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Distribution and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in the Gulf of Maine, USA

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Abstract

Alexandrium ostenfeldii is a thecate, mixotrophic dinoflagellate recently linked to a novel suite of toxins called spirolides. This study provides the first description of the regional distribution of *A. ostenfeldii* in the Gulf of Maine (GOM), and the first report and analysis of spirolide toxicity in *A. ostenfeldii* in waters south of Nova Scotia. Morphological examination of cells in field samples and of clonal cultures isolated from several stations in the GOM confirmed the presence of *A. ostenfeldii*. A genus-specific antibody probe, and an *A. ostenfeldii* species-specific oligonucleotide probe labeled these cells; a probe specific for the North American *A. fundyense/tamarense/catenella* species complex did not label *A. ostenfeldii* cells. Cell size ranged from 20 to nearly 90 μm, and most cells contained food vacuoles, with a total vacuole size from 1 to 48 μm.

The hydrographic forcings controlling the distribution of *A. ostenfeldii* in the GOM are quite similar to those acting on the *A. fundyense* population at the same time of the year. The highest concentrations of *A. ostenfeldii* were observed nearshore, to the east of Penobscot Bay, at times with an offshore-turning branch of high cell concentration to the south of Penobscot Bay. Casco Bay appears to be an area of accumulation for *A. ostenfeldii* cells advected toward shore from the core of the population to the northeast. Concentrations of *A. ostenfeldii* were generally higher at the surface than deeper, except at locations where the pooling of lower-salinity water at the surface may have led to the subduction of the population flowing in from the east.

PSP toxins were detected in field populations containing *A. ostenfeldii* and *A. fundyense*, but not in *A. ostenfeldii* cultures isolated from the GOM. Spirolide toxins were found in 36 of 60 field samples. More than 83% of samples containing *A. ostenfeldii* cells had one or more of spirolide congeners A, B, C2 and D2. The total concentration of spirolides per cell at each station where *A. ostenfeldii* was detected ranged from non-detectable to 282 fmol cell^{-1}. *A. ostenfeldii* cultures originating from different locations showed a great diversity in spirolide content and composition. All cultures contained spirolides, ranging from 28 to 113 fmol cell^{-1}. Spirolide congeners desMeC, C and D were present in some cultures, but were not detected in any of the field samples. Based on differences and similarities between their toxin profiles, five toxin phenotypes were identified. The highest per cell spirolide contents in the cultures were nearly two times lower than the highest levels observed in field samples. Currently there is no routine monitoring for spirolide toxins in shellfish in the

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region, but it may be necessary eventually to expand ongoing toxin monitoring in the GOM to include analysis for spirolides.

Keywords: *Alexandrium ostenfeldii*; Gulf of Maine; Spirolides; Harmful algal blooms; Dinoflagellates

1. Introduction

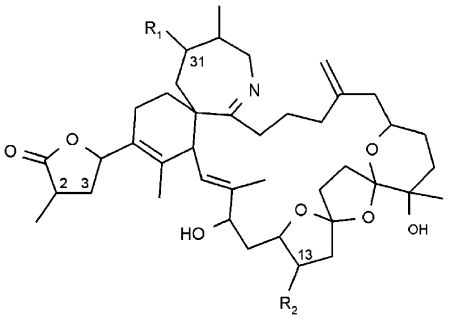
Alexandrium ostenfeldii is a thecate, mixotrophic dinoflagellate, recently associated with a novel suite of toxins called spirolides (Cembella et al., 2001). First described from Iceland by Paulsen in 1904, *A. ostenfeldii* was originally thought to be exclusively a cold-water organism, and was considered an "Arctic-boreal" species (Okolodkov and Dodge, 1996) but is now known to occur in temperate waters throughout the world. The species has been found in Europe, including in the Faroe Islands and Denmark (Moestrup and Hansen, 1988), Scotland (John et al., 2003), Norway (Balech and Tangen, 1985), Spain (Fraga and Sanchez, 1985) and Belgium (possibly synonymous with *Pyrodinium phoneus*) (Woloszynska and Conrad, 1939). In the north Pacific, *A. ostenfeldii* has been reported along the coast of Eastern Kamchatka (Konovalova, 1991) and near Tacoma, Washington (Balech, 1995) while in the southern Pacific the organism is known from New Zealand (MacKenzie et al., 1996). In the Mediterranean, *A. ostenfeldii* has been found in Alexandria Harbor, Egypt (Balech, 1995). *A. ostenfeldii* has also been reported in the western Atlantic, in the Gulf of St. Lawrence (Levasseur et al., 1997) and in Nova Scotia (Cembella et al., 1999a; Cembella et al., 1999b; Cembella et al., 2001). The first account of *A. ostenfeldii* in the Gulf of Maine (GOM) waters was in West Boothbay Harbor, Maine by Jacobson and Anderson (1996).

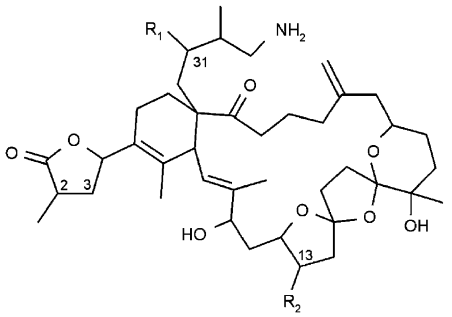
Spirolides are a group of macrocyclic imines (Fig. 1) first detected in the digestive glands of shellfish from the eastern coast of Nova Scotia in 1995 (Hu et al., 1995). The toxins are fast acting, causing rapid death in laboratory mice with neurotoxic symptoms when injected intraperitoneally or administered orally (Hu et al., 1996; Richard et al., 2000), but their toxicity to humans and mode of action are still under investigation. Several spirolide analogs have been isolated from shellfish and plankton, most of which are biologically active while others (spirolides E and F) seem to be inactive metabolites formed in shellfish (Hu et al., 2001). The structures of common known spirolides are presented in Fig. 1.

A. ostenfeldii has been shown to produce either saxitoxin derivatives, linked to paralytic shellfish poisoning (PSP) toxins, or spirolide toxins. Certain strains of *A. ostenfeldii* from Scandinavia may produce low amounts of both groups of toxins, although this requires confirmation (Cembella et al., 2001). In Denmark, *A. ostenfeldii* produces PSP toxins (Hansen et al., 1992), but some strains produce novel spirolides (MacKinnon et al., 2004). In New Zealand, however, this species typically produces saxitoxin derivatives (MacKenzie et al., 1996) but spirolide-producing isolates have not yet been found (Quilliam et al, unpublished data). In Nova Scotia, Canada, *A. ostenfeldii* produces spirolide toxins, with no evidence for saxitoxin production (Cembella et al., 2000b; Bauder et al., 2001; Cembella et al., 2001). The presence of spirolide toxins in plankton tow samples taken near the east coast of Scotland has not been conclusively linked to *A. ostenfeldii* (Ruehl et al., 2001), though the high coincidence of this species with spirolide occurrence makes a relationship likely. Recently, spirolides were detected in Norwegian mussels that tested positive in the lipophilic toxin mouse assay and in *A. ostenfeldii* from the same waters (Aasen et al., 2003).

The toxin composition of *A. ostenfeldii* isolates from a given region can vary greatly. Cultures created from cysts collected from the sediments around New Zealand had different PSP toxin compositions, ranging from undetectable PSP toxins, to almost exclusively saxitoxin, to high proportions of gonyautoxins (GTX3 and GTX5) relative to other derivatives (MacKenzie et al., 1996). These toxin profiles were stable with repeated testing over time. Similarly, along the eastern coast of Nova Scotia, spirolide composition in plankton samples from the field was similar over time and depth within a site, but differed greatly between sites (Cembella et al., 2000a).

Herein we present information on the distribution and toxin composition of *A. ostenfeldii* in the GOM. These data were obtained during the ECOHAB-GOM investigation of *A. fundyense*, under study because of its role as the causative organism of PSP





Spirolide	R1	R2		EMW
A	H	CH_3	$\Delta^{2,3}$	691.5
13-desMe-C	CH_3	H	$\Delta^{2,3}$	691.5
B	H	CH_3		693.5
C	CH_3	CH_3	$\Delta^{2,3}$	705.5
D	CH_3	CH_3		707.5
E	H	CH_3	$\Delta^{2,3}$	709.5
F	H	CH_3		711.5

Fig. 1. Chemical structures of common spirolides found in *Alexandrium ostenfeldii* and in shellfish contaminated by feeding upon this dinoflagellate. The $\Delta^{2,3}$ indicates that there is a double bond between carbons 2 and 3.

toxicity in the GOM. During the ECOHAB-GOM program it became evident that *A. ostenfeldii* co-occurs with, and is occasionally more abundant than, *A. fundyense* in the region. Because the GOM has a large and productive shellfish industry that is already subject to repeated seasonal closures due to PSP toxicity from *A. fundyense*, the toxin production of *A. ostenfeldii,* including toxin type, content, and composition was of great interest.

The co-occurrence of morphologically similar *Alexandrium* spp. initially led to problems with the identification and enumeration of the known PSP toxin producer, *A. fundyense*, by light microscopy and using an immunofluorescent probe. This issue was resolved when an rRNA probe, specific to *A. ostenfeldii* (AOST01) (John et al., 2003) became available as a complement to an *A. fundyense* rRNA probe (NA-1) (Scholin et al., 1994). Used in tandem, these probes allow discrimination between the two *Alexandrium* spp., even in the same sample (Anderson et al., 2005a). The distribution of *A. fundyense* in the GOM is presented elsewhere in this issue (Keafer et al., 2005a, b; Townsend et al., 2005).

This study provides the first description of the large-scale distribution of *A. ostenfeldii* in the GOM, and the first report and analysis of spirolide toxicity in *A. ostenfeldii* in GOM waters south of Nova Scotia.

2. Materials and methods

2.1. Distribution and abundance

Samples were collected during three ECOHAB-GOM cruises in the GOM in the springs of 2001 and 2003. The study area comprised a large region between Penobscot Bay to the east and Cape Elizabeth to the west (Fig. 2). In 2001, 270 stations were sampled from May 6 to 15 in two back-to-back cruises (Leg 1, May 5–11; Leg 2, May 11–15), and 172 stations were sampled from June 6 to 10. In 2003, approximately 80 stations were sampled three times in back-to-back cruises from May 28 to June 1. Surface data from the first cruise in 2003 are presented here.

Hydrographic data were collected using a Seabird 9/11 plus CTD mounted on a rosette with 10-L Niskin bottles that were used to collect water samples. Vertical profiles of temperature, salinity, fluorescence, and depth (pressure) were obtained at every station. Water samples were collected on the upcast at depths of 1, 5, 10, 20, 30, and 40 m. For plankton samples, 8-L of sea water from a 10-L Niskin bottle were concentrated by filtration through a 20-μm Nitex sieve (Nitex mesh, Sefar America, Inc., fitted at the end of a 3 in. diameter PVC tube) and backwashed with filtered seawater

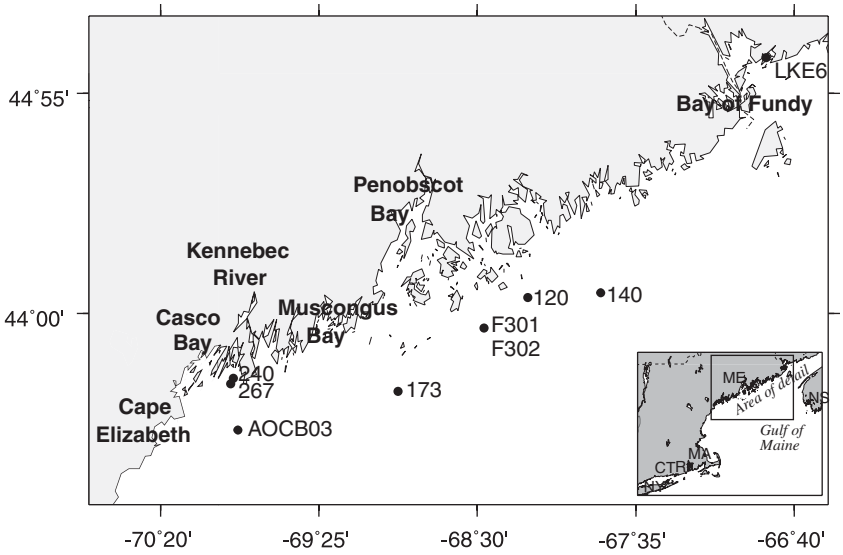


Fig. 2. Gulf of Maine, with inset showing greater region in which sampling area was located. Stations from which *Alexandrium ostenfeldii* cultures were isolated are labeled with the station number or clone designation.

(FSW) to a volume of 20 mL. For oligonucleotide probe labeling, one 5 mL subsample, equivalent to 2 L of whole seawater, was transferred into a 15-mL polypropylene centrifuge tube, brought to a volume of 14 mL with FSW, and preserved with 0.75 mL formalin (5% v/v, final concentration = 1.9% formaldehyde). These formalin-preserved samples were immediately centrifuged (5000*g*, 5 min, room temperature), aspirated to the cell pellet, and resuspended in 14 mL of ice-cold methanol to extract chlorophyll and stabilize rRNA. These samples were stored at −20 °C. The remainder of the 20 mL sample was taken for enumeration of *A. fundyense* using the sandwich hybridization assay (Anderson et al., 2005a).

For *A. ostenfeldii* counts, 7.4 mL of formalin/methanol preserved material (the concentrate from at least 1 L of whole water) was filtered and labeled using the AOST01 rRNA probe (John et al., 2003) following the dual-labeling method for whole cell counts described in Anderson et al. (2005a).

2.2. Culturing

Thirteen cultures of *A. ostenfeldii* were created by micropipette isolation of single vegetative cells from five stations in the GOM (Fig. 2) during the cruise in May 2001 (clones HT120B5, HT120B6, HT120B7, HT120D6, HT140E4, HT140E7, HT173B4, HT240D2, HT240D8, HT267B4, and HT267C2) and from one station during the cruise in June 2001 (clones F301 and F302). Clone AOCB03 originated from a single cyst isolated from sediments collected using a Craib Corer offshore of Casco Bay in February 1998 and germinated after several months of storage. Clone LK E6 was isolated from the Bay of Fundy in the summer of 2001.

Cultures were maintained in tubes with 25 mL of f/2 nutrient medium minus silicate (Guillard, 1975) at 10 °C and at a photon flux density of ca. 50 μmol m^{-2} s^{-1}, on a 14 h:10 h light:dark cycle.

2.3. Analysis of spirolide toxins

2.3.1. Field sample collection

During the cruise in June 2001, select stations in the western GOM were sampled for analysis of PSP toxins and spirolides. At these stations, 1800 L of water from a depth of approximately 1 m were pumped through a 33 cm-diameter, 20-μm plankton net, using a Lil' Giant submersible pump attached to a length of garden hose. The 20–60-μm fraction was concentrated using Nitex sieves. The concentrate was split into separate samples for PSP toxin analysis (concentrate from 1512 L), spirolide toxin analysis (from 216 L) and *Alexandrium* spp. counts (from 72 L). Samples for spirolide toxin analysis were centrifuged (5000*g*, 5 min, room temperature), aspirated to the cell pellet, and frozen at −20 °C until analysis.

Samples for *Alexandrium* spp. counts corresponding to the toxin samples (concentrate from 72 L)

were preserved, stored, probed and counted as described above, except that the concentrate equivalent to 2.48 L of whole seawater was examined. For reasons described in Section 4.2.2, the *A. ostenfeldii* concentrations determined from the 1 m Niskin bottle samples, instead of the *A. ostenfeldii* concentrations from the toxin samples, were used to determine toxin content per cell.

2.3.2. Culture sample collection

Clonal, non-axenic *A. ostenfeldii* cultures of 25 mL were maintained as described above. Growth rate was monitored once every 4 days by measuring chlorophyll fluorescence using a Turner Designs Fluorometer. Cultures were harvested during late exponential growth phase by centrifugation (5000*g*, 10 min, room temperature) and aspiration to a volume of 2 mL. For cell enumeration, three 25 μL subsamples were removed and each preserved in 960 μL filtered seawater plus 15 μL Utermöhl's iodine solution. *A. ostenfeldii* were counted in 100 μL of each subsample under a Nikon Labophot compound microscope. The remainder of the 2 mL sample was re-centrifuged (5000*g*, 10 min, room temperature), aspirated to a pellet, and stored at −20 °C until analysis. Cells per pellet ranged from 1.7×10^4 to 1.22×10^5. We did not observe excessive broken cells or empty theca in our cell counts, indicating that lysis of cells during the centrifugation steps was negligible. Dissolved spirolide toxins, released by cell lysis or by leaching into the growth medium, were not measured.

2.3.3. Spirolide toxin analysis by LC-MS

The algal cell pellet was extracted on ice in 100% MeOH by sonication using a microtip at 45% of maximum intensity (ca. 25 W) on the 50% pulse duty mode, until complete cell fragmentation was achieved, as determined by examination under phase-contrast microscopy. The sonicated suspension was centrifuged at 6675*g* for 15 min at 5 °C. The supernatant was retained and filtered by centrifugation through a 0.45-μm pore-size membrane filter (Ultrafree-MC, Durapore membrane, Millipore Ltd., Bedford, MA).

Spirolide toxins were analyzed by liquid chromatography-mass spectrometry (LC-MS), according to the method of Cembella et al. (2000b), using an Agilent (Palo Alto, CA) HP1100 LC system connected with a Perkin-Elmer SCIEX (Concord, Ontario) API-165 single quadrupole MS systems equipped with an ion-spray source (detection limit = 0.03 μM). Analyses were conducted in positive ion electrospray mode using a Turbo IonSpray interface, with drying nitrogen maintained at 350 °C and selected ion monitoring (SIM) of the $[M+H]^+$ ions. Chromatographic separations were performed on a 150 × 2 mm i.d. column packed with 3 μm BDS-Hypersil C8-silica with 0.2 mL/min of mobile phase composed of acetonitrile-water (35:65, v/v) with 2-mM ammonium formate and 50-mM formic acid. The column effluent was split, with 10% going to the mass spectrometer. Calibration was performed against standards of pure spirolides 13-desMethylC (desMeC), B and D. Relative molar response factors for other spirolides were assumed to be the same. Spirolide structure confirmations were performed using LC-MS/MS analyses using the same LC system connected with a Perkin-Elmer SCIEX API-4000 triple quadrupole MS system using nitrogen as collision gas.

2.4. Analysis of PSP toxins

2.4.1. Culture sample collection

Five cultures of *A. ostenfeldii* from the GOM were grown in 1 L of f/2 medium, minus silicate, in Fernbach flasks. Cultures were maintained at 10 °C at an irradiance of ca. 50 μmol $m^{-2}s^{-1}$, on a 14:10 h light:dark cycle. A 1 mL subsample was withdrawn from each flask approximately every 5 days for cell counts to monitor growth rate. Subsamples were preserved with 10 μL Utermöhl's iodine solution and counted under a Nikon Labophot microscope. When a culture reached mid- to late exponential growth phase, the entire culture was concentrated on a 13-μm Nitex sieve and backwashed into a 15 mL, disposable, polypropylene centrifuge tube. Samples were centrifuged (5000*g*, 10 min, room temperature), the supernatant aspirated, and 4 mL of cold 0.05 M acetic acid (HOAc) was added to the cell pellet. Three 25-μL samples were withdrawn from the resuspended pellet and each was diluted into 1.0 mL aliquot of FSW with 10 μL Utermöhl's iodine solution to enumerate cells in the slurry. Toxin extraction was completed by sonicating the cell suspension in an ice bath using a Branson sonic cell disruptor for 20 s, at a setting of 6 A. Samples were stored frozen at −20 °C prior to HPLC analysis. Each sample contained more than 1.1×10^6 cells.

2.4.2. PSP toxin analysis by HPLC

Prior to analysis, extracts were thawed, mixed and centrifuged (5000*g*, 10 min, room temperature).

The extract was partially cleaned by passing it through a preconditioned Millipore Sep-Pak Lite C18 cartridge, followed by filtration through a Millipore 1000 MW centrifugal filter device. Aliquots (100 μL) of the sample extract supernatant were loaded into auto-sampler vials and analyzed for PSP toxins by high-performance liquid chromatography (HPLC) (Waters) using the three-step isocratic elution method of Oshima (1995) with modification of the post column reaction (PCR) temperatures. For the C1–C4 toxins, the PCR temperature was maintained at 45 °C to achieve greater sensitivity for toxins C1 and C2. The detection limits for C1–C4 were 20, 7, 52, and 42 nM, respectively. Gonyautoxins (GTX) were analyzed at 35 °C to enhance the fluorescence response of GTX1, GTX4, and GTX6 (= B2). Detection limits for GTX 1–6 were 14, 8, 3, 14, 46, and 14 nM, respectively. The PCR temperature was maintained at 50 °C for the saxitoxin group, thus providing a better fluorescent signal for neosaxitoxin. The detection limits were 10 nM for saxitoxin, 22 nM for neosaxitoxin, 12 nM for decarbomoyl saxitoxin, 8 nM for decarbomoyl GTX 2, and 3 nM for decarbomoyl GTX3. External standard solutions, kindly provided by Y. Oshima (Tohoko University, Sendi, Japan), were run prior to sample analysis and after every fourth sample.

2.5. Morphology

Morphological species identification of cultures was confirmed by epifluorescence microscopy of cells stained with Calcofluor White MR2 (Polysciences, Warrington, PA #4359) which stains cellulose thecal plates (Fritz and Triemer, 1985). Cultures were preserved with 5% formalin (final concentration), and stored at 4 °C until analysis. Samples were centrifuged, aspirated to a pellet, resuspended in 1 mL FSW, and 5 μL of a 1.0-mg/mL solution of Calcofluor White was added. After staining for 10 min, the sample was aspirated to a pellet, and was resuspended in 10 mL of FSW for analysis. Subsamples of up to 1 mL were examined at 100–200 × under a Zeiss Axioskop microscope with a 100 W mercury lamp and a Zeiss #2 filter set (excitation 365 nm, emission 420 nm). Images were taken with a Zeiss MC 100 digital camera system.

The width at the cingulum, the number of food vacuoles, and the size of each food vacuole at its widest point were measured for 518 *A. ostenfeldii* cells in 56 plankton samples taken for toxin analysis during the cruises in May and June 2001. Cell size measurements were taken under tungsten light under a Zeiss Axioskop at 200 ×. Measurements were made with an ocular reticule, calibrated using a stage micrometer.

3. Results

3.1. Morphology

Morphological examination of cells in field samples and of clonal cultures isolated from the GOM confirmed the presence of *A. ostenfeldii* (Fig. 3A). Analysis of thecal plates stained with Calcofluor White (Fig. 3B, C) revealed the distinctive angular first apical (1′) plate and large ventral pore of *A. ostenfeldii* (Balech, 1995). Additionally, *A. ostenfeldii* cells in field samples were labeled by the genus-specific antibody probe (M8751-1), genus-specific oligonucleotide probe (AG), and *A. ostenfeldii* species-specific oligonucleotide probe (AOST01). Many positively labeled cells contained food vacuoles characteristic of *A. ostenfeldii* (Fig. 3D). *A. ostenfeldii* cells were not labeled by the NA-1 probe, specific for the North American *A. fundyense, A. tamarense, A. catenella* species complex (Anderson et al., 2005a).

3.2. Mixotrophic status

The average cell size, range of cell sizes, average number of food vacuoles per cell, average and range of sizes of food vacuoles, and total food vacuole size per cell are shown in Table 1. Cell size ranged from 20.0 to 87.8 μm, and total vacuole size was from 1.0 to 47.5 μm cell^{-1}. The average cell size, average food vacuole size, average number of food vacuoles per cell, and total size of food vacuoles per cell were all larger during the May 2001 cruise than during the June 2001 cruise, although none of these differences were statistically significant according to Student's *t*-tests. In May, 83.6% of cells contained one or more food vacuoles, while in June, 62.9% of cells showed this sign of mixotrophy.

3.3. Distribution and abundance

Concentrations of *A. ostenfeldii* and corresponding salinity contours from the May and June 2001 cruises are shown in Figs. 4–6. Additional hydrographic information and analysis of *A. fundyense* dynamics for all surveys are presented in Keafer et al. (2005b).

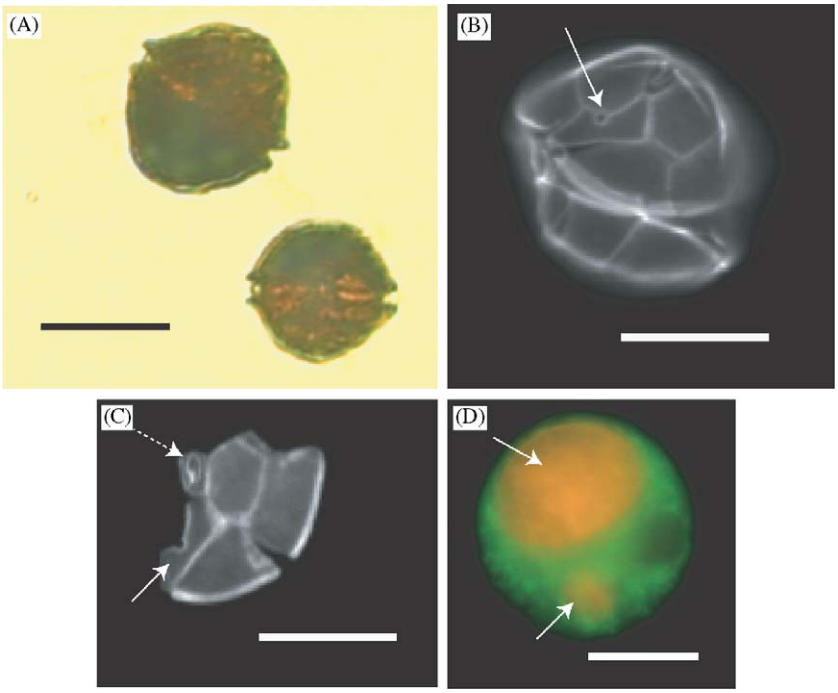


Fig. 3. *Alexandrium ostenfeldii*, scale bars are 20 μm: (A) Light micrograph of two cells from culture showing variability in cell size; (B) Epifluorescence micrograph *A. ostenfeldii* from culture stained with calcofluor white, showing thecal plates. Arrow points to characteristic 1′ apical plate and large ventral pore; (C) Epifluorescence micrograph of thecal plates stained with Calcofluor white. Dashed arrow points to apical pore complex, solid arrow points to 1′ apical plate and ventral pore; (D) Epifluorescence micrograph of AOST01-probed cell from field sample. Probe labels cytoplasm green, arrows indicate food vacuoles with red-orange chlorophyll autofluorescence.

Table 1
Measurements of cell size and mixotrophic status in *Alexandrium ostenfeldii* in Gulf of Maine field samples in May and June 2001: average cell size, range of cell sizes, food vacuole size, range of food vacuole sizes, average number of food vacuoles per cell, and average total food vacuole content per cell

	May 2001 ($n = 280$)	June 2001 ($n = 240$)
Mean cell size (μm at cingulum)	44.5±7.6	39.2±7.7
Range of cell sizes (μm at cingulum)	20.0—65.0	22.5–87.
Mean number food vacuoles per cell	1.7±1.4	1.0±1.0
Mean food vacuole size (μm at widest point)	13.6±7.7	9.1+6.1
Total food vacuole size per cell (μm)	25.1±17.0	13.3±9.4
Range of food vacuole sizes (μm)	1.0–40.0	1.0–47.5

In the first part of the survey (Leg I, May 6–11), there were two regions of relatively high cell concentration in the surface waters (Fig. 4A). In Casco Bay, a patch with higher cell concentrations (244 cells L^{-1}) was found inside the 29 isohaline (Fig. 4B). This patch was relatively isolated from the greater core of the regional population (285 cells L^{-1} maximum, and an average concentration of 100–200 cells L^{-1}) that was located in the northeast of the study area, inshore of the 32 psu isohaline. Another patch of moderate concentration (20–100 cells L^{-1}) was in waters of salinity greater than 32 psu, offshore to the northeast. The highest cell concentrations in the core population were centered between the 31 and 32 psu isohalines, just inshore of the edge of the eastern branch of the Maine Coastal Current (MCC), a relatively cold, high-salinity feature (Brooks, 1985; Lynch et al., 1997; Pettigrew et al., 1998) that moves in a southwest direction along the coast.

Cell abundance was generally lower at 10 m than at the surface throughout the study area (Fig. 4 C), particularly in the core of the population east of Penobscot Bay. In the nearshore region between

Penobscot Bay and the Kennebec River outflow, however, cell concentrations were higher at 10 m than at the surface (Fig. 4A, C). This occurred within a patch of relatively low-salinity (30–31 psu) surface waters overlying higher-salinity waters (31–32 psu) at 10 m (Fig. 4B, D). The population in Casco Bay was primarily at the surface, with fewer than 10 cells L^{-1} at a depth of 10 m. The station inside Casco Bay also shows a gradient in salinity, from 29 psu at the surface to 31 psu at 10 m (Fig. 4B, D).

A portion of the study area was re-sampled (Leg II) from May 11 to 15, 2001 (Fig. 5A, B). Several notable changes occurred in the distribution of the population during the interval between the surveys. The core of the population was still inshore of the 32 psu isohaline, which had moved away from the coast. The cell concentration in the core increased to more than 200 cells L^{-1}. The population expanded spatially offshore near Penobscot Bay and alongshore to the west, enhancing the connection between the eastern and Casco Bay populations. The maximum cell concentration at the surface in Casco Bay had increased from 244 to 574 cells L^{-1} over 7 days. As in Leg I, there was an area of lower concentration at the mouth of the Kennebec River.

By June 2001, there was a clear bifurcation of the population into a band along the coast and an offshore extension south of Penobscot Bay (Fig. 6A). Surface concentrations of *A. ostenfeldii* in Casco Bay had decreased by an order of magnitude and relatively high concentrations were observed in Muscongus Bay (>200 cells L^{-1}), resulting in an eastward shift of the high cell concentration in the western GOM. Densities of 100–200 cells L^{-1} were also centered further to the north and east in June, nearshore to Penobscot Bay. The offshore tongue of high *A. ostenfeldii* concentration was more defined in June, extending south from Penobscot Bay. The eastern edge of this tongue was contained by the 31.7 psu isohaline (Fig. 6B). The survey in June did not extend as far to the east as in May, so less information is available about connections with the population further "upstream" to the east.

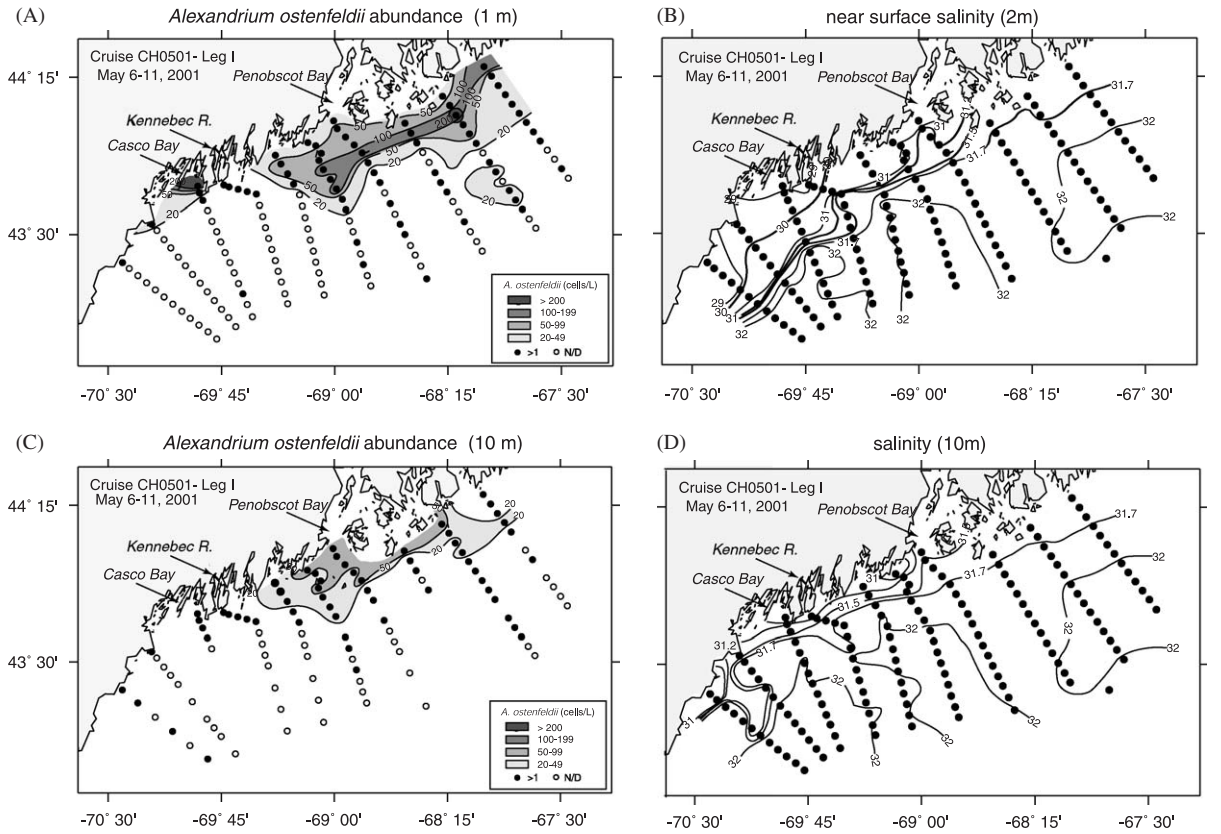


Fig. 4. May 2001 cruise (Leg I): (A) abundance of *Alexandrium ostenfeldii* cells at the surface; (B) near surface salinity; (C) abundance of *A. ostenfeldii* cells at 10 m; (D) salinity at 10 m.

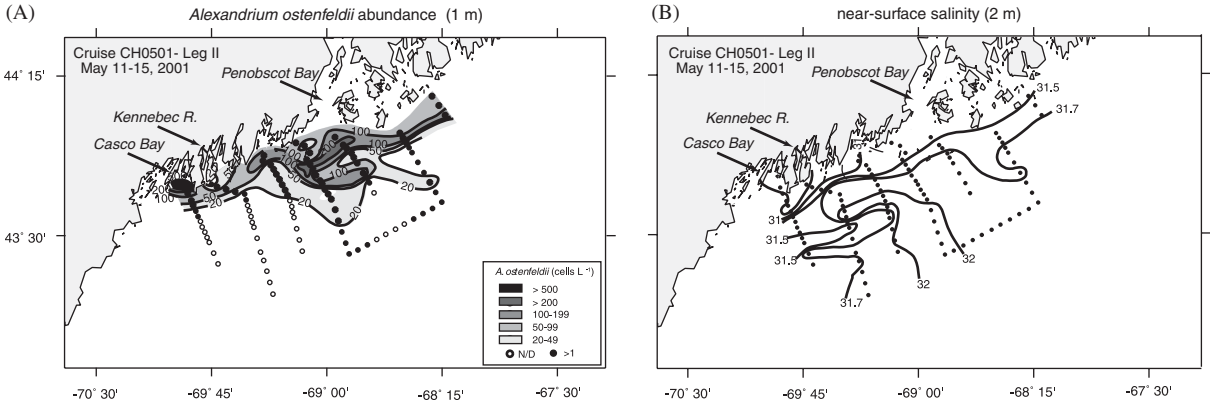


Fig. 5. May 2001 cruise (Leg II): (A) abundance of *Alexandrium ostenfeldii* cells at the surface; (B) near-surface salinity.

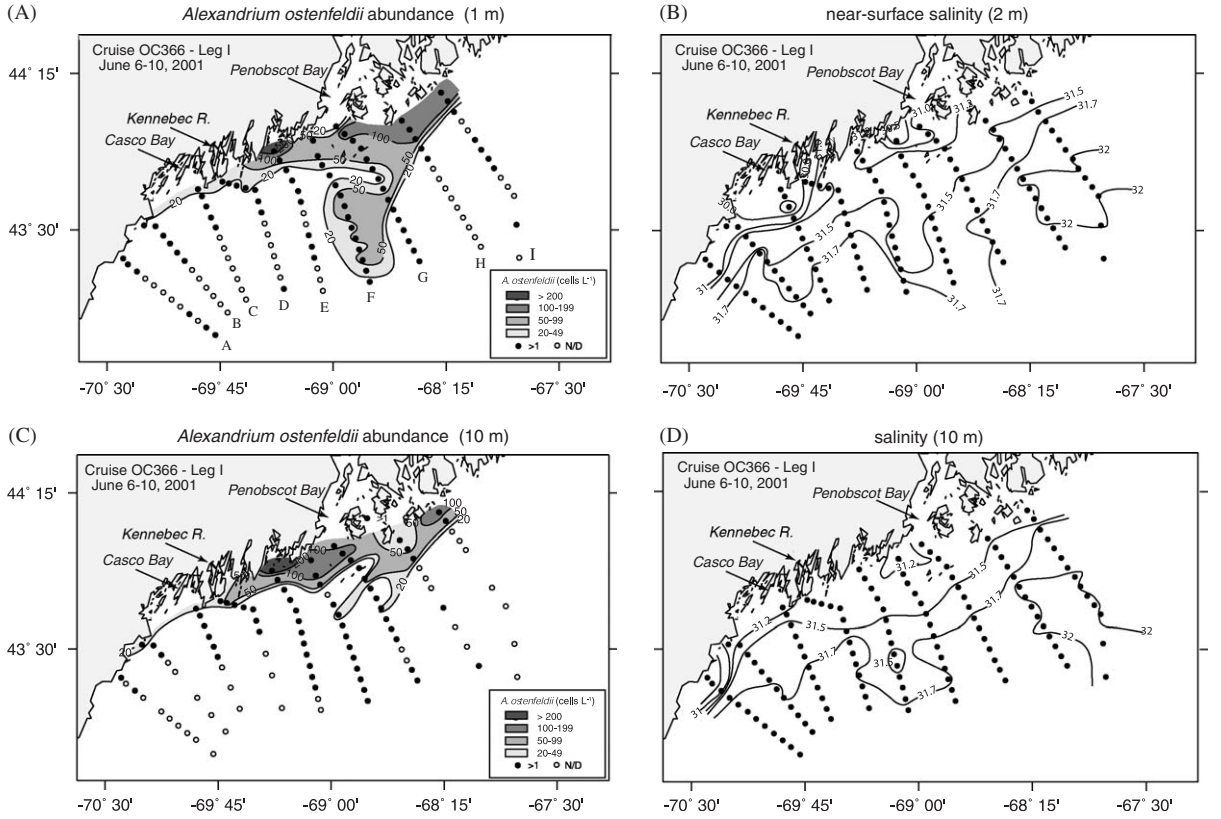


Fig. 6. June 2001 cruise: (A) abundance of *Alexandrium ostenfeldii* cells at the surface, with transects designated by letters A–I; (B) near surface salinity; (C) abundance of *A. ostenfeldii* cells at 10 m; (D) salinity at 10 m.

In June 2001, concentrations of *A. ostenfeldii* were sometimes higher at 10 m than at the surface (Fig. 6C), most notably at several stations in the western GOM, including in Casco Bay, and as in May, at the mouth of the Kennebec River, in Muscongus Bay, and along the western edge of Penobscot Bay. Along the eastern edge of Penobscot Bay, cell concentrations were lower at 10 m than at the surface. Cell concentrations were also lower at 10 m than at the surface in the offshore-extending tongue of the population.

In late May 2003 (Fig. 7A), regions of high cell concentration at the surface in Casco Bay (400 cells L^{-1}) and Muscongus Bay (361 cells L^{-1})

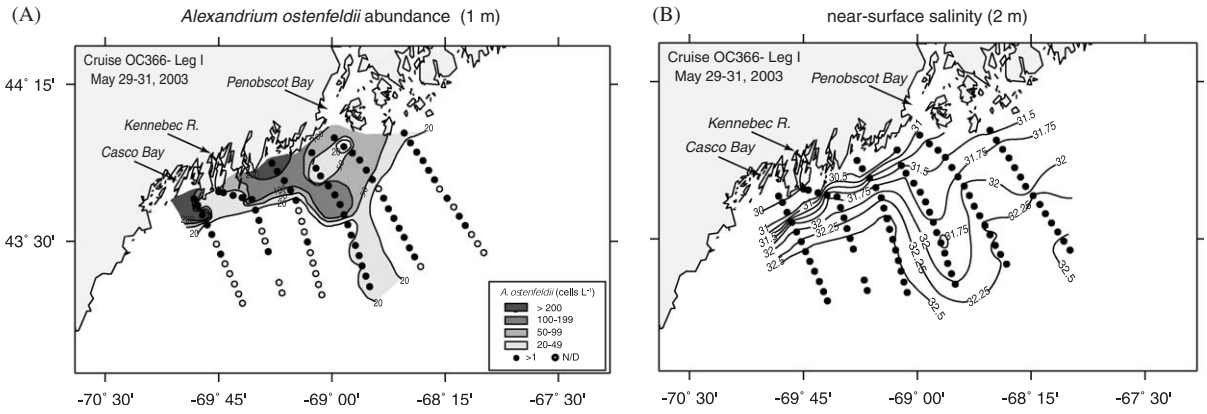


Fig. 7. May/June 2003 cruise (A) abundance of *Alexandrium ostenfeldii* cells at the surface; (B) near surface salinity.

were separated by an area of slightly lower concentration at the mouth of the Kennebec River. As in June 2001, a patch with high cell concentrations was located nearshore to Penobscot Bay, inside the 31.7 psu isohaline (Fig. 7B). An offshore branch of the *A. ostenfeldii* population extended south from Penobscot Bay, following an offshore extension of lower-salinity waters bordered by the 32 psu isohaline.

3.4. Spirolide toxins

3.4.1. Field samples

Spirolide toxins were found in 36 of 60 field samples from the June 2001 cruise. More than 83% of samples containing *A. ostenfeldii* cells had one or more of spirolides A, B, C2 and D2, where C2 and D2 designate unknown structural isomers of C and D. At six stations where *A. ostenfeldii* was present, no spirolide toxins were detected. At six stations where no *A. ostenfeldii* was found, spirolides were detected.

The total concentration of spirolides per cell at each station where *A. ostenfeldii* was detected ranged from non-detectable to 282 fmol cell^{-1} (Fig. 8). The highest cellular toxin contents (>100 fmol cell^{-1}) were found in the mid- to eastern portion of the study area, just offshore of Penobscot Bay.

The geographic distribution of spirolide profiles in field samples is shown in Fig. 9. Of the field samples that were positive for spirolides, 100% (36 samples) contained spirolide A. All samples that contained spirolide B also contained spirolide C2 (47%). Only 14% of spirolide-positive field samples had spirolide D2. For spirolide concentrations above the detection limit at stations where *A. ostenfeldii* was present, the content per cell of each spirolide congener ranged widely, from a high of 214 fmol cell^{-1} for spirolide A to a low of 1.3 fmol cell^{-1} for spirolide D2 (Fig. 8).

Analyses of the relationships between total toxin content, the toxin content of individual spirolide isomers, and the variables of cell size, average food vacuole size per cell, total food vacuole size per cell, and average number of food vacuoles per cell showed no discernable or significant correlations or trends, as determined by Student's *t*-tests. The use of data from only 11 stations may be responsible for this lack of correlation. Because of the high variability of cell and food vacuole size measurements, data from stations for which fewer than 10 cells were measured were not used in the analysis.

3.4.2. Cultures

A. ostenfeldii cultures originating from different locations within the GOM showed a great diversity in their spirolide content and composition (Fig. 10). All clones contained spirolides, with total concentrations ranging from 28 to 113 fmol cell^{-1}. The highest per cell spirolide contents in the cultures were nearly 2 times lower than the highest level observed in field samples.

Spirolide congeners desMeC, C and D were found in a few cultures, but were not detected in any of the field samples. Based on differences and similarities between their toxin profiles, the cultures may be separated into five broad categories, or spirolide phenotypes, shown in Table 2. Group 1 clones contain only spirolides C, C2, and D2. Group 2 clones contain largely spirolides A and C2, with lower levels of spirolides B, desMeC, and D2. Five of the cultures fall into Group 3, and also

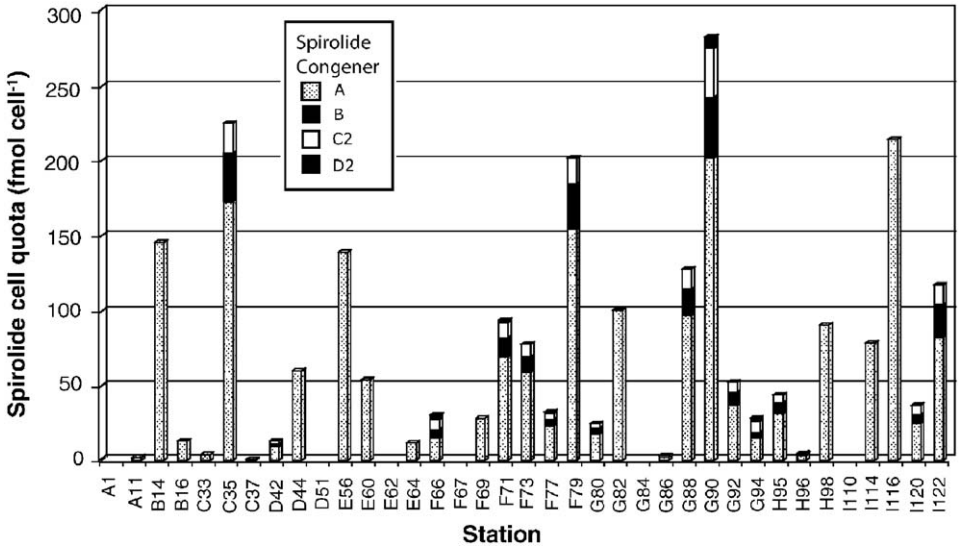


Fig. 8. Average cellular spirolide content and composition of *Alexandrium ostenfeldii* in field samples collected in the Gulf of Maine in June 2001. Locations of transect A–I are shown in Fig. 6.

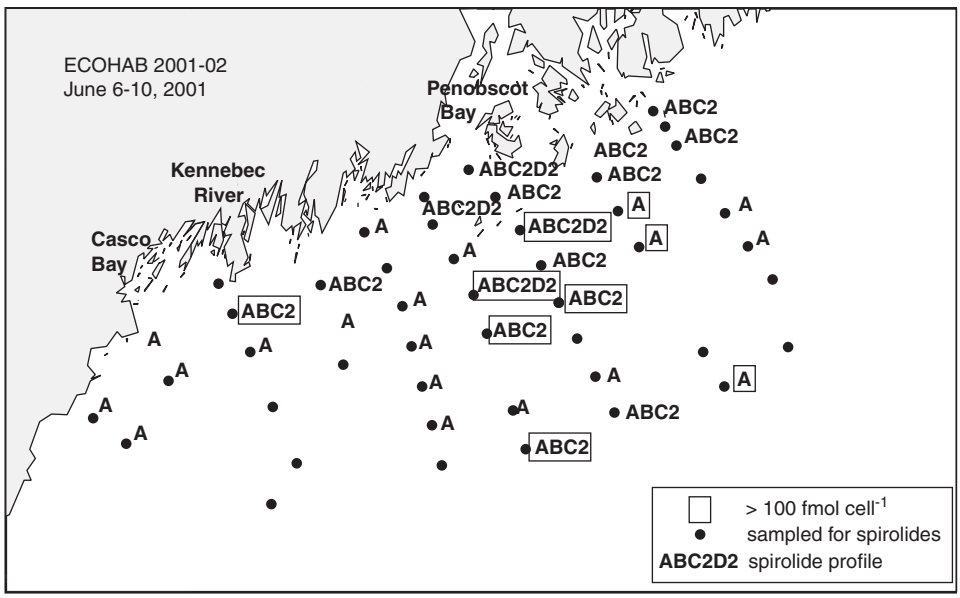


Fig. 9. Geographic distribution of spirolide profiles in field samples, June 2001.

contain primarily spirolides A and C2, and lower levels of B and D2, but no desMeC. Group 4 cultures have only spirolides A and B, while those in Group 5, made up of two replicates of the same clone, contain more than 80% spirolide A, with very small amounts of spirolides B and D.

As in the field samples, cultures isolated from throughout the study region had a variety of toxin profiles (Fig. 11). Multiple toxin phenotypes were isolated from a single sample taken at a single station. For example, multiple cultures isolated from stations 120 and 140 had both Groups 1 and 3 profiles, while cultures isolated from station 240/267 had spirolide profiles of Groups 2, 3, and 4. The spirolide toxin profiles of individual cultures were often different from the toxin profiles in field samples collected at the same site 1 month later (Figs. 9 and 11).

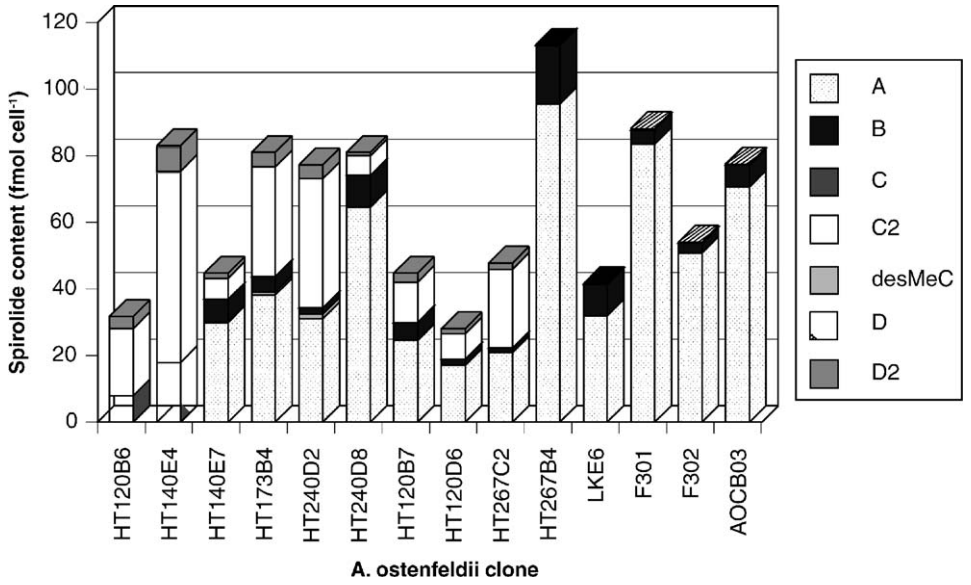


Fig. 10. Average cellular spirolide content and composition of clonal isolates of *Alexandrium ostenfeldii* from the Gulf of Maine.

3.5. PSP toxin measurements

3.5.1. Field samples

In June 2001, PSP toxins were detected in subsamples of the same field samples that were analyzed for spirolide toxins. In addition to *A. ostenfeldii*, cells of *A. fundyense*, a known producer of PSP toxins, were present in these samples. Thus, we cannot confirm nor reject PSP toxin production by *A. ostenfeldii* in the GOM based on field samples alone.

3.5.2. Cultures

Six of the *A. ostenfeldii* cultures (clones LKE6, HT120B6, HT140E7, HT173B4, HT240D8, and F301) were tested for PSP toxins using HPLC and found to be negative. A repeated analysis of clone HT 173 B4 confirmed a lack of PSP toxins.

4. Discussion

This study provides the first analysis of the large-scale distribution and of the toxin content and concentration of *A. ostenfeldii* in the GOM. *A. ostenfeldii* was widely distributed and abundant in the region, co-occurring with, and at times more abundant than, the PSP toxin producer, *A. fundyense*. While cultures of *A. ostenfeldii* isolated from the study area did not produce PSP toxins, cultures of *A. ostenfeldii* and plankton samples from the GOM contained a wide array of spirolide toxin congeners.

4.1. Distribution and abundance

A. ostenfeldii likely had been previously overlooked in the GOM due to the overlap in size range between *A. ostenfeldii* and *A. fundyense*. Application of molecular probe technology in cell counts has been key to distinguishing between *A. ostenfeldii* and other morphologically similar, globose phytoplankton species in the region (Anderson et al., 2005a). Additionally, *A. ostenfeldii* tends to form amorphous, round, temporary cysts when subjected to stress, such as during sample collection, making the species difficult to identify (Cembella et al., 2001).

Early in the ECOHAB-GOM program, a putative species-specific immunofluorescent probe was used to detect and enumerate *A. fundyense* in field samples. During the 1998 field season, we found that the immunofluorescent probe also labeled larger, rounder cells containing large "inclusion bodies" or food vacuoles (Anderson et al., 2005a). Investigation of the thecal plate morphology of these cells, presented here, revealed them to be *A. ostenfeldii*. Employment of an *A. ostenfeldii*-specific oligonucleotide probe in 2001 and thereafter allowed this first large-scale examination of the distribution and abundance of *A. ostenfeldii* in the GOM.

Table 2
Spirolide composition in clonal isolates of *Alexandrium ostenfeldii* from the Gulf of Maine

Clone	Spirolide congener concentration (mol fraction)							Group
	A	B	C	C2	desMeC	D	D2	
HT120B6	Nd	Nd	19.8	49.2	Nd	Nd	8.9	1
HT140E4	Nd	Nd	16.8	53.9	Nd	Nd	7.2	1
HT140E7	59.5	13.7	Nd	12.5	0.3	Nd	3	2
HT173B4	41.6	5.2	Nd	35.8	0.9	Nd	4.8	2
HT240D2	36.6	2.3	Nd	45.6	1.6	Nd	4.9	2
HT240D8	72.1	10.7	Nd	6.5	Nd	Nd	1.2	3
HT120B7	48.1	10.6	Nd	23.7	Nd	Nd	5.5	3
HT120D6	56.8	6.1	Nd	25.3	Nd	Nd	5.1	3
HT120B5	40.6	10.6	Nd	27.5	Nd	Nd	7	3
HT267C2	38.4	2.3	Nd	43.3	Nd	Nd	3.4	3
HT267B4	48.2	8.9	Nd	Nd	Nd	Nd	Nd	4
LKE6	58.8	17.6	Nd	Nd	Nd	Nd	Nd	4
F302	81	4.7	Nd	Nd	Nd	0.5	Nd	5
F301	82.6	4.2	Nd	Nd	Nd	0.4	Nd	5
AOCB03	91	Nd	8.6	Nd	Nd	0.3	Nd	5

Clones are separated into five phenotypic groups, based on spirolide profile.Nd = not detected (<0.2 fmol cell^{-1}).

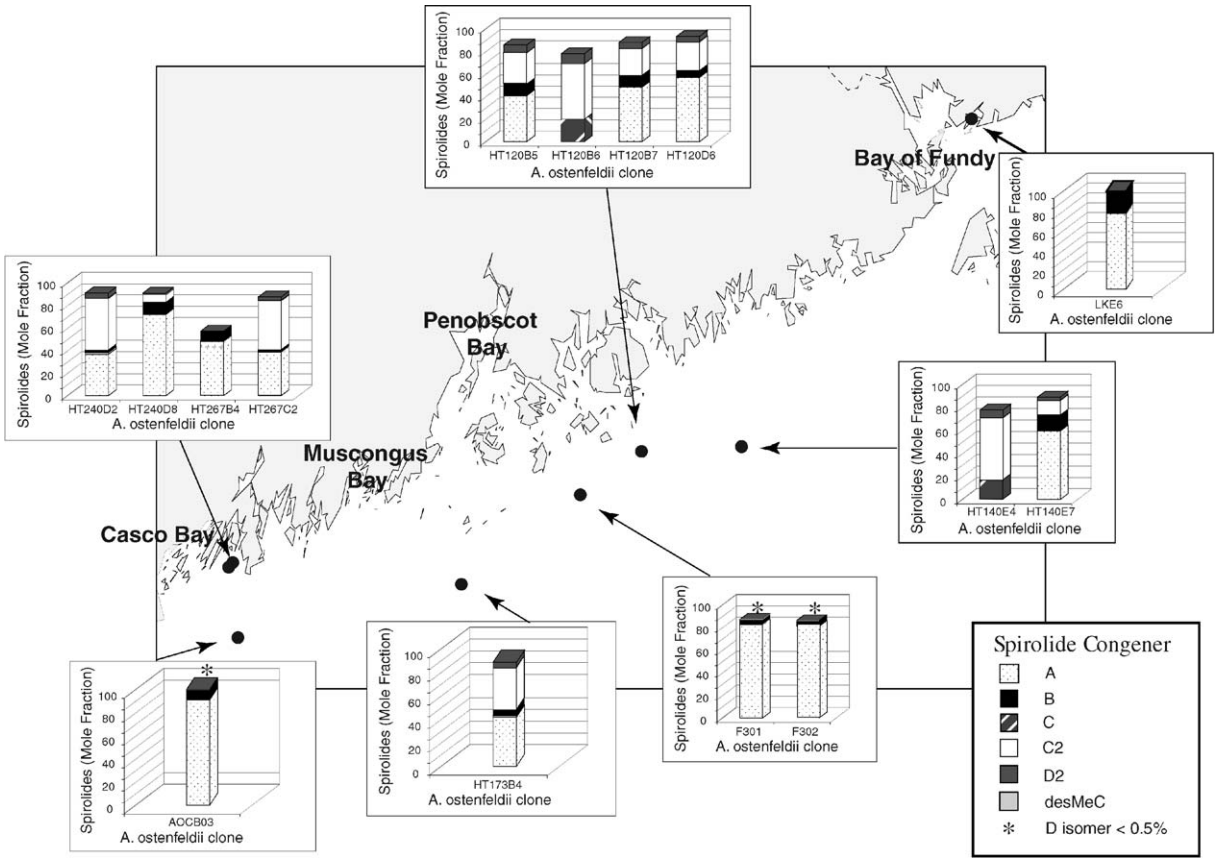


Fig. 11. Geographic distribution of spirolide profiles (mole fraction of each congener) of clonal isolates of *Alexandrium ostenfeldii* from the Gulf of Maine.

There are remarkable similarities between the distributions of *A. ostenfeldii* observed during three surveys in 2001 and one survey in 2003. During all four surveys, patches with relatively high cell concentration were detected nearshore in Casco Bay and/or Muscongus Bay, separated to a greater or lesser extent by an area of low cell concentration at the mouth of the Kennebec River. The core of the population extended alongshore in the northeast of our study area, across the mouth of Penobscot Bay. In June 2001 and 2003, a tongue of the population extended offshore to the south or southeast of Penobscot Bay. During all cruises in both years, *A. ostenfeldii* was at its lowest concentrations or was not detected offshore in the western- and eastern-most regions of the study area.

The core of the *A. ostenfeldii* population generally was observed inshore of the eastern branch of the MCC (EMCC), possibly kept near the coast by this mass of colder, high-salinity (32 psu) water. The EMCC may veer offshore to the south, giving rise to an offshore branch of the *A. ostenfeldii* population, as was seen in June 2001.

The hydrographic forcings controlling the distribution of *A. ostenfeldii* in the GOM were quite similar to those acting on the *A. fundyense* population at the same time of year. Keafer et al. (2005b) and Townsend et al. (2005) present discussions of the interactions between *A. fundyense* distributions and hydrographic conditions in the GOM. Casco Bay appears to be an area of accumulation for *A. ostenfeldii* cells advected toward shore from the core of the population located to the east in the "upstream" waters of the coastal flow, as is the case for *A. fundyense* (Keafer et al., 2005a). Inputs to Casco Bay may come in pulses, rather than continuously, as seen in the disconnect between the Casco Bay patch and the northern core of the population during Leg I of the cruise in May 2001, and the reconnection between the populations just a few days later during Leg II. More frequent sampling is required to confirm this hypothesis. The two fold increase in the population in Casco Bay seen during this seven-day interval is likely due to a combination of in situ growth and accumulation of cells flowing in from the northeast.

The higher cell concentrations in the eastern portion of our study area might suggest that the "source" of the GOM population lies to the east, perhaps in the Bay of Fundy. This is thought to be the case for *A. fundyense* in the region (Anderson et al., 2005b; McGillicuddy et al., 2005). Nearshore, eastern populations of *A. fundyense* serve as a source for blooms observed in the western GOM (Keafer et al., 2005b). At this time we have no information either about the distribution of cysts of *A. ostenfeldii* that may seed blooms in the region, or about the early spring distribution of vegetative cells in the GOM. Peaks in *A. ostenfeldii* population density in the western GOM may occur earlier in the spring, a phenomenon not captured by our sampling regime in 2001 and 2003.

In May and June 2001, concentrations of *A. ostenfeldii* were generally higher at the surface than in deeper waters to the north and east of Penobscot Bay, and in the offshore portion of the population in June. At the mouth of Penobscot Bay, Muscongus Bay, and near the outflow of the Kennebec River, cell concentrations were sometimes higher at 10 m than at the surface, a phenomenon more pronounced during June. The pooling of lower salinity water at the surface in this area may have lead to the subduction of the population flowing in from the east (Keafer et al., 2005b). Alternatively, *A. ostenfeldii* may reside lower in the water column in June, seeking cooler optimal temperatures as the surface waters warm. The mixotrophic *A. ostenfeldii* may also move deeper in the late spring to follow sinking prey populations, particularly as nutrients are stripped from the near-surface waters in the late spring (Love et al., 2005).

Non-quantitative observations during the ECOHAB cruises in the western GOM in 1998 and 2000 indicated that *A. ostenfeldii* were most abundant in mid to late April, and decreased in abundance through May and into June as water temperatures increased. These observations in the field match our laboratory data. Strains of *A. ostenfeldii* isolated from the GOM grow slowly at 10 °C under low light ($\sim$50 μmol s^{-1} m^{-2}) and very poorly at 15 °C or under higher light ($>$50 μmol s^{-1} m^{-2}) (K. Gribble, unpubl. obs.) In this way, GOM strains appear to inhabit a similar temperature niche to that of *A. ostenfeldii* strains in Nova Scotian waters, some of which were isolated from the field at temperatures of 5 °C, grew well at 14 °C under low light, but had poor temperature tolerance for growth above 16 °C (Cembella et al., 2000b). Both Nova Scotian and GOM strains contrast with *A. ostenfeldii* from Denmark, which has positive growth rates from 11 to 23 °C and optimal growth at 20 °C. Cultures of *A. ostenfeldii* in New Zealand also seem tolerant of higher temperatures, and can be maintained at 17 °C under high irradiance (MacKenzie et al., 1996).

Strains of *A. fundyense* from the GOM, on the other hand, grow well from 5–23 °C (Anderson et al., 2005b; Stock et al., 2005) and strains from Cape Cod salt ponds exhibit positive photosynthetic capacity and growth at irradiances from 50 to $>1000\,\mu mol\,s^{-1}\,m^{-2}$ (Glibert et al., 1988). The greater temperature and irradiance ranges of *A. fundyense* relative to *A. ostenfeldii* in the GOM may contribute to differences in the distributions of the two species.

4.2. Toxin content and composition

Spirolide content and composition varied widely both in field samples and in cultures of *A. ostenfeldii* from the GOM. These results are in keeping with the variability in toxin production exhibited by *A. ostenfeldii* in other areas. In coastal Nova Scotia, spirolide profiles were quite different between two sites, and although the profile at each site remained relatively constant with water depth and over the season (Cembella et al., 2000a) the toxin profile varied between years at one of the sites (Cembella et al., 2001). A culture of *A. ostenfeldii* from Denmark had a markedly different spirolide profile than those in field samples from Nova Scotia (Cembella et al., 1997). A large-scale sampling effort along the coast of Scotland revealed a wide variety of spirolide profiles in field samples from different locations (Ruehl et al., 2001). In cultures of *A. ostenfeldii* from New Zealand, no spirolide toxins were detected, but there were considerable differences in PSP toxin profiles between isolates from different locations (MacKenzie et al., 1996).

Little work has been done to directly investigate mechanisms controlling differences in toxin production in *A. ostenfeldii.* Evidence from the current and previous studies of *A. ostenfeldii* (Maclean et al., 2003), and from analyses of toxin production by other species suggests that both genetics and environmental factors play a role in determining toxin content and composition.

4.2.1. Cultures

Cultures of *A. ostenfeldii* isolated from the GOM showed great diversity in their spirolide content and concentration. These cultures were grown simultaneously and under the same conditions, and were harvested at late exponential phase, though growth rate varied from culture to culture. *A. ostenfeldii* is mixotrophic (Jacobson and Anderson, 1996), but can be maintained completely autotrophically in the laboratory. All cultures were grown autotrophically, with no evidence of mixotrophy or cannibalism within any culture.

Given the similar environmental conditions and physiological states of the cultures, differences in spirolide profiles and contents reflect genetic differences between the strains. Such a high level of diversity in the population of *A. ostenfeldii* in the GOM is striking. Spirolide profiles differed not only between separate areas of the GOM, but even between clones isolated from a single site. Four clones from the same station in Casco Bay, isolated from two water samples taken 1 day apart, exhibited three distinct spirolide profiles. Four clones isolated from a single water sample at Station 120 had two different profiles, matching the two profiles of the clones isolated from one sample taken at nearby Station 140. Multiple toxin phenotypes isolated from the same site indicates great genetic diversity within the population. The current study shows at least five different toxin phenotypes in isolates from the region.

MacKenzie et al. (1996) found PSP profiles of *A. ostenfeldii* cultures from New Zealand remained stable with repeated analysis over time. Further work will be done to determine the consistency of spirolide profiles in GOM isolates over time and with different environmental conditions. The lack of PSP toxins in *A. ostenfeldii* in the GOM is not unexpected, given that PSP toxins have not been detected in isolates in the neighboring waters of Nova Scotia (A. Cembella and M.A. Quilliam, unpublished data).

4.2.2. Field samples

Concentrations of *A. ostenfeldii* cells in the samples taken for toxin measurements were, on average, approximately 2–3 times lower than in the corresponding Niskin bottle samples taken at the same stations and times. *A. ostenfeldii* are known to form pellicle or temporary cysts when subjected to agitation (Cembella et al., 2001). The extreme stress of passage through the submersible pump at high flow rate, collection in a 20-μm plankton net, and sieving onto a 20-μm Nitex sieve may have disrupted a large portion of *A. ostenfeldii* cells or made these cells impermeable to the AOST01 oligonucleotide probe, and thus undetectable. For these reasons, concentrations of *A. ostenfeldii* cells determined from the 1-m Niskin bottle samples were used to determine cellular toxin content. If the cells

were present in the toxin samples but not counted in the Niskin samples, the corresponding toxin content calculations would be overestimates. Conversely, if some cells were destroyed during the toxin sample concentration process, but counted in the Niskin samples, the toxin calculations would be underestimates. Nevertheless, these values give an idea of the relative toxicity of plankton samples in different parts of the GOM.

Field samples represent a composite profile of all the toxin phenotypes at a given station.

In field samples, differences in spirolide content and concentration are likely due to genetic variability within the population, overlain by influences of environmental conditions. Thus, spirolide toxin profiles may not easily be used in the field as "tracers" for water masses or distinct populations of *A. ostenfeldii*.

Variability in the physiology of *A. ostenfeldii* at different stations may account for some of the differences in spirolide content and concentration in field samples. Differences in concentrations of inorganic nutrients across the study area (Love et al., 2005) could lead to higher or lower growth rates in *A. ostenfeldii*, a factor shown to effect toxin production in other *Alexandrium* species (Hall et al., 1982; Boyer et al., 1987; Anderson et al., 1990b). In batch cultures of *A. ostenfeldii* from Nova Scotia, variation in environmental conditions of irradiance, salinity and inorganic nitrogen led to little change in spirolide profile, but greatly affected total spirolide concentrations. High concentrations of inorganic nitrogen, high salinity, and moderate light levels all independently increased the total spirolide concentration (Maclean et al., 2003). Photoperiod also has been shown to influence the rate of production and content per cell of spirolide toxins in *A. ostenfeldii* from Nova Scotia (John et al., 2000).

Additionally, differences in the mixotrophic status of *A. ostenfeldii* or in the prey species available for consumption could influence toxin production. There are several examples of toxin production changing with varying nutrient status or environmental conditions in toxic phototrophic, mixotrophic, and heterotrophic dinoflagellates. In batch cultures of the PSP toxin producer, *A. fundyense*, both phosphate limitation and low temperatures increase toxin concentration per cell, whereas nitrate limitation reduces total cellular toxin concentration relative to a nutrient-replete control (Hall et al., 1982; Boyer et al., 1987; Anderson et al., 1990b). In semi-continuous cultures of *A. fundyense*, toxin composition varies systematically with growth rate and with the type of nutrient stress (Anderson et al., 1990a). Phosphate limitation in the toxic haptophyte, *Prymnesium,* results in an increase in prymnesiotoxin hemolytic activity, but when nutrient-limited cultures are fed bacteria, thereby supplementing phosphate, toxicity decreases (Legrand et al., 2001). The heterotroph, *Pfiesteria piscida*, has toxic and non-toxic stages depending on food source, with icthyotoxicity only elicited by exposure to fish prey (Burkholder and Glasgow Jr., 1997). Thus, we may expect that there is some influence of feeding history on the toxin production of *A. ostenfeldii* in the field.

The lack of detectable spirolide toxins at six stations where *A. ostenfeldii* was present may, in some cases, be due to low concentrations of cells at these stations producing levels of spirolides below the limit of detection by LC-MS. In other cases, however, cell numbers were very high (19,000 cells per sample), yet no toxin was detected. The *A. ostenfeldii* at these stations may not have been producing spirolides, or unknown problems with sample collection, storage or preparation may explain the observed lack of toxicity.

At five stations, spirolide toxins were found where no cells were detected. In these locations, *A. ostenfeldii* may have been present, but at concentrations below our detection limit of 1 cell L^{-1}. We also may have detected spirolides that had moved into higher trophic levels, as predators consumed *A. ostenfeldii* and concentrated the toxins (Turner et al., 2000; Doucette et al., 2005; Turner et al., 2005).

4.2.3. Differences between field samples and cultures

Cultured *A. ostenfeldii* contained desMeC, C, and D spirolides, though field samples did not have these congeners. Since all of the cultures expressing these additional congeners were isolated in May 2001, and all of the field samples for spirolide toxins were taken in June 2001, this difference could indicate a shift from one set of genotypes to another between the two cruises. That is, we may have sampled different populations in May than in June. Alternatively, conditions in culture may have allowed expression of the additional spirolides, while environmental conditions in situ did not. Field sampling simultaneous with isolations to establish cultures is needed to determine which of these explanations is correct.

5. Conclusions

Alexandrium ostenfeldii had a noteworthy presence in coastal GOM waters during the Spring. This species co-occurred with *A. fundyense,* the major PSP toxin-producer in this region, and the distribution of both species appeared to be subject to many of the same physical forcings. However, given the differences between *A. ostenfeldii* and *A. fundyense* in physiology and in the types of toxins produced, it is important that the ecology and human health impacts of these two species be studied separately.

While *A. ostenfeldii* does not contribute to PSP toxicity in the region, we have shown that it produces an array of spirolide toxins. Currently there is no routine monitoring for spirolide toxins in shellfish in the GOM, nor in Canadian waters to the north, but results of ongoing animal studies may change this in the future. Spirolides are clearly a group of potent neurotoxins, the pharmacology of which is still under investigation. Spirolides injected intraperitoneally into animals result in rapid death. Although spirolides have also been shown to be toxic to mice in oral feeding studies, it is not clear if spirolides would affect humans at the concentrations that typically accumulate in shellfish. It may be necessary eventually to expand ongoing shellfish toxin monitoring in the GOM to include analysis for spirolides.

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