic character of different *Alexandrium* spp. isolates (Cembella et al., 1987). Only in the past decade have variations in toxin composition been observed during nutrient-limited growth. This occurs once cells adapt to low nutritional environment, as in very old batch cultures (Boczar et al., 1988) or in semi-continuous cultures (Anderson et al., 1990a; MacIntyre et al., 1997). During this Casco Bay study, significant changes were observed in the toxin composition of *A. fundyense* cells throughout the bloom. Since toxin composition is a measure of the relative abundance of different STX derivatives, it is less susceptible to errors due to low cell or toxin concentrations, as was observed for toxin content. Trends in the toxin profiles were examined through time and between stations (inshore vs. offshore) using two methods: first as changes in the relative proportions of each toxin derivative (mol% total toxin), and second as changes in the ratios of the molar toxin content of each derivative. With this combined approach, trends were identified that are generally consistent with data from N-limited laboratory cultures.

Table 1 summarizes toxicity variation results obtained for different strains of *fundyense/tamarense* using either N- or P-deficient batch or semi-continuous cultures. As observed throughout all the N-stressed laboratory studies (N-starved and N-limited), the total toxin content (fmol $cell^{-1}$) decreased significantly, as did the absolute concentration of each STX derivative (Boczar et al., 1988; Anderson et al., 1990b; MacIntyre et al., 1997; Poulton, 2001; S. Dyhrman, unpubl.). In contrast, during P-limited semi-continuous studies, the total toxin content and concentration of each STX derivative increases (Anderson et al., 1990a; Taroncher-Oldenburg, 1998). When examining the differences in toxin composition during the N-limited laboratory studies, trends in the relative abundances (mol% total toxin; Table 1) of the different STX derivatives were less apparent than when compared to the field data. In the laboratory studies, mol% STX decreased whereas the percentage of GTX 1,4 increased for all N-limited experiments. During the two P-limited studies, the trends in mol% total toxin were nearly identical, the strongest indicator being the large increase in mol% GTX 2,3. As for potential indicators of N-limitation, only the increasing trend in the relative abundance of GTX 1,4 with nutrient limitation can be identified, since a decreasing trend in mol% STX occurs during both N- and P-limitation.

Another method of examining changes in toxin composition uses ratios of the molar toxin content of different STX derivatives. In the laboratory studies shown in Table 1, a few distinctive trends were identified using toxin ratios during both N- and P-limitation. Dramatic increases in NEO:STX, GTX1,4:STX, C1,2:STX and GTX2,3:STX were observed in all N-limited laboratory studies. A much smaller increase was observed in GTX1,4:NEO in most N-limited studies. During P-limitation, GTX2,3:STX and GTX2,3:NEO increased the most, with only minor increases in NEO:STX, GTX1,4:STX and C1,2:STX. Since large increases in GTX2,3:STX occurred during both N- and P-limitation this ratio is inconclusive as a potential indicator for a specific nutrient. However, increases in NEO:STX, GTX1,4:STX, C1,2:STX and potentially GTX1,4:NEO all appear to be indicators of N-limitation of *A. fundyense* strains from the Gulf of Maine and Casco Bay.

Table 1
Summary of N- and P-batch or semi-continuous data from the literature and our laboratory showing increasing (**U**) or decreasing (**D**) trends in toxin content and or composition

	Nitrogen starvation or limitation							Phosphate limitation	
	Anderson et al. (1990a) Semi-continuous	Boczar et al. (1988) Batch culture	MacIntyre et al. (1997) Mesocosm	MacIntyre et al. (1997) Semi-continuous	Dyhrman, 2000 (unpub.) Semi-continuous	Poulton (2001) Mesocosm	Poulton (2001) Field	Anderson et al. (1990a) Semi-continuous	Taroncher–Oldenburg (1998) Semi-continuous
Alexandrium species	*A. fundyense*	*A. tamarense*	*A. tamarense*	*A. tamarense*	*A. fundyense*	*A. fundyense*	*Alexandrium*	*A. fundyense*	*A. fundyense*
Location	GOM		GSL	GSL	CB	CB	CB	GOM	GOM
fmol/cell (TOTAL)	**D** (80–20)	**D** (30–2)	**D** (250–75)	**D** (250–80)	**D** (150–50)	**D** (125–60)	N/A	**U** (250–1000)	**U** (130–550)
STX	**D**	**D**	**D**	**D**	**D**	**D**	N/A	**U**	**U**
NEO	**D**	**D**	**D**	**D**	**D**	**D**	N/A	**U**	**U**
C 1,2	**D**	–	**D**	**D**	**D**	**D**	N/A	**U**	**U**
GTX 1,4	**D**	–	–	–	**D**	NC	N/A	**U**	**U**
GTX 2,3	**D**	–	–	–	**D**	**D**	N/A	**U**	**U**
Mole % total toxin									
STX	**D** (20–0)	**D** (40–2)	**D** (18–4)	**D** (17–2)	**D** (15–3)	**D** (20–7)	**D** (10–0)	**D** (30–10)	**D** (20–10)
NEO	**D** (25–8)	**U** (10–45)	**U** (30–40)	**U** (25–30)	**D** (25–18)	**U** (15–25)	**D** (30–15)	NC	**D** (20–10)
C 1,2	**U** (20–50)	–	NC	**U** (55–65)	**U** (15–30)	**D** (30–20)	**D** or NC	NC	NC
GTX 1,4	**U** (12–35)	–	–	–	**U** (15–20)	**U** (10–20)	**U** (25–45)	NC	NC
GTX 2,3	**D** (14–2)	–	–	–	**D** (35–25)	NC	NC	**U** (35–65)	**U** (15–50)
GTX 5	NC	–	–	–	–	–	–	–	
Toxin ratios									
NEO:STX	**U** (1–20)	**U**—(0.5–1)	N/A	N/A	**U** (1–8)	**U** (1–4)	**U** (3–7)	**U**—(0.4–1)	**U** (0.6–1)
GTX 1,4:STX	**U** (1–50)	–	–	–	**U** (1–10)	**U** (0.5–3)	**U** (6–15)	**U**—(0.4–0.8)	NC
C 1,2:STX	**U** (2–120)	–	N/A	N/A	**U** (1–12)	**U** (1.5–5)	**U**—(0.5–1.5)	**U**—(0.2–1)	NC
GTX 2,3:STX	**U** (1–10)	–	–	–	**U** (2–10)	**U** (1–4)	**U** (4–12)	**U** (2–6)	**U** (4–9)
GTX 1,4:NEO	**U** (0.5–4)	–	–	–	**U**—(0.7–1.2)	NC	**U** (1–4)	NC	NC
C 1,2:NEO	**U** (2–8)	–	N/A	N/A	**U**—(0.5–1.5)	**D**—(2–1)	NC	NC	NC
GTX 2,3:NEO	NC	–	–	–	NC	**D**—(1.6–0.8)	**U**—(1–2)	**U** (3–9)	NC
C 1,2:GTX 1,4	**U** (1–3)	–	–	–	**U**—(0.5–1.5)	**D**—(3–1)	NC	NC	**U** (4.5–7)
C 1,2:GTX 2,3	**U** (1–7)	–	–	–	**U**—(0.5–1.5)	NC	NC	NC	NC

D = decreasing trend as limitation becomes more severe (— = weak trend); **U** = increasing trend as limitation becomes more severe (— = weak trend); NC = no change; N/A = not applicable; CB = Casco Bay, Maine; GOM = Gulf of Maine; GSL = Gulf of St. Lawrence; ( ) = Denotes actual range in values from the literature or observed (where applicable).

During the 1998 Casco Bay field season, large changes in relative proportions (mol% total toxin) and toxin ratios were observed. Throughout most of the stations the increasing mol% of GTX1,4 and decreasing mol% of STX were consistent with previous studies using N-limited cultures (Table 1). The other relative toxin abundances, such as C 1,2 and GTX 2,3 did not vary significantly. Overall the relative proportions of different STX derivatives to the total toxin alone do not provide enough information or criteria to determine *A. fundyense* nutrient status in the field. Since information is lost when relative proportions are calculated, such as when toxin content changes, a more logical measure of toxin composition uses more robust values of toxin concentration, such as, absolute concentrations (pmol toxin l^{-1} or fmol $cell^{-1}$) of the different STX derivatives (Taroncher-Oldenburg et al., 1999). The combined approach using both relative proportions and toxin ratios is useful when examining changes in physiology or attempting to identify physiological indicators of nutrient stress.

Consistent with the N-limited culture experiments in Table 1, large increases in the molar toxin ratios, NEO:STX, GTX1,4:STX, GTX2,3:STX and GTX1,4:NEO (Figs. 8–10) were observed during the latter portion of the Casco Bay field season. This coincided with a period of very low N:P ratios (0–4; Fig. 4C) and low or undetectable DIN concentrations in surface waters that indicate potentially N-limiting conditions (Fig. 4B). Overall, the field toxin composition data are generally consistent with N-limited populations of *A. fundyense*. Trends in three of the four ratios that are indicative of N-limiting conditions in laboratory cultures were observed in the field populations. In particular, GTX1,4:STX increased dramatically, and this ratio has only been observed with N-limitation.

One inconsistency between the laboratory culture results and the Casco Bay field data relates to the low abundance of C 1,2 toxins in the field samples. During N-replete conditions, isolates from Casco Bay or the Gulf of Maine typically contain 20–30% C-toxins or between 30 and 40 fmol $cell^{-1}$. Furthermore, mol% C 1,2 typically increases during semi-continuous N-limitation (Table 1; Anderson et al., 1990a; MacIntyre et al., 1997; S. Dyhrman, unpubl.), yet low C 1,2 concentrations were continually observed in the field samples. Although interconversions via hydrolysis do occur, they appear unlikely since the hydrolyzed products of C 1 and C 2 toxins are GTX 2 and GTX 3, and the abundance of GTX 2,3 was not elevated compared to N-replete conditions. There is a clear need for further investigation on how Casco Bay isolates vary their toxicity (and particularly the C toxins) in response to N-limitation, and how genetically similar isolates from Casco Bay are to natural populations through time in the field.

Several toxins appear to be more abundant at inshore stations compared to offshore stations. This inshore/offshore trend was observed in the mol% STX distribution, as well as in C 1,2. Using a Tukey's multiple comparison test, the offshore station, D1 was significantly different ($P<0.05$) from the inshore station, D12, where the mol% STX was approximately 10% inshore and 5% offshore. Since inshore stations are potentially exposed to greater quantities of nutrients due to riverine inputs, and STX is known to increase as nutrient availability increases (Table 1), the observed differences between inshore and offshore toxicity may be nutrient-driven.

Some of the stations (e.g., Cundy's Harbor, Lumbo's Hole) were in sheltered areas with limited exchange with the adjacent coastal waters except under certain circumstances, e.g. winds directed to the SE (Janzen et al., 2005), and thus it is reasonable to expect that the repeated sampling followed the same population through time. Once populations have been delivered into Casco Bay from the adjacent coastal waters during downwelling-favorable conditions, some of that population may be retained within Casco Bay because it is out of the coastal flow, trapped by the freshwater flow from the Kennebec River plume and onshore winds, e.g., directed to the NW (Keafer et al., 2005b; Janzen et al., 2005). Thus, trends in toxin composition observed at those sites (Figs. 7 and 10) were similar to those observed for the more-exposed, offshore transect stations because of a common origin and common nutrient conditions but the composition can diverge over time as the inshore population becomes isolated from the coastal flow. This is consistent with the similarities in toxin composition observed for six different Casco Bay isolates of *A. fundyense* that we analyzed, and with toxicity patterns observed in a larger study of *Alexandrium* isolates in the Gulf of Maine (Anderson et al., 1994).

Overall the evidence provided indicates that *A. fundyense* cells became progressively N-limited during the bloom season. The rapid decline in DIN

in surface waters and the changes in the toxin composition profile and cell abundance all are consistent with progressive N-limitation, with one exception, namely the relatively low abundance of C toxins in the field. Since most of the trends observed were similar for both the on- and offshore cell populations, this suggests that populations throughout the region may have had similar nutritional histories.

One confounding factor that may have an influence on the data presented here is the cross-reactivity that is now known to occur (Anderson et al., 2005) with antibody M-8751-1 and *A. ostenfeldii* cells. Our methods assumed that all *Alexandrium* cells counted were capable of PSP toxin production, so samples containing significant numbers of *A. ostenfeldii* cells would have low calculated toxin quotas. *A. ostenfeldii*, which is now known to co-occur with *A. fundyense* in the Gulf of Maine (Gribble et al., 2005), is a producer of the recently identified spirolide class of algal toxins, but is not yet associated with PSP toxin synthesis in N. American waters (Cembella et al., 2000; Gribble et al., 2005). It is difficult to assess retrospectively the extent of *A. ostenfeldii* influence on estimates of *A. fundyense* abundance. Anderson et al. (2005, their Fig. 6) have compared abundance data generated by an oligonucleotide probe (demonstrated to label *A. fundyense* and not *A. ostenfeldii*) with results from antibody (M8751-1) staining for samples collected in the Casco Bay region during the spring, 2000. A regression analysis of oligonucleotide- vs. antibody-based counts showed a slope of 0.9041 and an r^2 value of 0.75. Thus the antibody tended to overestimate *A. fundyense* abundance due to its cross-reactivity with *A. ostenfeldii*, but the influence of the latter species was not large. This view is supported by the general agreement between the timing of peak antibody-based *A. fundyense* estimates and the peak in 1998 shellfish toxicity at moored mussel sites near Casco Bay coincident with this study (Keafer et al., 2005b). Nevertheless, the low and highly variable toxin content values observed at certain phases of the bloom and that precluded conclusions about nutrient status may reflect the inaccuracies with our *A. fundyense* cell counts. We note, however, that our conclusions regarding progressive N-limitation of *A. fundyense* cells in 1998 are predominantly based on measurements of toxin composition, which would not be affected in any way by the presence or absence of *A. ostenfeldii*.

5. Conclusions

The observed trends in the toxin ratios for *A. fundyense* were generally indicative of N-limitation as bloom termination occurred at the end of the field season. These changes in toxicity patterns are dependent on the initial toxin profile of the strain from the region of interest, and these toxins vary to different degrees with a given stress. For example, the *A. fundyense* isolates from the Casco Bay region produce five dominant toxins (>15–20%), whereas a Gulf of St. Lawrence *A. tamarense* isolate has only three major toxins; so changes in toxin composition will most likely differ in magnitude. Therefore, prior to using these indicators in the field, it is necessary to characterize the response of regional isolates to a suite of nutritional stresses.

This initial examination of toxicity in *A. fundyense* field populations has raised many questions regarding physiological responses to environmental stress. Additional field and laboratory studies are needed, for example how the role of other physical factors such as temperature impact the utility of toxins, in order to verify the results presented here, which are compelling, but not conclusive. One key issue that needs more focus in future field studies is the ability to accurately determine toxin content when cells and toxicity are both low in concentration. Further method development and careful sample processing are required in order to obtain accurate cell counts and toxin measurements.

In conclusion, this study examined for the first time changes in both toxin concentration and composition throughout an *A. fundyense* bloom. Many environmental factors, such as temperature, stratification, and nutrient concentration, changed dramatically during the bloom season, yet it was still possible to discern patterns in toxin composition that were generally consistent with progressive N-limitation. That this was possible in a dynamic coastal environment is truly remarkable, and this type of study should therefore be repeated, both in the Casco Bay region and elsewhere. Further efforts are clearly needed to prove the validity of what appears to be a robust indicator of N-limitation for toxic *Alexandrium* species in natural waters.

Acknowledgments

We thank the shipboard and technical assistance of Kristin Gribble, Sonya Dyhrman, Jim Churchill, Jeff Turner, Dave Lawrence, Dave Kulis, Ted

Loder, Robert Brudrow, Becky Love, Mario Sengco and the crew of the RV *Gulf Challenger* from the University of New Hampshire, Durham NH. This research was supported by the US ECOHAB-GOM (Ecology and Oceanography of Harmful Algal Blooms-Gulf of Maine) Program National Science Foundation Grant OCE – 9808173 and the EPA Star Graduate Fellowship (# U-915038-01-0). Contribution #156 from the US ECOHAB Program, sponsored by NOAA, NSF, EPA, NASA and ONR.

References

Adachi, M., Sako, Y., Ishida, Y., Anderson, D.M., Reguera, B., 1993. Cross-reactivity of five monoclonal antibodies to various isolates of *Alexandrium* as determined by an indirect immunofluorescence method. Nippon Suisan Gakkaishi 59 (10), 1807.

Anderson, D.M., 1990. Toxin variability in *Alexandrium* species. In: Graneli, E., Sundström, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier, New York, pp. 41–51.

Anderson, D.M., 1997. Bloom dynamics of toxic *Alexandrium* species in the northeastern U.S. Limnology and Oceanography 42, 1009–1022.

Anderson, D.M., 1998. Physiology and bloom dynamics of toxic *Alexandrium* species, with emphasis on life cycle transitions. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms, vol. 4. Springer, Berlin, pp. 29–48.

Anderson, D.M., Lindquist, N.L., 1985. Time-course measurements of phosphorus depletion and cyst formation in the dinoflagellate *Gonyaulax tamarensis*. Journal of Experimental Marine Biology and Ecology 86, 1–13.

Anderson, D.M., Kulis, D.M., Binder, B.J., 1984. Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: cyst yield in batch cultures. Journal of Phycology 20, 418–425.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., 1990a. Toxin composition variations in one isolate of the dinoflagellate *Alexandrium fundyense*. Toxicon 28 (8), 885–893.

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990b. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Marine Biology 104, 511–524.

Anderson, D.M., Kulis, D.M., Doucette, G.J., Gallagher, J.C., Balech, E., 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Marine Biology 120, 467–478.

Anderson, D.M., Kulis, D.M., Keafer, B.A., 1999. Detection of the toxic dinoflagellate *Alexandrium fundyense* (dinophyceae) with oligonucleotide and antibody probes: variability in labeling intensity with physiological condition. Journal of Phycology 35, 870–883.

Anderson, D.M., Kulis, D.M., Keafer, B.A., Gribble, K.E., Marin, R., Scholin, C.A., 2005. Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.015].

Boczar, B.A., Beitler, M.K., Liston, J., Sullivan, J.J., Cattolico, R.A., 1988. Paralytic shellfish toxins in *Protogonyaulax tamarensis* and *Protogonyaulax catenella* in axenic culture. Plant Physiology 88, 1285–1290.

Boyer, G.L., Sullivan, J.J., Andersen, R.J., Harrison, P.J., Taylor, F.J.R., 1987. Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. Marine Biology 96, 123–128.

Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms, vol. 41. Springer, Berlin, pp. 381–403.

Cembella, A.D., Sullivan, J.J., Boyer, G.L., Taylor, F.J.R., Andersen, R.J., 1987. Variation in paralytic shellfish toxin composition within the *Protogonyaulax tamarensis/catenella* species complex: red tide dinoflagellates. Biochemical Systematics and Ecology 15, 171–186.

Cembella, A.D., Lewis, N.I., Quilliam, M.A., 2000. The marine dinoflagellate *Alexandrium ostenfeldii* (dinophyceae) as the causative organism of spirolide shellfish toxins. Phycologia 39, 67–74.

Churchill, J.H., Pettigrew, N.R., Signell, R.P., 2005. Structure and variability of the Western Maine Coastal Current. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.019].

Doucette, G.J., Turner, J.T., Powell, C.L., Keafer, B.A., Anderson, D.M., 2005. Trophic accumulation of PSP toxins in zooplankton during *Alexandrium* blooms in Casco Bay, Gulf of Maine, April–June, 1998. I. Toxin levels in *A. fundyense* and zooplankton size fractions. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.031].

Estrada, M., Berdalet, E., 1998. Effects of turbulence on phytoplankton. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms, vol. G 41. Springer, Berlin.

Etheridge S.M., Roesler, C., 2005. Effects of temperature, irradiance, and salinity on photosynthesis, growth rates, total toxicity, and toxin composition for *Alexandrium fundyense* isolates from the Gulf of Maine and Bay of Fundy. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.026].

Flynn, K., Franco, J.M., Fernandez, P., Reguera, B., Zapata, M., Wood, G.J., Flynn, K.J., 1994. Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen or phosphorous stress. Marine Ecology Progress Series 111, 99–109.

Flynn, K., Jones, K.J., Flynn, K.J., 1996. Comparisons among species of *Alexandrium* (dinophyceae) grown in nitrogen- or phosphorous-limiting batch culture. Marine Biology 126, 9–18.

Franks, P.J.S., Anderson, D.M., 1992. Alongshore transport of a toxic phytoplankton bloomin a buoyancy current: *Alexandrium tamarense* in the Gulf of Maine. Marine Biology 112, 153–164.

Gribble, K.E., Keafer, B.A., Quilliam, M., Cembella, A.D., Kulis, D.M., Anderson, D.M., 2005. Distribution and toxicity of *Alexandrium ostenfeldii* (dinophyceae) in the Gulf of Maine, USA. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.018].

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.),

Culture of Marine Invertebrate Animals. Plenum Publishing Corp., New York, pp. 29–60.
Hall, S., 1982. Toxins and toxicity of *Protogonyaulax* from the Northeast Pacific. Ph.D. Thesis, University of Alaska, Fairbanks, p. 196.
Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 79–99.
Janzen, C.D., Churchill, J.H., Pettigrew, N.R., 2005. Observations of bay/shelf exchange between eastern Casco Bay and the western Gulf of Maine. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.032].
Keafer, B.A., Churchill, J.H., McGillicuddy, D.J., Anderson, D.M., 2005a. Bloom development and transport of toxic *Alexandrium fundyense* populations within a coastal plume in the Gulf of Maine. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.016].
Keafer, B.A., Churchill, J.H., Anderson, D.M., 2005b. Blooms of the toxic dinoflagellate, *Alexandrium fundyense*, in the Casco Bay region of the western Gulf of Maine: Advection from offshore source populations and interactions with the Kennebec River plume. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.017].
Love, R.C., Loder, T.C., Keafer. B.A., 2005. Nutrient conditions during *Alexandrium fundyense* blooms in the western Gulf of Maine, USA. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.030].
MacIntyre, J.G., Cullen, J.J., Cembella, A.D., 1997. Vertical migration, nutrition and toxicity in the dinoflagellate *Alexandrium tamarense*. Marine Ecology Progress Series 148, 201–216.
Martin, J.L., Page, F.H., Hanke, A., Strain, P.M., LeGresley, M.M., 2005. *Alexandrium fundyense* vertical distribution patterns during 1982, 2001, and 2002 in the offshore Bay of Fundy, Eastern Canada. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.010].
Martorano, C.D., 1997. Nutrient dynamics during blooms of *Alexandrium* spp. in the southwestern Gulf of Maine. M.Sc. Thesis, University of New Hampshire, Durham, NH, p. 132.
Matsuda, A., Nishijima, T., Fukai, K., 1996. Effects of nitrogen deficiency on the PSP production by *Alexandrium catenella* under axenic cultures. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. UNESCO, pp. 305–308.
Ogata, T., Ishimaru, T., Kodama, M., 1987. Effect of water temperature and light intensity on growth rate and toxicity change in *Protogonyaulax tamarensis*. Marine Biology 95, 217–220.
Oshima, Y., 1995. Post-column derivatization HPLC methods for paralytic shellfish poisons. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae, IOC Manuals and Guides. UNESCO. No. 33, pp. 81–94.
Oshima, Y.S., Sugino, K., Yasumoto, T., 1989. Latest advances in HPLC analysis of paralytic shellfish toxins. In: Mycotoxins and Phycotoxins. Proceedings of the Seventh IUPAC Symposium.
Oshima, Y., Blackburn, S.I., Hallegraeff, G.M., 1993. Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. Marine Biology 116, 471–476.
Parkhill, J.P., Cembella, A.D., 1999. Effects of salinity, light and inorganic nitrogen on growth and toxigenicity of the marine dinoflagellate *Alexandrium tamarense* from northeastern Canada. Journal of Plankton Research 21, 939–955.
Poulton, N.J., 2001. Physiological and behavioral diagnostics of nitrogen limitation for the toxic dinoflagellate *Alexandrium fundyense*. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, p. 246.
Redfield, A.C., 1958. The biological control of chemical factors in the environment. American Scientist 46, 205–221.
Rhee, G.Y., 1978. Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. Limnology and Oceanography 23, 10–25.
Sako, Y., Adachi, M., Ishida, Y., 1993. Preparation and characterization of monoclonal antibodies to *Alexandrium* species. In: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms of the Sea. Elsevier, Amsterdam, pp. 87–93.
Sako, Y., Naya, N., Yoshida, T., Uchida, C.H., Ishida, Y., 1995. Studies on stability and heredity of PSP toxin composition in the toxic dinoflagellate *Alexandrium*. In: Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Lavoisier, pp. 345–350.
Scholin, C.A., Hallegraeff, G.M., Anderson, D.M., 1995. Molecular evolution of the *Alexandrium tamarense* 'species complex' (dinophyceae): dispersal in the North American and West Pacific regions. Journal of Phycology 34, 472–485.
Taroncher-Oldenburg, G., 1998. Cell cycle dynamics and physiology of saxitoxin Biosynthesis in *Alexandrium fundyense* (dinophyceae). Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, p. 205.
Taroncher-Oldenburg, G., Kulis, D.M., Anderson, D.M., 1999. Coupling of saxitoxin biosynthesis to the G1 phase of the cell cycle in the dinoflagellate *Alexandrium fundyense*: temperature and nutrient effects. Natural Toxins 7, 207–219.
Townsend, D.W., Pettigrew, N.R., Thomas, A.C., 2001. Offshore blooms of the red tide dinoflagellate, *Alexandrium* sp., in the Gulf of Maine. Continental Shelf Research 21, 347–369.
Townsend, D.W., Bennett, S.L., Thomas, M.A., 2005. Diel vertical distributions of the red tide dinoflagellate *Alexandrium* spp. in the Gulf of Maine. Deep-Sea Research II, this issue [doi:10.1016/j.dsr2.2005.06.027].
Turner, J.T., Doucette, G.J., Powell, C.L., Kulis, D.M., Keafer, B.A., Anderson, D.M., 2000. Accumulation of red tide toxins in larger fractions of zooplankton assemblages from Massachusetts Bay, USA. Marine Ecology Progress Series 203, 95–107.
Valderrama, J.C., 1981. The simultaneous analysis of total nitrogen and total phosphorous in natural waters. Marine Chemistry 10, 109–122.
White, A.W., 1978. Salinity effects on growth and toxin content of *Gonyaulax excavata*, a marine dinoflagellate causing paralytic shellfish poisoning. Journal of Phycology 14, 475–479.