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Association of the gonyaulacoid dinoflagellate *Alexandrium ostenfeldii* with spirolide toxins in size-fractionated plankton

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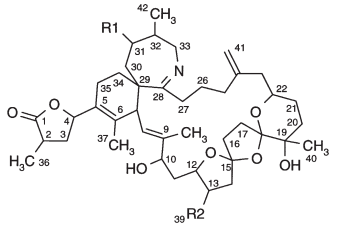
Spirolides are pharmacologically active macrocyclic imines discovered in shellfish and plankton size-fractions collected from near-surface waters along the southeastern coast of Nova Scotia (NS), Canada. These compounds are most prevalent in the water column within plankton size-fractions from 21 to 56 μm during late spring. Analysis of bulk plankton material by liquid chromatography combined with ion-spray mass spectrometry indicated that gonyaulacoid dinoflagellates were dominant members of the plankton assemblage in these fractions when spirolides were abundant at Graves Shoal, NS, but that spirolides disappeared from the water column after a shift to diatom dominance. Spirolide composition in the plankton was rather consistent over time and among different size-fractions within a site, but the profile was radically different between sites in Nova Scotia. At Ship Harbour, NS, spirolides A, B, C and desmethyl-C were the major constituents, whereas at Graves Shoal, the primary spirolide derivatives were B, D and an isomer D2. Correlation analysis of spirolide abundance and the occurrence of particular taxa in size-fractionated plankton showed that the highest correlations were with Alexandrium species ($r^2 = 0.93$) and GB42 cells ($r^2 = 0.83$). These latter cells are now recognized as athecate forms of gonyaulacoid dinoflagellates, primarily of Alexandrium ostenfeldii. By analysis of size-fractionated plankton and comparison with cultured plankton isolates from spirolide-rich areas, we have substantiated the primary role of A. ostenfeldii as the source of spirolides in the water column.

INTRODUCTION

In the early 1990s, an unusual toxin syndrome was first associated with extracts of shellfish from certain aquaculture sites along the southeastern coast of Nova Scotia (NS), Canada (Cembella *et al.*, 1998). Potent pharmacological activity, expressed as neuro-convulsions and rapid death (within minutes) after intraperitoneal injection of lipophilic extracts into laboratory mice used for routine shellfish toxin testing, was typically confined to late spring–early summer (May–June). The seasonal but annual recurrence of this phenomenon, the association of toxicity only with the viscera of scallops and mussels, and the apparently restricted geographical distribution, suggested a planktonic vector linked to suspension feeding by bivalve molluscs.

A novel group of macrocyclic imines, termed spirolides, isolated from scallop and mussel viscera during these toxic episodes (Hu *et al.*, 1995, 1996), was found to be responsible for the toxicity in mice. Several derivatives have now been structurally characterized from shellfish and planktonic sources (Hu *et al.*, 2001). Spirolides A, B, C and D and their desmethyl derivatives are biologically active (Figure 1), whereas E and F appear to be inactive degradation products in shellfish (Hu *et al.*, 1996).

Despite the circumstantial association of spirolides with a planktonic origin, the identification of the causative organism remained problematic for several years. The occurrence of spirolides in shellfish viscera typically coincided with the period after the decline of the spring bloom of centric diatoms (*Skeletonema*, *Thalassiosira*, etc.), when thecate dinoflagellates dominated in the upper water



	R1	R2		MW
A	H	CH_3	$\Delta^{2,3}$	691.5
desMeC	CH_3	H	$\Delta^{2,3}$	691.5
B	H	CH_3		693.5
desMeD	CH_3	H		693.5
C	CH_3	CH_3	$\Delta^{2,3}$	705.5
D	CH_3	CH_3		707.5

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

column. This thecate dinoflagellate assemblage was generally a heterogeneous mixture of *Gonyaulax* spp., *Alexandrium* spp., *Scrippsiella trochoidea*, *Protoperidinium brevipes*, and *Dinophysis* spp., accompanied by tintinnids (Cembella *et al.*, 1998). Also present were high numbers of golden-pigmented spherical cells of 42 μm diameter (GB42), which were subsequently identified as athecate cells of gonyaulacoid dinoflagellates, primarily *Alexandrium ostenfeldii* (Paulsen) Balech *et* Tangen (Cembella *et al.*, 2000).

Spirolides in the plankton were initially detected in bulk concentrates and later in pooled micropipette isolates of individual cells, tentatively identified as *Alexandrium* spp., from field populations in Nova Scotia (Cembella *et al.*, 1999). The extraction of spirolides from clones of *A. ostenfeldii* in unialgal culture (Cembella *et al.*, 2000) confirmed this species as the source of the compounds. The current study is a retrospective analysis of size-fractionated field plankton collected from two key sites characterized by high seasonal abundance of spirolides in the water column. The objective was to determine the relationship between plankton and spirolide composition in particular size-fractions. This study is the first concerted attempt to describe the spatio-temporal distribution of dinoflagellate metabolites that are presumably derived via polyketide biosynthesis in various plankton size classes from different sites.

METHOD

At irregular intervals during June and July 1996, bulk plankton samples were collected by pumping large volumes (>3 × 10^3 l) of near-surface water (from 3 m depth) at Graves Shoal and Ship Harbour, two key shellfish aquaculture sites on the eastern coast of Nova Scotia. Plankton samples for spirolide analysis were concentrated by pumping with a high-volume (200 l min^{-1}) centrifugal pump into a conical Nitex plankton net (0.5 × 2.5 m) of 20 μm mesh. The contents of the cod end were size fractionated through a stacked series of Nitex screens (95, 76, 56, 44, 26 and 21 μm). Live cells from the 21–26 and 26–44 μm fractions were retained for culturing, and a sample of each size-fraction was diluted 1:10 in filtered seawater and preserved in 2% formalin–acetic acid (FAA) for plankton identification by optical microscopy at ×250. Vertical plankton net (20 μm mesh) tows were also taken at each site from a depth of 15 m and analysed by microscopy for qualitative plankton composition.

Physical data (temperature, salinity, σ_t) on the stratification of the water column were obtained by a conductivity–temperature–depth (CTD) sensor (Applied Microsystems STD-12 Plus). A vertical CTD cast was made at each sampling site and time interval, to a depth of 20 m at Graves Shoal and 15 m at Ship Harbour.

Concentrated plankton samples were extracted by sonication in 10:1 (wet weight:volume) 100% methanol. After centrifugation at 5000 *g* for 15 min, 0.5 ml of supernatant was centrifuged through a 0.45 μm spin-filter cartridge (Ultrafree-MC, Millipore).

Spirolide composition was determined by liquid chromatography with ion-spray mass spectrometry (LC-MS) on a Hewlett-Packard (Palo Alto, CA) HP1090 LC system coupled to a Perkin-Elmer/SCIEX (Concord, Canada) API-III+ triple quadrupole MS system. Analyses were conducted in positive ion mode using selected ion monitoring of the $[M+H]^+$ ions. Chromatographic separation of spirolides was carried out using a 250 × 2.1 mm i.d. column packed with 5 μm Vydac 201TP (C18) and 0.2 ml min^{-1} of mobile phase composed of acetonitrile/water (35:65, v/v) with 0.1% (v/v) trifluoroacetic acid. The column effluent was split, with only 10% going to the mass spectrometer. Calibration was performed against standards of pure spirolides B and D prepared from scallop viscera. Relative molar response factors for other spirolides were assumed to be the same.

RESULTS

Plankton composition and distribution

According to weekly mouse toxicity assays of extracts of shellfish viscera from Nova Scotia (S. Hancock, Canadian Food Inspection Agency, Dartmouth, NS), the symptoms of spirolide toxicity were most evident during May and June, 1996. During peak toxicity in June, mouse death times after intraperitoneal injection were typically <8 min. This period coincided with the initiation of stratification of the water column at Graves Shoal, NS (Figure 2). The density structure was dependent upon late vernal warming and the effect of fresh water run-off, although limited input of freshwater and substantial exchange with adjacent coastal waters tended to minimize the salinity-dependent stratification at Graves Shoal.

In May and early June, the identifiable net plankton

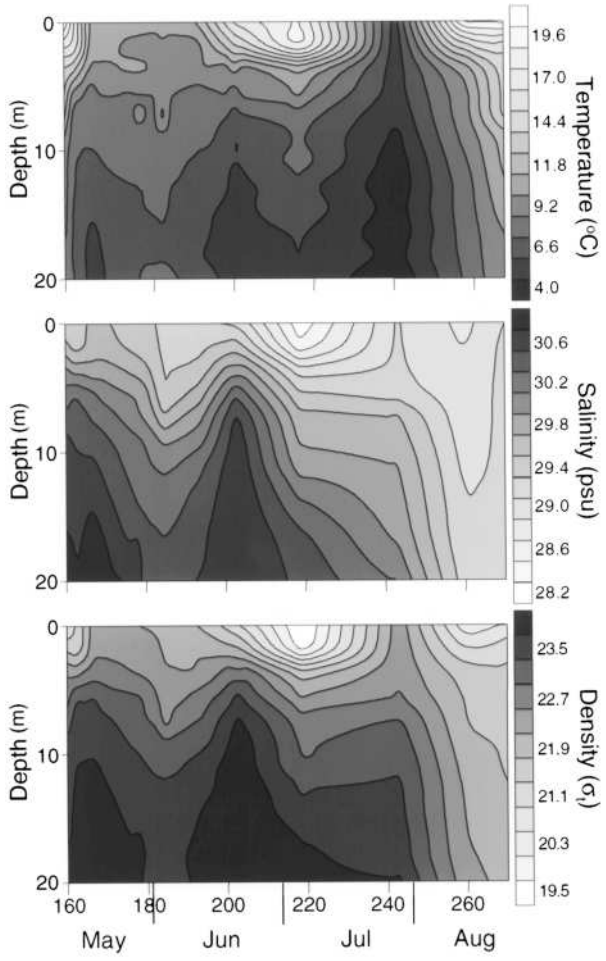


Fig. 2. Physical data on the stratification of the water column at Graves Shoal, NS, during late spring and summer, 1996 (Julian days). Variables are temperature (°C), salinity (p.s.u.) and density (σ_t).

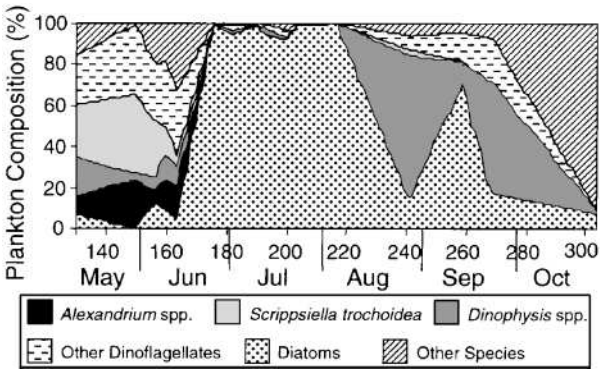


Fig. 3. Plankton composition (% numerical abundance) in vertical net tows (>20 μm) from 15 m depth at Graves Shoal, NS, from spring to autumn, 1996 (Julian days). Dominant taxa among the dinoflagellates are indicated as genus or species.

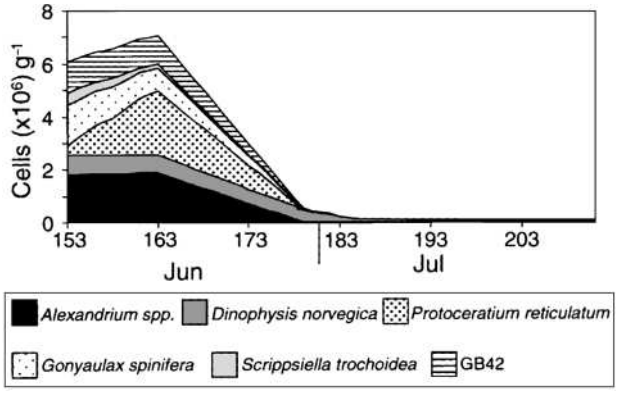


Fig. 4. Abundance of major dinoflagellates in the 26–44 μm size-fraction from 3 m depth collected when spirolides were present in the water column at Graves Shoal, 1996 (Julian days). Note that for this filled-area plot, the concentration of cells represented for each taxon is not cumulative, but is indicated by the difference in the area between adjacent taxa above and below.

composition (>20<150 μm) was dominated by thecate dinoflagellates (Figure 3), primarily *S. trochoidea*, *Alexandrium* spp. (*tamarense* and *ostenfeldii*) and *Dinophysis* spp., with minor contributions by *Gonyaulax alaskensis*, *Gonyaulax spinifera*, *Protoceratium reticulatum* (= *Gonyaulax grindleyi)*, *Protoperidinium* spp., *Amylax triacantha*, *Heterocapsa triquetra*, *Cachonina niei* and *Ceratium longipes*. Other dinoflagellates, including *Prorocentrum lima*, *Prorocentrum gracile* and *Dissodium Sp.*, were found occasionally, while the non-thecate dinoflagellate *Gyrodinium spirale* was common but in low abundance in samples during this period. By the end of June, coinciding with the disappearance of spirolides from the water column and shellfish viscera, diatoms such as *Chaetoceros laciniosus* and *Skeletonema costatum* had assumed dominance in the water column. Spirolides did not recur in the plankton even after a shift back to flagellate dominance with the reappearance of *Dinophysis norvegica* and *S. trochoidea* in late August. Significantly, *Alexandrium* was not present in vertical net tows after the initial bloom in May to early June.

At Graves Shoal, the general distributional pattern among the key dinoflagellate species found in the net tows, dominated by *S. trochoidea*, *Alexandrium* spp. and other thecate dinoflagellates, was also reflected in the composition of the 26–44 μm size-fraction analysed for spirolides (Figure 4). During the peak in spirolides in early June, the smallest size-fraction (21–26 μm) contained primarily *S. trochoidea*, *Protoperidinium brevipes*, *Alexandrium* spp. and smaller morphotypes resembling GB42 cells. At this time, in the 44–56 μm size-fraction, *Alexandrium* spp. (6.5×10^5 cells g^{-1} wet weight of plankton) co-dominated with mollusc eggs/larvae, along with lesser amounts of *Protoceratium reticulatum*, the tintinnid *Tintinnopsis* sp. and GB42 cells. The larger size-fractions (56–76, 76–95 and >95 μm) contained relatively few *Alexandrium* cells (<0.35×10^5 cells g^{-1} wet weight). In the 56–76 μm fraction, *Tintinnopsis* was

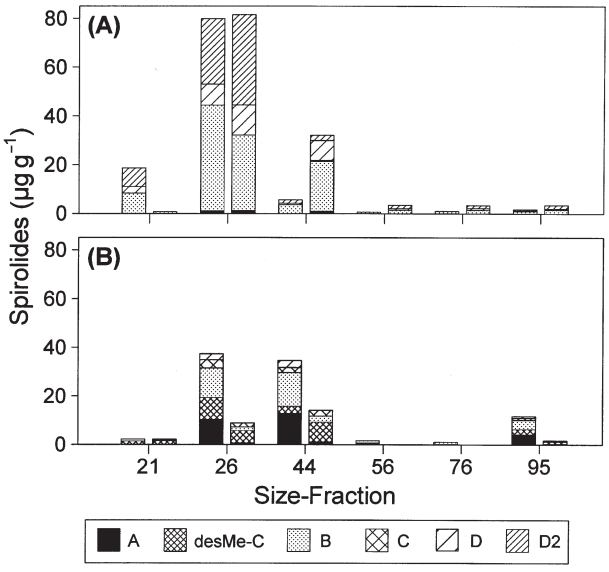


Fig. 5. Spirolide concentration (µg g^{-1}) in plankton size-fractions from 3 m depth collected at (**A**) Graves Shoal (2 June; 12 June, 1996) and (**B**) Ship Harbour (10 June; 18 June, 1996). Missing values indicate that no spirolides were detected by LC-MS.

common, but the two largest fractions were typically dominated by the silicoflagellate *Distephanus speculum*, large dinoflagellates including *Ceratium longipes* and *C. fusus*, the diatom *Chaetoceros* sp., and miscellaneous zooplankton, such as copepod nauplii and mollusc larvae.

Ship Harbour was sampled only twice in mid-June when spirolides were in abundance in the water column. Similar to mouse bioassay results of samples from Graves Shoal, spirolide toxicity in shellfish viscera coincided with stratification in the water column at Ship Harbour. However, the surface stratification was very pronounced at Ship Harbour, with a well developed pycnocline between 2 and 3 m depth (data not shown). Compared to Graves Shoal, Ship Harbour receives a more substantial freshwater run-off during the spring.

Although the most prevalent dinoflagellates (*Alexandrium*, *Dinophysis*, *Gonyaulax*, *Scrippsiella*) in the vertical net tows and the 26–44 µm size-fraction collected at 3 m were similar to those found at Graves Shoal, diatoms were dominant at Ship Harbour. By 10 June, diatoms comprised >82% of the net plankton, and >95% of the total by 18 June, when spirolides were last detected at this site (data not shown). In contrast to Graves Shoal, during the spirolide peak at Ship Harbour (10 June), cells of *Chaetoceros* species were more than an order of magnitude more abundant than those of any other species in all size-fractions. *Alexandrium* cells were common in the smaller size-fractions, 4.4×10^5 cells g^{-1} (21–26 µm), 1.2×10^6 cells g^{-1} (26–44 µm) and 4.5×10^5 cells g^{-1} (44–56 µm), respectively, but were present in only insignificant numbers in the 56–76 and 76–95 µm fractions. The fraction >95 µm consisted mainly of long chains of *Chaetoceros* (8.0×10^7 cells g^{-1}), but also contained substantial numbers of *Alexandrium* cells (3.0×10^5 cells g^{-1}). The tintinnid *Helicostomella* sp. was present, but other potential grazers upon large thecate dinoflagellates, such as rotifers and copepods, were absent or in very low abundance. The plankton data from Ship Harbour have not been plotted as a time series, as net tows and pump sampling were performed on only two dates, which, nevertheless did correspond to high abundance of both spirolides and *A. ostenfeldii* cells.

Spirolide composition of size-fractionated plankton

Detectable levels of spirolides in the plankton were found on only a few occasions at Graves Shoal and Ship Harbour. At both sites, the highest spirolide levels were typically found in the 26–44 and 44–56 µm size-fractions (Figure 5). On 10 June 1996, the spirolide content of the >95 µm fraction was anomalously high at Ship Harbour. During the peak of toxicity, on a weight-normalized basis (per gram wet weight), the total yield of spirolides from the 26–44 µm fraction was more than twice as high at Graves Shoal than at Ship Harbour. For the 26–44 µm fraction, the total spirolide content of *Alexandrium* cells at Graves Shoal was roughly constant: 4.27×10^5 µg $cell^{-1}$ on 2 June and 4.31×10^5 µg $cell^{-1}$ on 12 June. In contrast, in the same fraction from Ship Harbour, the spirolide content per *Alexandrium* cell fell from 3.16×10^5 µg on 10 June to 1.32×10^5 µg on 18 June.

The spirolide composition of the plankton (%) was dramatically different between the two sites (Figure 6). At Graves Shoal, the primary spirolide derivatives were B, D and as yet undescribed isomer D2, whereas at Ship Harbour, size-fractions were rich in spirolides A, B and desmethyl-C, with C and D often present as minor constituents. When values for total spirolides of <3.6 µg g^{-1} were eliminated from comparison (because of possible problems with detection limits of analogues in low abundance), the spirolide profiles among the size-fractions collected on different dates from Graves Shoal were remarkably consistent. A similar analysis of the profiles from Ship Harbour revealed considerably more heterogeneity among different dates and fractions.

Figure 7 shows the correlation (Pearson product-moment r^2; $n = 24$) between the abundance of particular plankton taxa and total spirolides (micrograms) and total paralytic shellfish poisoning (PSP) toxins (micrograms of saxitoxin equivalents) at Graves Shoal. The data from all size-fractions from all dates for which spirolides were detected are included in this analysis. The data on PSP toxin content (Cembella *et al.*, in prep.) are only

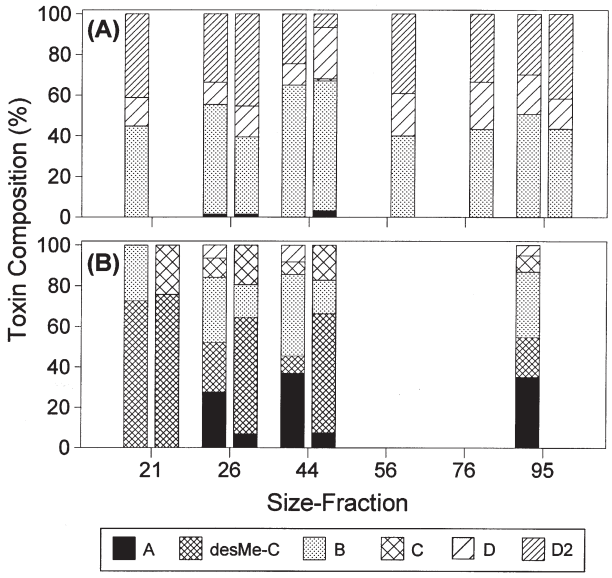


Fig. 6. Relative spirolide composition (%) in plankton size-fractions from 3 m depth collected at (**A**) Graves Shoal (2 June; 12 June, 1996) and (**B**) Ship Harbour (10 June; 18 June, 1996). Values are not shown if the total spirolide concentration was at trace levels (<1.66 μg g^{-1} wet weight).

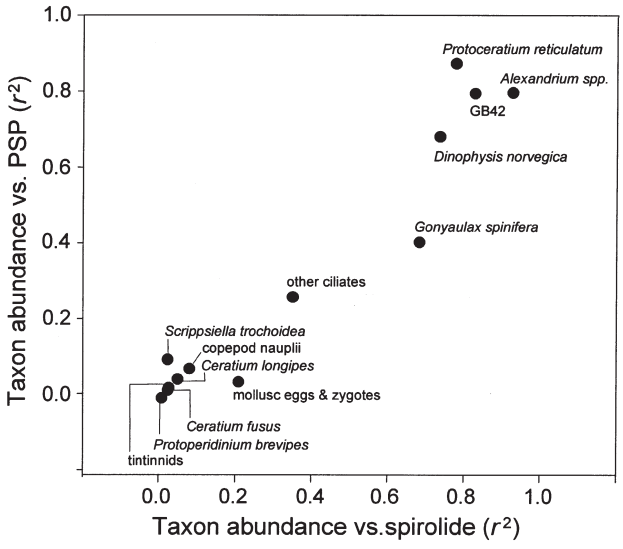


Fig. 7. Plot of correlation coefficients (Pearson product-moment: r^2 ; n = 24) between the abundance of particular plankton taxa versus total spirolides and versus paralytic shellfish poisoning (PSP) toxins at Graves Shoal, NS. The data from all size-fractions for all dates for which spirolides were detected are included in this analysis.

considered here to indicate the co-occurrence of these toxins with spirolides, and not to imply a causal relationship. Spirolide levels were highly correlated with the abundance of *Alexandrium* spp. (r^2 = 0.93), GB42 cells (r^2 = 0.83) and *P. reticulatum* (r^2 = 0.78) at this site. Certain gonyaulacoid dinoflagellates, such as *G. spinifera* and *G. alaskensis*, occurred commonly but not invariably in spirolide-rich samples from Ship Harbour and Graves Shoal. A known producer of diarrhoetic shellfish poisoning (DSP) toxins, *D. norvegica*, appeared to correlate moderately well with both PSP toxins and spirolides, but the absence of this species in many samples containing spirolides effectively rules it out as a causative agent. Other dinoflagellates, including *Protoperidinium brevipes* and *S. trochoidea*, which co-occurred during periods of high spirolide levels at Nova Scotia sites in previous years, were poorly correlated (r^2 < 0.02) with spirolides in plankton fractions from Graves Shoal in 1996.

DISCUSSION

We are now confident in attributing the proximal source of spirolides in Nova Scotia waters primarily (if not exclusively) to *A. ostenfeldii*. This conclusion is consistent with many lines of independent evidence: (1) the high correlation of *Alexandrium* cell abundance with spirolide concentrations in field samples from multiple sites; (2) the production of spirolides by a unialgal isolate of *A. ostenfeldii* from Ship Harbour, NS (Cembella *et al.*, 2000), with a toxin profile consistent with that of micropipette-isolated cells and bulk extracts of mixed plankton assemblages from this site (Cembella *et al.*, 1999); (3) the lack of spirolide production by cultured isolates of *A. tamarense* and other thecate dinoflagellates, such as *P. reticulatum*, which were highly correlated with the abundance of spirolides at field sites in NS; (4) the detection of spirolides in isolates of *A. ostenfeldii* from Limfjord, Denmark (Cembella *et al.*, 1998); and (5) the strong association of GB42 cells with *A. ostenfeldii*.

The abundance of GB42 cells, previously identified as derived from thecate dinoflagellates but with the thecae absent (possibly pellicular cysts) (Cembella *et al.*, 1998, 2000), suggests that many such cells may be created as artifacts in the process of pumping and size fractionation. The use of high-volume pumping through plankton nets is an efficient means of harvesting biomass for toxin extraction and analysis of other chemical constituents from bulk plankton samples. This method is particularly effective in preparing cell concentrates when *in situ* concentrations of target species for cell isolation for toxin micro-analysis and culture are low (<1000 cells l^{-1}). Nevertheless, the formation of significant numbers of pellicular cysts indicates that due caution must be exercised in the handling of cells to avoid cell damage and consequent loss of intracellular toxins. In the case of spirolides, evidence that the cell quota of spirolides is similar in harvested cultures of *A. ostenfeldii* and field plankton (Cembella *et al.*, 1999) and that little toxin is lost to the medium in batch culture growth of this species

(John, *et al.*, 2001) indicates that this may not be a significant problem.

We initially hypothesized that the high level of spirolides in the plankton size-fraction >95 μm might be evidence of accumulation by microzooplankton grazers and potential transfer through marine food webs. Ingestion by micrograzers could explain the higher levels of spirolides found in the largest size-fraction than in the 56–76 and 76–95 μm fractions. *Alexandrium* spp. are grazed upon by a variety of copepod species in the North Atlantic [reviewed by (Turner *et al.*, 1998)]. Tintinnids have also been observed to graze upon toxic *Alexandrium* cells on the Atlantic coast of Canada (Prakash *et al.*, 1971), although grazing experiments with tintinnids from northern Europe indicated that at least some toxigenic strains of *A. ostenfeldii* repel tintinnid grazers (Hansen *et al.*, 1992). Micrograzers, including rotifers, copepods (adults and nauplii) and tintinnids (*Tintinnopsis* spp.), were an important component of the vertical net tow (>20 μm) at Graves Shoal during the peak of spirolide toxicity, but there was only a low correlation ($r^2 < 0.15$) of spirolide levels with the abundance of microzooplankton in all size-fractions (Figure 7). Furthermore, high levels of spirolides were not observed in the >95 μm size-fraction from Ship Harbour, even though the numerical abundance of tintinnids was high. For example, the tintinnid *Helicostomella* comprised 8.4% of the total plankton, whereas species of the centric diatom *Chaetoceros* were >85% of this size-fraction. The poor correlation of microzooplankton with spirolide concentrations and the appearance of *Alexandrium* cells in the >95 μm fraction suggest that some spirolide-producing cells became trapped in the matrix of chain-forming centric diatoms (particularly *Chaetoceros* spp.) during fractionation and sample concentration.

As in other cold water environments where *A. ostenfeldii* blooms occur, such as Oslofjord, Norway (Balech and Tangen, 1985) and the lower St Lawrence estuary in eastern Canada (Levasseur *et al.*, 1998), in Nova Scotia waters, this species is maximally abundant in late spring. Graves Shoal and Ship Harbour are geographically separated by <150 km, but differ markedly in hydrographic characteristics: the former is a coastal embayment with considerable mixing of open coastal waters along the Scotian Shelf, whereas the latter is a classic estuarine fjord with a well developed sill. Nevertheless, in both hydrographic regimes, the appearance of *A. ostenfeldii* in the pelagic zone is associated with the development of a stable pycnocline and stratification of surface waters. Given the prominent hydrographic differences between Ship Harbour and Graves Shoal, the development of *A. ostenfeldii* blooms seems to be more related to the formation of the pycnocline than the direct effect of either salinity or temperature on cell growth or excystment of benthic cysts. This is also consistent with the development of blooms of *A. tamarense* in the lower St Lawrence estuary in eastern Canada (Therriault *et al.*, 1985). In the absence of a comprehensive benthic cyst survey in coastal Nova Scotia embayments, it has not yet been possible to establish whether the simultaneous appearance of *A. ostenfeldii* at multiple sites along the east coast of Nova Scotia is due to coincident excystment of indigenous populations into each water mass or to the introduction of vegetative cells to the upper water column via entrainment from outer coastal waters.

The marked and relatively stable differences in spirolide profile in the plankton from Ship Harbour and Graves Shoal suggest that the respective *A. ostenfeldii* blooms represent distinct populations. Such geographical variation in toxin expression has also been described for *Alexandrium* populations from northeastern North America that produce PSP toxins (Anderson *et al.*, 1994). In the case of spirolides in bulk plankton samples, it is not yet clear whether these differences are derived from a single genotype or if they represent a mixture of variants each expressing a different but characteristic spirolide profile within a given geographical population. Further clonal isolates from multiple sites are required to resolve this issue.

The link between the occurrence of *A. ostenfeldii* and spirolides in Nova Scotia has now been completely substantiated. Yet little is known about the population dynamics of this species in coastal waters, and the kinetics and effects of spirolides in marine food webs remain to be determined. In any case, the restricted temporal distribution of spirolide-producing *A. ostenfeldii* cells to late spring suggests that increased vigilance in monitoring *A. ostenfeldii* cell numbers and spirolide toxicity in shellfish is warranted during this period. Further studies are intended to address the vertical distribution of spirolides in the water column and details of the temporal changes in spirolide concentration in the plankton and in shellfish.

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