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Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal waters

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Blooms of the toxic dinoflagellate *Alexandrium tamarense* (Lebour) Balech, a known producer of potent neurotoxins associated with paralytic shellfish poisoning (PSP), are common annual events along the Scottish east coast. The co-occurrence of a second *Alexandrium* species, *A. ostenfeldii* (Paulsen) Balech & Tangen is reported in this study from waters of the Scottish east coast. The latter species has been suspected to be an alternative source of PSP toxins in northern Europe. Recent identification of toxic macrocyclic imines known as spirolides in *A. ostenfeldii* indicates a potential new challenge for monitoring toxic *Alexandrium* species and their respective toxins in natural populations. In mixed phytoplankton assemblages, *Alexandrium* species are difficult to discriminate accurately by conventional light microscopy. Species-specific rRNA probes based upon 18S and 28S ribosomal DNA sequences were developed for *A. ostenfeldii* and tested by dot-blot and fluorescence *in situ* hybridization (FISH) techniques. Hybridization patterns of *A. ostenfeldii* probes for cultured *Alexandrium* isolates, and cells from field populations from the Scottish east coast, were compared with those of rDNA probes for *A. tamarense* and a universal dinoflagellate probe. *Alexandrium* cell numbers in field samples determined by whole-cell *in situ* hybridization were much lower than those determined by optical microscopy with the Utermöhl method involving sedimentation chambers, but the results were highly correlated (e.g. $r^2 = 0.94$; $n = 6$ for *A. tamarense*). Determination of spirolides and PSP toxins by instrumental analysis on board ship demonstrated the presence of both toxin groups in plankton assemblages collected from surface waters near the Orkney Islands, and confirmed the association of *A. ostenfeldii* with spirolides in northern Europe. These results show that rRNA probes for *A. tamarense* and *A. ostenfeldii* are useful, albeit only semi-quantitative, tools to detect and discriminate these species in field studies.

Key words: *Alexandrium tamarense*, *Alexandrium ostenfeldii*, molecular probes, phycotoxins, toxic algae

Introduction

Harmful algal blooms are a common occurrence in northern European waters, causing a wide variety of environmental and public health problems, including massive fish mortalities, seafood poisoning in humans, and biofouling of beaches and fishing gear (see references in Granéli *et al.*, 1990). On a global basis, there is some evidence for an increase in the frequency, intensity and distribution of harmful events associated with algal blooms (Smayda, 1990; Hallegraeff, 1993). In any case, it is beyond dispute that the social and economic consequences have become more severe in the last several decades, concomitant with increased exploitation of non-traditional seafood products, fisheries stocks and aquaculture species.

Among the several dozen reported toxigenic species of phytoplankton, those belonging to the marine dinoflagellate genus *Alexandrium* Halim (Balech, 1995) are perhaps the most thoroughly investigated for their toxic properties. *Alexandrium* species are frequently implicated as the cause of paralytic shellfish poisoning (PSP) in human consumers of contaminated seafood. The taxonomic history of *Alexandrium* is long and complex, with many issues remaining unresolved. *Alexandrium ostenfeldii* was first described (as *Goniodoma*) from Iceland and other locations in Scandinavia (Paulsen, 1904, 1949), but inadequacies in the plate

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tabulation warranted a redescription from Norwegian specimens (Balech & Tangen, 1985). The best-known species of the genus, *Alexandrium tamarense* (Lebour) Balech, was first described as *Gonyaulax tamarensis* from the Tamar estuary near Plymouth, UK (Lebour, 1925). This species is now widely reported from temperate to sub-Arctic regions, and even tropical latitudes (Taylor, 1984). Braarud (1945) recognized three varieties of *G. tamarensis* (var. *tamarensis*; var. *globosa*; and var. *excavata*) from Norwegian waters. Later, these varieties were assigned specific status within the genus *Gonyaulax* Diesing. In his classic monograph on the morphology of *Alexandrium,* Balech (1995) considers 'excavatum' to be a minor variant of *Alexandrium tamarense* and 'globosa' as synonymous with *Alexandrium ostenfeldii* (Paulsen) Balech & Tangen.

Although *A. ostenfeldii* has been suspected to be a possible source of PSP toxicity in Norwegian shellfish (Balech & Tangen, 1985), this has not been definitively established because of a temporal overlap with the presence of *A. tamarense* blooms in locations such as Oslofjord. Certain cultured strains of *A. ostenfeldii* from Limfjord, Denmark were found to produce low levels of PSP toxins (Hansen *et al*., 1992), and a few isolates from New Zealand were shown to be very toxic (Mackenzie *et al*., 1996).

A group of novel marine toxins, macrocyclic imines known as spirolides, was isolated and characterized from shellfish (Hu *et al*., 1995, 1996) and later from plankton (Cembella *et al*., 1998, 1999) collected from the coastal waters of Nova Scotia, Canada. The causative organism of spirolide toxicity in shellfish was recently identified as *A. ostenfeldii* (Paulsen) Balech & Tangen (Cembella *et al*., 2000), and certain isolates of this species can produce a wide diversity of spirolides in unialgal batch culture (Hu *et al*., 2001). The association of spirolides with *A. ostenfeldii* is important, given that this species is broadly distributed, particularly in north temperate latitudes. In northern Europe, cultured isolates from Danish waters have also been found to produce these toxins (Cembella *et al*., 2000; A.D. Cembella & M.A. Quilliam, unpublished data).

Under casual microscopic observation, such as is routinely performed in monitoring programmes for harmful algae, cells of *A. ostenfeldii* are difficult to discriminate reliably from those of *A. tamarense*. The vegetative cells of the former species are typically larger and more 'globose' than those of the latter (Balech, 1995; Cembella *et al*., 2000), but there is considerable variation in gross morphology among cells of these species. Key diagnostic features, such as the size and shape of the ventral pore at the margin of the first apical (1′) thecal plate, must be examined individually for each specimen – a tedious procedure. Thus even in the absence of other *Alexandrium* taxa, a species-specific probe for *A. ostenfeldii* would be a useful complement for conventional monitoring of phytoplankton.

Since populations of these *Alexandrium* species may coincide in nature (Cembella *et al*., 1998; Levasseur *et al*., 1998), whereas their toxic properties are very divergent, more reliable and rapid methods for species discrimination are desirable. *Alexandrium tamarense* is frequently dominant when the species co-occur and is better known. As a consequence, it is likely that the relative abundance of *A. ostenfeldii,* and hence the risk of spirolide toxicity, has been underestimated in field samples.

The application of taxon-specific nucleic acid probes is one emerging method for discriminating among phytoplankton species. At least in principle, such probes can be used for identifying, quantifying and mapping the distribution of various taxa in natural plankton populations, but these techniques have not been widely applied. Nucleic acid probes have been developed for a broad range of algae and certain hierarchical groups (e.g. Lange *et al*., 1996; Scholin *et al*., 1996; Miller & Scholin, 1998; Simon *et al*., 2000), and in some cases they have been used to determine the abundance and diversity of Prymnesiophyceae (Moon-van der Staay *et al*., 2000) and Bolidophyceae (Heterokonta) (Guillou *et al*., 1999) in field samples. A few molecular probes have also been developed for toxic microalgal taxa, including both diatoms and dinoflagellates (Scholin *et al*., 1996, 1997; Miller & Scholin, 1998; Simon *et al*., 1997). Species-specific rRNA probes for the potentially toxic pennate diatom *Pseudo-nitzschia australis* (Bacillariophyceae) have been used to discriminate this species from other co-occurring *Pseudo-nitzschia* species in culture and field samples, and to quantify cells of these respective taxa (Scholin *et al*., 1996; Miller & Scholin, 1998; Orsini *et al*., 2002). To date, only a single rRNA probe has been published for the genus *Alexandrium* (Miller & Scholin, 1998). This probe is specific for the North American clade of *A. tamarense,* but more rRNA probes for this group and other geographic clades of the species are under development (U. John *et al*., unpublished).

The study reported here represents the first concerted attempt to apply molecular probes for the discrimination of these *Alexandrium* species in natural populations, combined with shipboard analysis of the respective toxin composition of these taxa. We present the results of the application of these taxon-specific probes to discriminate between cells *A. ostenfeldii* and *A. tamarense* in field plankton samples collected off the Scottish

coast near the Orkney Islands, where annual blooms of toxic dinoflagellates are known to occur (Medlin *et al*., 1998; Higman *et al*., 2001).

Materials and methods

Field sampling

The cruise track of the research vessel Heincke in May 2000 extended over an area of 420 × 105 km, from the Orkney Islands to the Firth of Forth along the east coast of Scotland. In the present study, six sampling sites were selected along the coast between Aberdeen and Edinburgh (Fig. 1). For cell identification and counts by optical microscopy, surface seawater samples were collected by pumping from a buoy, with the hose orifice fixed at 1 m depth. Taxonomic samples were preserved with formalin (2% final concentration). Parallel samples for nucleic acid probing were also collected via this pumping method. For toxin analysis, plankton samples (non-quantitative) were obtained with a plankton net (20 μm mesh size) from sub-surface water (approximately 1–3 m depth). Net planktonic material was concentrated by application of low vacuum onto 0.45 μm PTFE filters (50 mm diameter) and rinsed with 0.2 μm-filtered seawater.

Plankton identification and counting

Plankton identification and quantitation was performed by the inverted-microscope method (Utermöhl, 1958) with 25 ml sedimentation chambers. Critical identification of *Alexandrium* cells was carried out on a Leitz Fluovert FS inverted microscope equipped with epifluorescence optics, after the direct addition of the optical brightener calcofluor (0.002% final concentration) (Fritz & Triemer, 1985) to the formalin-fixed samples. Species of *Alexandrium* were discriminated by size, shape, and characteristic thecal features, including presence or absence of the ventral pore on the first apical (1′) plate, form of the apical pore complex (APC), and the shape of the sixth precingular (6″) and posterior sulcal plates (Kofoid notation). *Alexandrium* cells were identified in two steps: by bright-field light microscopy, and then by epifluorescence after calcofluor staining of the thecal plates. As a check on the accuracy of the identifications and counts by the Utermöhl method, for a number of net tow samples, 100 *Alexandrium* cells randomly found in the observation field were carefully identified under epifluorescence microscopy, by manually rotating each cell until the ventral pore could be observed (× 320 magnification).

Cultures and growth conditions

For validation of selectivity and sensitivity, oligonucleotide probes were tested with unialgal cultured strains of various taxa from a range of geographical locations. A list of the cultured algal strains used in this study is presented in Table 1. Unialgal cultures were grown in 500 ml Erlenmeyer flasks in IMR1/2 growth medium (Eppley *et al*., 1967) supplemented with 10 nM selenite (for *Alexandrium tamarense, A. catenella, A. fundyense, A. pseudogonyaulax, A. taylori, A. minutum* and *A. lusitanicum*) or K medium (Keller *et al*., 1987) (for *A. affine, A. ostenfeldii* and *Thalassiosira rotula*). All cultures were maintained at 15°C in a controlled growth

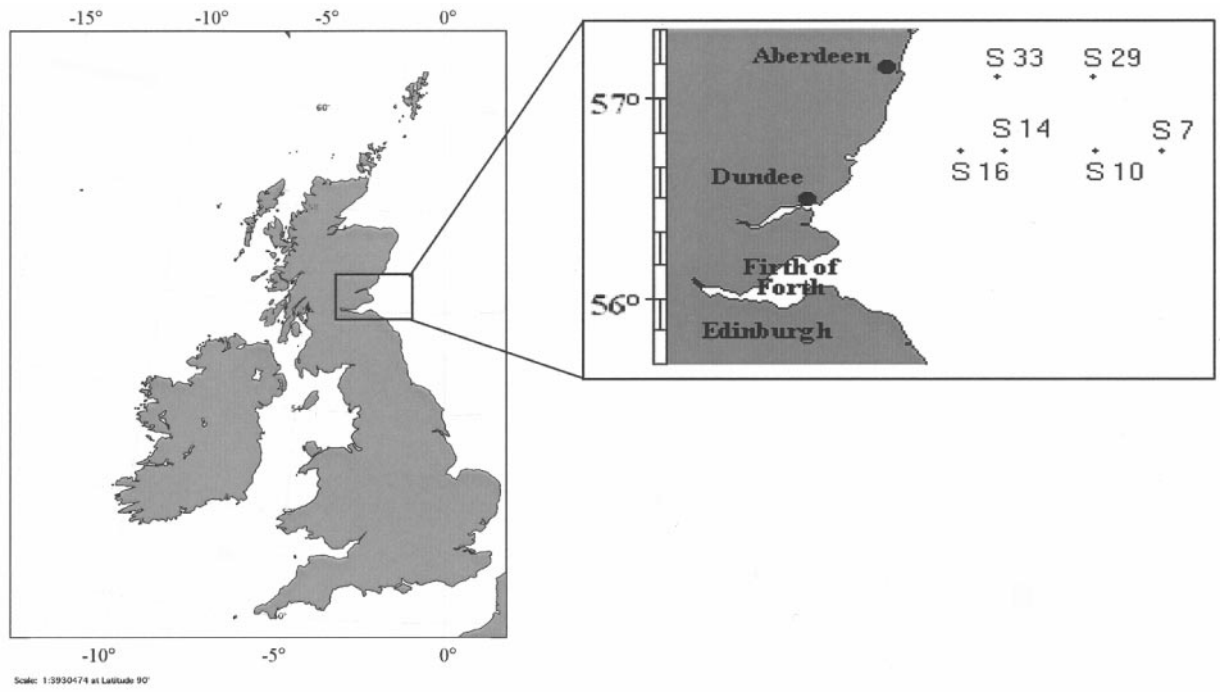

Fig. 1. Map showing the British Isles and an expanded view of the Scottish east coast surveyed during the research cruise of the RV *Heincke* in May 2000. Sample stations (S) considered in this study are numbered.

Table 1. Designation and geographical origin of strains used in this study

Species	Strain	Origin	Collector
Alexandrium affine	CCMP 112	Ria de Vigo (Spain)	I. Bravo
Alexandrium catenella	BAH ME 255	Tarragona (Spain)	M. Delgado
	BAH ME 217	Tarragona (Spain)	M. Delgado
Alexandrium fundyense	GT 7	Bay of Fundy, New Brunswick (Canada)	A. White
	CCMP 1719	Portsmouth, MA (USA)	D. Kulis
Alexandrium lusitanicum	BAH ME 91	Laguna Obidos (Portugal)	E. Silva e Sousa
Alexandrium minutum	Al3T	Gulf of Trieste (Italy)	A. Beran
	Al5T	Gulf of Trieste (Italy)	A. Beran
Alexandrium ostenfeldii	BAH ME 136	Timaru (New Zealand)	N. Berkett
	AOSH1	Ship Harbour, Nova Scotia (Canada)	N. Lewis
	K0324	Limfjord (Denmark)	P.J. Hansen
	K0287	Limfjord (Denmark)	P.J. Hansen
Alexandrium pseudogonyaulax	AP2T	Gulf of Trieste (Italy)	A. Beran
Alexandrium tamarense	GTTP01	Perch Pond, Falmouth, MA (USA)	D. Kulis
	NEPCC 407	English Bay, Vancouver, British Columbia (Canada)	A. Cembella
	AL18b	St Lawrence estuary, Quebec (Canada)	A. Cembella
	OF 84423.3	Ofunato Bay (Japan)	M. Kodama
	BAH ME 181	Orkney Islands (UK)	M. Elbrächter
	BAH ME 182	Orkney Islands (UK)	M. Elbrächter
	SZN 019	Gulf of Naples (Italy)	M. Montresor
	SZN 021	Gulf of Naples (Italy)	M. Montresor
	31/4	Cork Harbour (Ireland)	W. Higman
	CCMP 115	Tamar estuary (UK)	I. Adams
Alexandrium taylori	Ay1T	Lagoon of Marano (Italy)	A. Beran
	Ay2T	Lagoon of Marano (Italy)	A. Beran
Thalassosira rotula	CCAP 1085/4	Fishguard (UK)	L.K. Medlin

chamber with a 14:10 h light:dark photocycle, at a photon flux density of 150 μmol m^{-2} s^{-1}, except for *A. ostenfeldii* and *T. rotula* (90 μmol m^{-2} s^{-1}).

DNA preparation

DNA extractions were made from 500 ml of culture in logarithmic growth phase, using a PAN Plant kit (PAN Biotech, Aldenach, Germany) according to the manufacturer's instructions, with minor modifications as follows. Cultures were filtered onto 47 mm, 3 μm pore-size polycarbonate filters (Isopore, Millipore, Bedford, MA, USA), then the cells were washed from the filter into 1.5 ml reaction tubes with 400 μl preheated (65°C) lysis buffer. Thirty microlitres of proteinase K (10 mg ml^{-1}) were added, followed by 90 min incubation at 65°C in a thermo-shaker. After cell lysis, 40 μl RNase A (10 mg ml^{-1}) was added and the extract incubated at room temperature for 30 min. Extraction and cleaning of the genomic DNA was performed on a silica membrane supplied with the kit. DNA concentration was measured spectrophotometrically at 260 nm, and DNA quality was verified by agarose gel electrophoresis.

PCR amplification of rRNA genes and sequencing

PCR amplification of the 18S rDNA gene and the D1/D2 region of the 28S rDNA gene was done in a thermocycler (MWG, Ebersberg, Germany) with the primers 1F and 1528R for 18S (Chesnick *et al*., 1997), and Dir1F and D2CR for 28S (Scholin *et al*., 1994), respectively. Conditions for PCR were as described in Chesnick *et al*. (1997) and Medlin *et al*. (1998) for the 18S and 28S rRNA genes, respectively. Up to three PCR products were pooled and cleaned with a PCR purification kit (Qiagen, Hilden, Germany) and sequenced with the Long Read kit (Biozym, Hessisch Oldendorf, Germany) and a LiCor 4000L automatic sequencer (MWG, Ebersberg, Germany), with the same primers as in the PCR for the 28S rRNA and internal primers for the 18S rRNA gene (Elwood *et al*., 1985). All sequencing primers were infrared-labelled. Sequences were compiled by DNASIS (Amersham, Freiburg, Germany). Sequence alignment was done with CLUSTAL software, and improved by eye for the 28S sequence and for the 18S sequence. The neighbor-joining tree option in the ARB program (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/) was used to identify the clade containing *A. ostenfeldii* from both the 18S and 28S rRNA tree for selected probe development.

Design of oligonucleotide probes

Ribosomal RNA oligonucleotide probes were designed with the ARB software package, according to Simon *et al*. (2000). Databases consisting of more than 450 published and unpublished algal 18S rRNA sequences and 150 28S rRNA sequences were consulted. Two functional probes were developed for *A. ostenfeldii*: AOST01 was targeted to the 28S rRNA, and AOST02 to the 18S rRNA of this species (Table 2). For comparison, a specific probe for the entire *Alexandrium tamarense/fundyense/catenella* species complex was selected from the 18S rRNA gene, and one probe was also selected from the 28S rRNA gene for the toxic North American

Table 2. Summary data of oligonucleotide probes used in this study

	Standardized probe name[a]	Specific for:	Probe sequence [5′–3′]	*In situ* conditions	Dot-blot conditions
DINO1[b]	S-C-DINO-1404 (*A. tamarense*)-a-A-20	Dinoflagellates (18S rRNA)	CCTCAAACTTCCTTGCITTA	20% formamide	55°C, 2× SSC, 0.1% SDS
ATAM01	S-S-A.tam-0775 (*A. tamarense*)-a-A-18	*A. tamarense* (18S rRNA) species complex	TTCAAGGCCAAACACCTG	20% formamide	56°C, 1× SSC, 0.1% SDS
ATNA02	L-St-At.NA-373 (*A. tamarense*)-a-A-18	*A. tamarense* (28S rRNA)– North American/Orkney strains	AACACTCCCACCAAGCAA	15% formamide	56°C, 1× SSC, 0.1% SDS
AOST01	L-S-A.ost-484 (*A. ostenfeldii*)-a-A-18	*A. ostenfeldii* (28S rRNA)	ATTCCAATGCCCACAGGC	20% formamide	55°C, 1× SSC, 0.1% SDS
AOST02	L-S-A.ost-0232 (*A. ostenfeldii*)-a-A-18	*A. ostenfeldii* (18S rDNA)	CACCAAGGTTCCAAGCAG	20% formamide	55°C, 1× SSC, 0.1% SDS

[a]Alm *et al.* (1996); [b]Groben *et al.* (2002 in preparation).

clade of the *A. tamarense* group (John *et al.*, 2002, submitted). The 28S rRNA probe for *A. tamarense* was shifted four bases from that published by Scholin *et al.* (1997) because of a potential hairpin loop in the previously published sequence. A Dinophyceae-specific probe, DINO01 (Groben *et al.*, in preparation), was used as a positive control in dot-blot and whole-cell hybridization experiments.

DNA dot-blot hybridization

Unmodified oligonucleotides were supplied by MWG-Biotech (Ebersberg, Germany) and labelled with digoxigenin (DIG) for non-radioactive DNA dot-blot experiments, using the 3′ Oligonucleotide Tailing Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Approximately 100 ng of amplified PCR product per sample was denatured for 10 min at 95°C, spotted onto a positively charged nylon membrane (Roche, Mannheim, Germany) and fixed by 90 s exposure of both sides of the membrane to standard ultraviolet illumination. Four hours of pre-hybridization followed by overnight hybridization were done in roller tubes in 10 ml hybridization buffer (5× sodium-sodium citrate (SSC), 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) sodium dodecylsulfate (SDS), 1% (w/v) blocking reagent (Roche, Mannheim, Germany), 0.1 mg ml^{-1} Poly A) in a hybridization oven with probe-dependent temperatures (Table 2). For hybridization, 0.1 pmol ml^{-1} DIG-labelled probe was added to the hybridization buffer. Washing of filters consisted of two 5-min washes in 2× SSC/0.1% SDS or 1× SSC/0.1% SDS, according to the different probes (Table 2) at room temperature, and two 15-min washes at hybridization temperature. Detection was performed with the DIG Luminescent Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and membranes were exposed to X-ray film (Amersham, Freiburg, Germany) for 1–3 h.

In situ *hybridization of whole dinoflagellate cells*

Whole cells from laboratory cultures were hybridized with fluorescein isothiocyanate (FITC)-labelled probes (Interactiva, Ulm, Germany) by a modified protocol from Scholin *et al.* (1996) and examined by epifluorescence microscopy. Cells were carefully filtered onto white 47 mm diameter (3.0 μm pore size) polycarbonate membranes (Isopore, Millipore, Bedford, MA, USA) in a standard filtration unit (Millipore, Bedford, MA, USA) under < 100 mmHg vacuum to prevent cell damage. Freshly prepared saline EtOH fixative (25 ml 100% ethanol, 2 ml Milli-Q water, 3 ml 25× SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris/HCl, pH 7.8)) was added to the filtration unit and cells were fixed for 1 h at room temperature. The fixative was filtered through and the filter incubated with hybridization buffer (5× SET buffer, 0.1% IGEPAL-CA630, 30 ng ml^{-1} Poly A) for 5 min at room temperature. The rinsed filter was cut into pieces; each piece was put onto a microscope slide and hybridized separately with a different probe. Sixty microlitres hybridization buffer containing 10–20% formamide with FITC-labelled probes (5 ng μl^{-1}) was applied directly onto the filter pieces and incubated for 1 h at 50–55°C in a humid chamber in the dark (Table 2). The filters were rinsed for 5 min in 100 μl of 1× SET buffer under the same conditions to remove excess unbound probe, before mounting in a mixture of CitiFluor (Citifluor, London, UK) as an anti-fade agent, with 4′,6′-diamidino-2-phenylindole (DAPI) (1 μg ml^{-1}) as a counterstain. The coverslip was sealed with clear nail varnish and the slides analysed with an Axioskop 20 epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with Zeiss filter sets 02 (DAPI) and 09 (FITC). Photographs were taken on 1600 ASA Fuji colour print film.

For the first tests of the rRNA probes, approximately 100 ng μl^{-1} of 27 different PCR products from target and non-target species were pipetted onto a positively charged nylon membrane. The DINO01 probe was used as a positive control.

Probe application to field samples

One litre of seawater collected by pumping from a buoy (see *Field sampling* protocol) at 1 m depth from each of six stations was filtered onto a 47 mm diameter (3 μm pore size) white polycarbonate filter (Isopore, Millipore, Bedford, MA, USA) on board ship. The cells were fixed

for *in situ* hybridization as described above. Filters were stored dry at 4°C for analysis in the laboratory within 2 weeks. For analysis, half of a filter was cut into four equal-sized pieces and hybridized with different probes (Table 2). Samples were analysed as described above with a Zeiss Axioskop 20 epifluorescence microscope (Zeiss, Oberkochen, Germany). The entire filter slice was scanned at $\times 400$ magnification and all positive signals were counted in each quadrant.

Extraction of algal toxins

For spirolide analysis, filtered planktonic material collected by 20 μm plankton net tow was extracted for 1 min in a 2 ml Eppendorf vial containing 1.0 ml methanol:water (1:1 v:v) with a Sonopuls HM 70 ultrasonicator (Bandelin, Germany). Crude extracts were centrifuged for 10 min (2980 *g*) and the supernatant was passed through a 0.45 μm nylon filter (Rotilab, Carl Roth, Karlsruhe, Germany).

For PSP toxin determination, plankton samples were extracted on board ship for subsequent analysis. Plankton cells were extracted from filter slices by adding 1.0 ml of 0.03 N acetic acid and ultrasonicating for 10 min. The sonicate was centrifuged for 10 min (2980 *g*), and passed through a 0.45 μm nylon filter (Rotilab, Carl Roth, Karlsruhe, Germany; 10 mm diameter). All crude extracts were analysed on board, then stored at −28°C and re-analysed in the laboratory within 3 months of the research cruise.

Liquid chromatography/mass spectrometry determination of spirolides

Plankton extracts were directly analysed on board ship by liquid chromatography with mass spectrometric detection (LC-MS) using a PE Series 200 quaternary pump and a PE Series 200 autosampler (Perkin-Elmer, Langen, Germany), according to minor modifications of the analytical method published in Cembella *et al.* (1999). Briefly, the separation of spirolides was carried out on a reversed-phase column (Luna 3 μm C18, 150×4.60 mm ID, Phenomenex, Aschaffenburg, Germany) with an ammonium formate/formic acid buffer system and acetonitrile/methanol as the mobile phase (Hummert *et al.*, 2002 submitted). Acetonitrile and methanol (Baker, Deventer, The Netherlands) were high-performance liquid chromatography (HPLC) grade. Water was purified to HPLC grade with a Millipore-Q RG ultra-pure water system (Millipore, Milford, USA). The quality of all other reagents was at least analytical grade (p.a.).

Spirolides were detected by an API 165 mass spectrometer equipped with an atmospheric pressure ionization (API) source operating in turbo ion spray (TIS) mode (Applied Biosystems, Concord, Canada). Selected ion monitoring (SIM) mode was used for the determination of spirolides, according to the published mass/charge (m/z) values of known spirolides (Hu *et al.*, 1995, 1996; Cembella *et al.*, 1999, 2000), whereby $m/z = 692.5$, 694.5, 706.5, 708.5, 710.5 and 712.5 $[M+H]^+$ were monitored.

In addition, spirolides found in field samples were characterized by LC/MS/MS with an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Canada) (Hummert *et al.*, 2002 submitted). The spirolide content at each sampling site was calculated by integration of all chromatogram peaks that were clearly identified as spirolides: m/z 692.5 and 706.5, corresponding to spirolides A/desmethyl spirolide C and spirolide C, respectively. The only available quantitative standard for spirolides was a calibration solution containing spirolide D (2.0 μg ml^{-1}) obtained from the Institute for Marine Biosciences, NRC, Halifax, Canada. Spirolide concentrations were determined from a calibration curve prepared with spirolide D, assuming equal molar response factors for the other spirolides.

Liquid chromatography/fluorescence determination of PSP toxins

PSP toxins in plankton samples were analysed by HPLC, with reference to a well-established method (Hummert *et al.*, 1997; Yu *et al.*, 1998) based on ion-pair chromatography with octane sulphonic acid, post-column oxidation with periodic acid, and fluorescence detection. Certified quantitative standards (NRC-PSP-1B) for saxitoxin (STX), neosaxitoxin (NEO) and gonyautoxins (GTX1, GTX2, GTX3 and GTX4), also containing unquantified traces of decarbamoyl derivatives (dcGTX2 and dcGTX3), were purchased from the Institute for Marine Biosciences, National Research Council, Halifax, Canada. Calibration curves of different PSP toxins were used to calculate the respective toxin concentrations in sample extracts.

Results

Morphology of A. ostenfeldii *and* A. tamarense

The basic thecal plate tabulation for both *A. ostenfeldii* and *A. tamarense,* according to the modified Kofoid notation applied to *Alexandrium* (Balech, 1995), is expressed as: Po, 4′, 6″, 6c, 9–10s, 5‴ and 2⁗. Detailed descriptions of these respective species from various locations are available in the literature (Balech, 1995; Balech & Tangen, 1985; Jensen & Moestrup, 1997; Moestrup & Hansen, 1988). In general, field specimens of these species from the east coast of Scotland are consistent with the description of material from other North Atlantic areas, including the coast of Spain (Balech, 1995), Norway (Balech & Tangen, 1985), and Nova Scotia, Canada (Cembella *et al.*, 2000).

Alexandrium ostenfeldii is morphologically similar to *A. tamarense* and the use of gross morphological characteristics to distinguish these species is often confounded, particularly after time in culture. For example, in field material from the waters near the Orkney Islands, *A. tamarense* was often found in chains of two cells (in culture up to four cells), but no chains of *A. ostenfeldii* were observed. In culture, a small fraction of *A. ostenfeldii* produced

chains of two cells, particularly when growth was rapid. Vegetative cells of *A. ostenfeldii* are typically larger than those of *A. tamarense,* but the size ranges overlap. In field specimens from the Orkney Islands, most *A. ostenfeldii* cells were >45 μm, and only 10–15% of cells were <40 μm in trans-diameter. *Alexandrium tamarense* cells from this area were typically <35 μm, although 15–20% of cells were larger, to a maximum of 47 μm. In cultured isolates from the Orkney Islands, the mean diameter of *A. ostenfeldii* cells was <35 μm; this size reduction in culture was also described by Cembella *et al.* (2000) from cultured Nova Scotian populations. Representative specimens of these species from northern Europe stained with calcofluor and viewed under epifluorescence microscopy (Fig. 2) and scanning electron microscopy (Fig. 3) are shown photographically. For samples from the Scottish east coast, the percentage of *A. ostenfeldii* cells in relation to the total count of *Alexandrium* cells determined by careful observation of calcofluor-stained specimens under epifluorescence microscopy was similar to that determined by the Utermöhl technique.

As noted by Braarud (1945), for *A. ostenfeldii* (as *Gonyaulax tamarensis* var. *globosa*) and in the later redescription (Balech & Tangen, 1985) from Norway, cells tend to be 'globose' and less angular than those of *A. tamarense*. In Orkney specimens of the former species, the sulcus is not markedly indented, and in contrast to *A. tamarense* cells, there are no clearly visible sulcal lists. In *A. tamarense,* the sulcal lists of most cells are visible even under low magnification ($\times 100$) in the inverted microscope. In comparison with *A. tamarense,* cells of *A. ostenfeldii* from field samples have a rather delicate theca, with ill-defined plate sutures, and a shallow cingulum. *Alexandrium tamarense* from the Orkney Islands does have the deeply indented sulcal region and angular features characteristic of most specimens of *Alexandrium* cf. *excavatum*

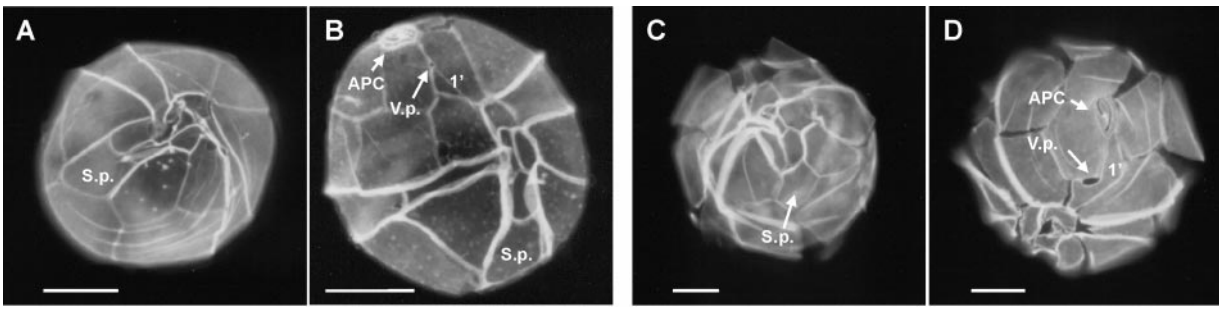

Fig. 2. Epifluorescence micrographs of calcofluor-stained thecal plates of *Alexandrium tamarense* (*A, B*) and *A. ostenfeldii* (*C, D*). APC, apical pore complex; V.p., ventral pore; 1′, first apical plate; S.p., posterior sulcal plate. Scale bar represents 10 μm.

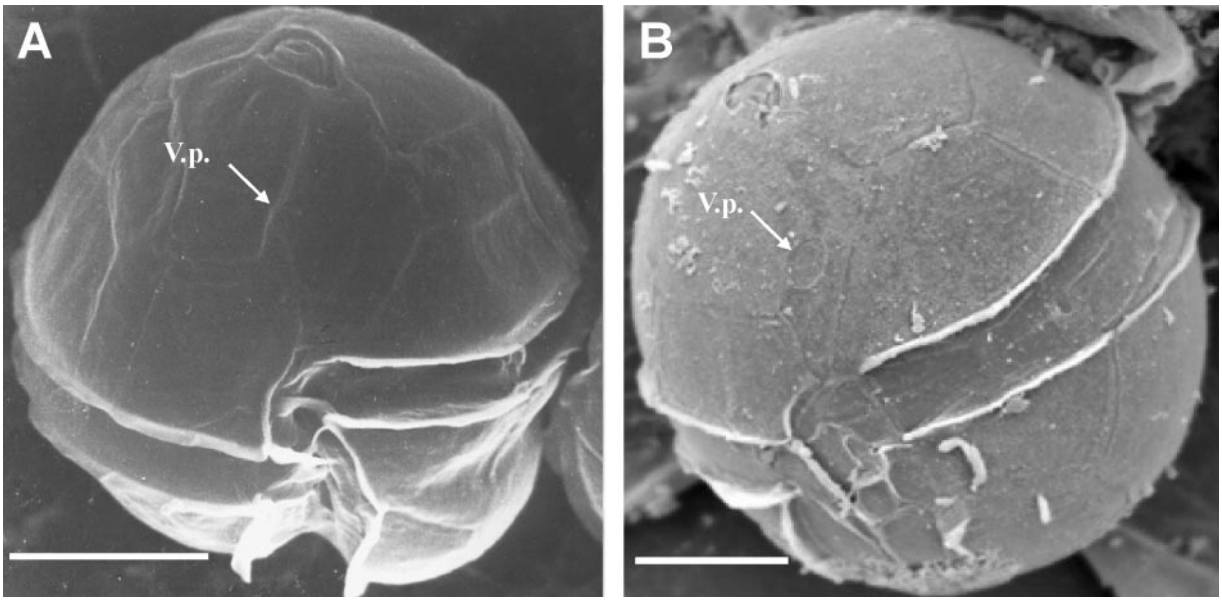

Fig. 3. Scanning electron micrographs of entire vegetative cells of *Alexandrium tamarense* (*A*) and *A. ostenfeldii* (*B*) from northern European waters. V.p., ventral pore. Note that the V.p. is partially occluded by membranaceous material in these specimens. Scale bar represents 10 μm.

sensu Balech (1995) from the Estuary and Gulf of St Lawrence in eastern Canada (Destombe & Cembella, 1990).

These species may be discriminated by careful observations of the details of particular thecal plates. Both species exhibit a ventral pore on the margin of the first apical (1′) plate, but the pore is large and kidney-shaped in *A. ostenfeldii* and smaller and circular in *A. tamarense*. In field material from the east coast of Scotland, these ventral pores are easily distinguished at high magnification ($> \times 250$) of calcofluor-stained cells under epifluorescence microscopy. The ventral pore may be occluded by membranaceous material, particularly in cultured cells, but the margin can usually be visualized after fluorescence staining (Fig. 2). Fixation and preparation for scanning electron microscopy can result in occlusion to the extent that only the faint outline of the ventral pore (indicated as V.p.) remains visible (Fig. 3).

In typical specimens of *A. ostenfeldii* from the Orkney Islands, the sixth precingular (6″) plate is much wider than long, whereas in *A. tamarense* the dimensions are more or less equal. In *A. minutum,* which was also present in very low numbers in several samples, the 6″ plate is narrow. In *A. ostenfeldii,* the 2‴′ plate is extremely large and regularly shaped – more or less rectangular with only small deviations at the sutures where it joins the posterior sulcal (S.p.) plate and the 1‴′ plate, making it slightly pentagonal. In *A. tamarense,* the 2‴′ plate is more asymmetrical.

Dot-blots

Both the *Alexandrium ostenfeldii*-specific probes (AOST1 and AOST2) only gave clearly positive hybridization signals with the target amplicons of the four *A. ostenfeldii* cultured strains in the first line of the dot-blots (Fig. 4). However, very weak and non-specific signals were detected for the AOST1 probe in PCR products from other dinoflagellates used as negative controls only after 90 min exposure time during autoradiography. All attempts to increase the stringency, such as lowering the probe concentration, raising the hybridization temperature and decreasing the salt concentration of the wash buffers, did not remove these weak signals when the membranes were subjected to prolonged exposure. However, in all cases the weakly negative signals could be clearly distinguished from the strong positive signals of the target samples.

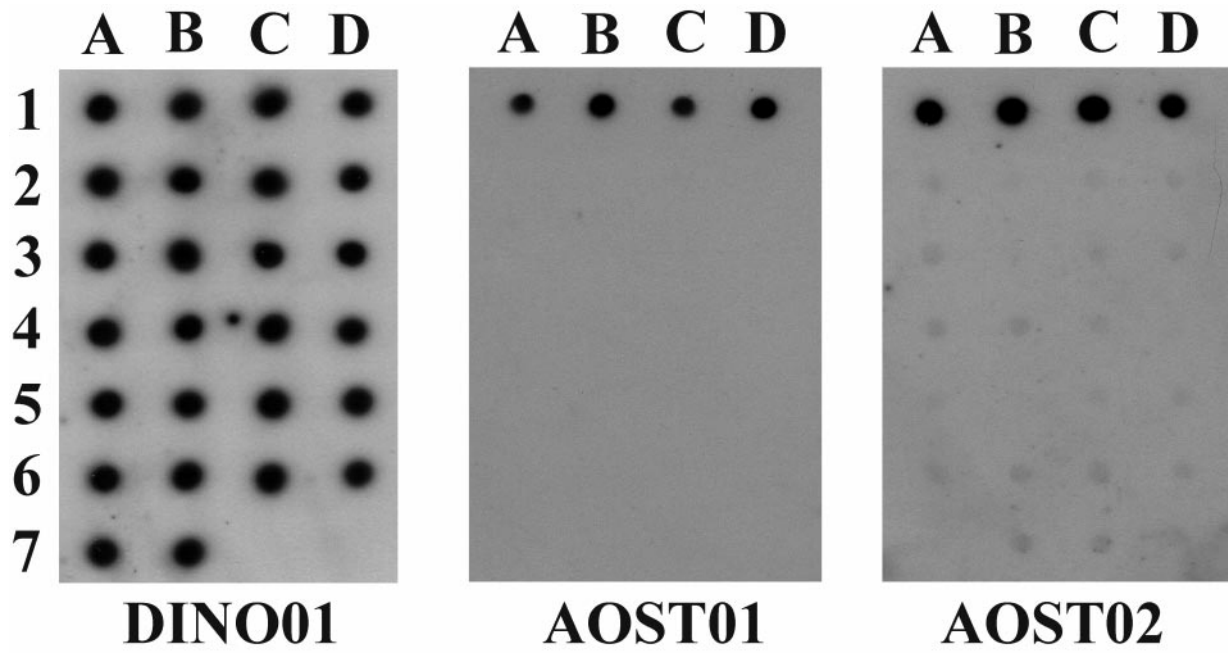

Fig. 4. Specificity of the rRNA probes tested by dot-blot hybridization with 26 strains of *Alexandrium* species and a strain of the diatom *Thalassiosira rotula*. PCR products of the 18S rDNA or 28S rDNA gene were bound to the nylon membrane and hybridized with 18S rDNA (DINO01; AOST02) or 28S rDNA (AOST01) digoxigenin-labelled probes. The dot-blot array is arranged as follows: A1, *A. ostenfeldii* (BAHME136); B1, *A. ostenfeldii* (K0287); C1, *A. ostenfeldii* (K0324); D1, *A. ostenfeldii* (AOSH1); A2, *A. tamarense* (CCMP407); B2, *A. tamarense* (AL18b); C2, *A. tamarense* (OF84423.3); D2, *A. tamarense* (GTPP01); A3, *A. fundyense* (GT7); B3, *A. fundyense* (CCMP1719); C3, *A. tamarense* (BAHME181); D3, *A. tamarense* (BAHME182); A4, *A. tamarense* (SZNB19); B4, *A. tamarense* (SZNB21); C4, *A. tamarense* (31/4); D4, *A. tamarense* (CCMP115); A5, *A. catenella* (BAHME255); B5, *A. catenella* (BAHME217); C5, *A. minutum* (AL5T); D5, *A. minutum* (AL3T); A6, *A. lusitanicum* (GTPORT); B6, *A. taylori* (AY2T); C6, *A. taylori* (AY1T); D6, *A. pseudogonyaulax* (AP2T); A7, *A. affine* (CCMP112); B7, *Prorocentrum minimum* (BAHME137); C7, *Thalassiosira rotula*. Exposure times vary between 1 and 2 h.

Fluorescence in situ *hybridization (FISH)*

Whole-cell hybridization experiments with FITC-labelled rRNA probes were carried out with two cultured strains of *A. ostenfeldii* (AOSH1, K0287) and *A. tamarense* (GTTP01, BAHME181), and one strain each of *A. lusitanicum* (BAHME91), *A. taylori* (AY1T), *A. pseudogonyaulax* (AP2T) and *A. affine* (CCMP112) as controls. These strains were used to find the most stringent hybridization conditions under which the probes were specific for the target cells. All *Alexandrium* strains exhibited a clearly positive (green) signal after hybridization with the dinoflagellate-specific probe (DINO01) (Fig. 5; A1 – D2). In contrast, the probes AOST1 and AOST2 gave positive signals only when *A. ostenfeldii* cells were present. All controls were clearly negative and showed only a weak yellowish colour due to residual pigment autofluorescence.

For whole-cell hybridizations from field material, half of the filter was divided into four equal slices and probed as follows: quadrant 1, Dinophyceae-specific probe (DINO01); quadrant 2, *Alexandrium tamarense* (specific for entire species complex) (ATAM01); quadrant 3, *Alexandrium tamarense* (specific for the North American clade) (ATNA02); and quadrant 4, *Alexandrium ostenfeldii* (AOST01). The DINO01 probe detected dinoflagellates in all assayed samples (Fig. 6). A specific probe for *A. ostenfeldii* (AOST01) gave positive signals for all samples within which spirolides were found (Fig. 7), whereas the probes for *A. tamarense* (ATAM01 and ATNA02) yielded positive results for all samples containing PSP toxins (Fig. 8).

The total dinoflagellate concentrations in the samples determined from counts performed by the *in situ* hybridization technique (DINO01 probe) and Utermöhl techniques differed substantially, with consistently higher concentrations found by the latter method. Furthermore, the cell concentrations of *Alexandrium* spp. determined by the Utermöhl method were always much higher than those detected by *in situ* hybridization, by up to an order of magnitude. At all six sample stations assayed with the FISH probes, more *A. tamarense* cells were detected than those of *A. ostenfeldii,* with both the *in situ* hybridization and Utermöhl

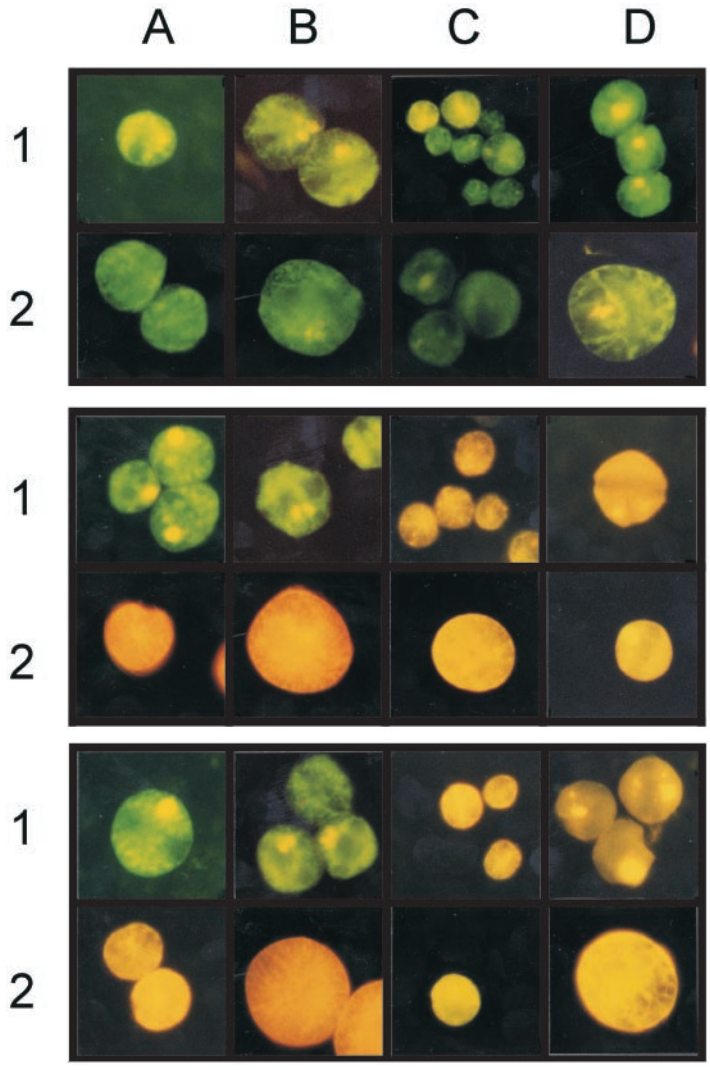

Fig. 5. Array of whole-cell hybridizations from eight *Alexandrium* strains representing six species. Dinoflagellates were hybridized with three different fluorescein-labelled rRNA probes: DINO01, AOST01 and AOST02. A1, *A. ostenfeldii* (AOSH1); B1, *A. ostenfeldii* (K0287); C1, *A. lusitanicum* (BAHME 91); D1, *A. tamarense* (BAH ME181); A2, *A. tamarense* (GTPP01); B2, *A. taylori* (Ay2T); C2, *A. pseudogonyaulax* (AP2T); D2, *A. affine* (CCMP 112).

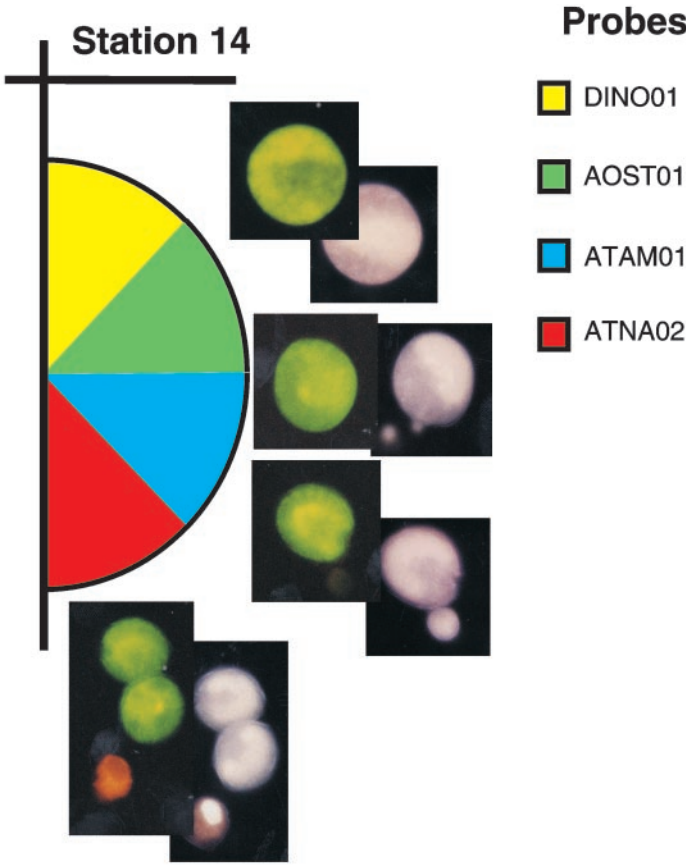

Fig. 6. An example of whole-cell hybridization of plankton samples from station 14. The semicircle represents half of the polycarbonate filter that was labelled. Quadrants labelled with different fluorescence-labelled rRNA probes are indicated in different colours as follows: DINO01 (yellow), AOST01 (green), ATAM01 (blue), ATNA02 (red). Positive hybridization (greenish signal) is shown for all probes adjacent to the same specimens counterstained with DAPI.

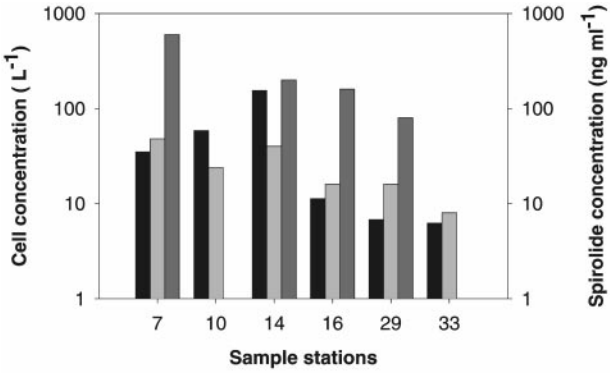

Fig. 7. Variation in concentration of *A. ostenfeldii* cells, counted as fixed cells in a Utermöhl chamber (dark grey bars) versus with FITC-labelled rRNA probes for *in situ* hybridization (light grey bars), at six sampling stations. Total spirolide concentration (ng ml^{-1}) (black bars) at the respective sampling stations is presented for comparison.

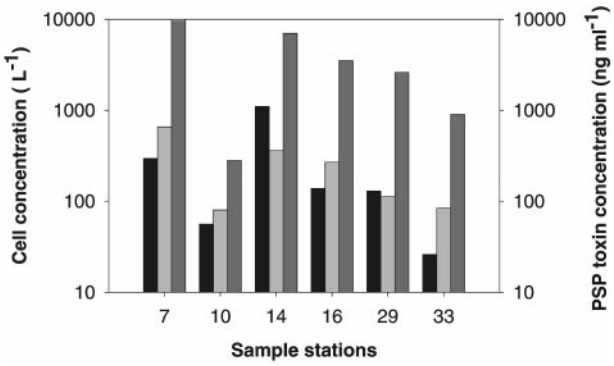

Fig. 8. Variation in concentration of *A. tamarense* cells, counted as fixed cells in a Utermöhl chamber (dark grey bars) versus with FITC-labelled rRNA probes for *in situ* hybridization (light grey bars), at six sampling stations. Total PSP toxin concentration (ng ml^{-1}) (black bars) at the respective sampling stations is presented for comparison.

counting methods (Figs 7, 8). For *A. tamarense,* the same quantitative trend was apparent with both counting methods at all stations – highest cell concentrations were found at station 7 and lowest concentrations at station 10. Counts performed by these alternative techniques were highly correlated (Pearson correlation coefficient; $r^2 = 0.94$; $n = 6$). The same covariance trend ($r^2 = 0.67$; $n = 6$) was observed for *A. ostenfeldii,* with two obvious exceptions: at stations 10 and 33, no *A. ostenfeldii* cells were detected in the Utermöhl counting chamber (25 ml), but this species was detected by *in situ* hybridization.

Analysis of spirolides and PSP toxins

Spirolides were detected at all sampling sites, with the highest concentration of total spirolides found at station 14 (155 ng ml^{-1} net-plankton extract). Only three main spirolide compounds (at m/z 692.5 and m/z 706.5) were present in sufficient amounts for identification and quantitation. Ion m/z 692.5

$[M+H]^+$ refers to two different spirolides, designated as spirolide A and des-methyl C, both with MW 691.5. Ion m/z 692.5 was not detected at station 29. Ion m/z 706.5 $[M+H]^+$ refers to spirolide C, which was the major derivative at all sampling sites. Two unconfirmed 'spirolide-like' compounds with molecular weight 691.5 were present only in trace amounts. There was an obvious discrepancy between detected spirolide concentration and the abundance of *A. ostenfeldii* cells counted by the Utermöhl technique ($r^2 = 0.01$; $n = 6$). For example, at stations 10 and 33, no *A. ostenfeldii* cells were counted (Fig. 7), but 59 and 7 ng spirolides ml^{-1} net-plankton extract, respectively, were detected at those stations. The trend in *A. ostenfeldii* cell abundance determined by the FISH probe tracked the spirolide concentration better than did the Utermöhl method (Fig. 7) ($r^2 = 0.37$; $n = 6$).

The total PSP toxin content in field samples determined by the HPLC technique generally followed a trend similar to that of the cell abundance of the putative toxigenic species, although correlations were not high for either counting method (Fig. 8). For example, in comparing PSP toxin concentration versus Utermöhl counts, the correlation ($r^2 = 0.56$; $n = 6$) was similar to that for the FISH probe counts ($r^2 = 0.43$; $n = 6$). The highest concentrations of total PSP toxins were measured at station 7 and station 14 (295 ng ml^{-1} and 1100 ng ml^{-1} of plankton extract, respectively); however, the concentration of *A. tamarense* cells was much higher at station 7 than at station 14 (10 400 versus 7000 cells l^{-1}, respectively). An additional potential source of PSP toxins, *A. minutum* cells, was also present but in low abundance (10 cells l^{-1} at station 7 and 400 cells l^{-1} at station 14).

Discussion

The current study based upon samples collected during the RV *Heincke* cruise along the east coast of Scotland represents the first large-scale integrated field observations of the co-distribution of *Alexandrium ostenfeldii* and *A. tamarense,* and their respective toxins, spirolides and PSP toxins. These observations are significant, particularly given the long historical reports of the occurrence of these species in northern European waters (Paulsen, 1904, 1949; Lebour, 1925; Braarud, 1945; Balech & Tangen, 1985) and the recent discovery of spirolides in cultured isolates from Denmark (Cembella *et al.*, 2000). The distribution of PSP toxicity on the Scottish east coast, circumstantially linked to blooms of *A. tamarense* via a density-driven circulation model (Brown *et al.*, 2001), has been associated with occasional outbreaks of human illnesses in the region.

Prior research raised suspicions that *A. ostenfeldii* might pose some risk of causing shellfish toxicity in northern Europe, owing to the presence of PSP toxins (Balech & Tangen, 1985). More recent findings of low PSP toxicity of cultured isolates of *A. ostenfeldii* from the region (Hansen *et al.*, 1992; A. Cembella, unpublished data) have tended to reduce this concern. Nevertheless, an early report linking the presence of *Pyrodinium phoneus* (Woloszynska & Conrad, 1939) (probably a description of *A. ostenfeldii*) with symptoms of human intoxication resembling those of PSP, after consumption of mussels from canals in Belgium, suggests that this risk should not be ignored.

Spirolides are novel toxins with unknown toxicological effects on marine ecosystems and human consumers of shellfish. The use of direct LC/MS analysis on board ship has proved to be an effective research tool for surveying the distribution of spirolides in the water column. We assume that the reason why spirolides have not been detected before in European waters is because there have been no previous efforts to analyse plankton or shellfish samples for the presence of these compounds. Analytical standards are not readily available and detection relies on sophisticated analysis by LC/MS, a technology available in only a few research laboratories. Thus the occurrence of spirolides in the plankton, now known to be associated with *A. ostenfeldii,* is most likely not a new phenomenon, but only a newly discovered one.

Emerging evidence of the high potency of spirolides (Richard *et al.*, 2002), even relative to other phycotoxins, and the accumulation of spirolides in bivalve shellfish in Atlantic Canada (Hu *et al.*, 1995), underscores the importance of monitoring for these compounds and the causative organism(s) in the water column, especially in shellfish harvesting areas. In most toxic phytoplankton monitoring programmes, *A. ostenfeldii* has been of marginal interest due to its typically weak ability to produce PSP toxins, at least in north temperate waters. During a previous cruise of the RV *Heincke* in 1997, substantial concentrations of *A. ostenfeldii* cells were observed along the east coast of Scotland (M. Elbrächter, unpublished observation). Now that the spirolide-producing capability of this species has been clearly demonstrated along both the European and North American Atlantic coasts, the requirements for monitoring *A. ostenfeldii* have fundamentally changed.

To date, there are only a few *A. ostenfeldii* strains in culture collections, perhaps as a reflection of the difficulty in the successful isolation and maintenance of strains in clonal culture (Cembella *et al.*, 2000). Few data are available on the occurrence of

natural bloom populations in the field. As a consequence, the biogeographical distribution of *A. ostenfeldii* and its spirolide-producing capabilities remain cryptic.

In contrast to *A. ostenfeldii,* shellfish toxicity derived from *A. tamarense* populations in European waters has been a well-known phenomenon for many decades (Ayres, 1975). Yet there have been few concerted studies on the biogeographical distribution of blooms of this species in relation to the occurrence of PSP toxins in the water column. In the current study, application of HPLC-FD to field populations yielded convincing links between the presence of PSP toxins and toxic dinoflagellate cells, while providing information on toxin composition.

In general, the autecological requirements of *A. tamarense* and *A. ostenfeldii* are somewhat different with respect to temperature and light. *Alexandrium ostenfeldii* clones from the northwest Atlantic tend to thrive in colder waters accompanied by lower light intensities than do members of the *A. tamarense* species complex (Cembella *et al*., 2000). In Atlantic Canada, *A. ostenfeldii* occurs frequently as a subdominant species within phytoplankton assemblages together with other toxigenic *Alexandrium* species (Cembella *et al*., 1998). While isolates from Danish coastal waters appear to grow well in culture at elevated temperatures (Jensen & Moestrup, 1997), *A. ostenfeldii* tends to bloom earlier than does *A. tamarense* in northern Europe, although there may be some overlap (Balech & Tangen, 1985). In this study, the co-occurrence of these two species has now been substantiated for the Orkney Islands, as well.

As noted previously, co-occurrence of *Alexandrium* species in northern Europe (Braarud, 1945; Balech & Tangen, 1985), now known to exhibit different cellular toxicity and toxin spectra, complicates the interpretation of toxic plankton monitoring data based on cell counting and identification by conventional optical microscopy. According to Cembella *et al.* (2000), based largely upon experience in Atlantic Canada, *A. ostenfeldii* is under-reported, particularly in routine phytoplankton monitoring programmes. This is due to morphological similarities with other toxigenic species such as *A. tamarense*. Thus, the successful application of FITC-labelled rRNA probes that are differentially specific for *A. ostenfeldii* versus *A. tamarense* cells is a major advance. The validity of this molecular probe approach, by comparison with thecal staining and epifluorescence microscopy, and the use of scanning electron microscopy – both techniques that are complex and often impractical to apply for routine analysis – has now been demonstrated with field populations of *Alexandrium* from European waters. Use of molecular probes as taxon-specific tools for the discrimination of *Alexandrium* species has the additional advantage that it does not require sophisticated taxonomic expertise for species recognition. For all *Alexandrium* species (but particularly *A. ostenfeldii*), the thecal plates, the primary diagnostic criteria, are rather fragile and readily shed in fixed samples, and with that, most of the distinguishing cell features are lost.

The co-occurrence of toxic and non-toxic strains of *A. tamarense* and *A. ostenfeldii* is especially problematic for toxin risk assessment in areas where no toxin monitoring is done. The fact that these species may be capable of synthesizing chemically unrelated families of marine phycotoxins poses an additional complexity for effective plankton monitoring based upon counts of total *Alexandrium* cells. For example, *A. ostenfeldii* alone could cause spirolide toxicity in shellfish during periods when toxigenic blooms of *A. tamarense* are absent, but this lipophilic toxicity would remain undetected by the routine mouse bioassay for PSP toxins, which involves an aqueous acid extraction. Furthermore, in mixed populations with *A. tamarense,* where *A. ostenfeldii* cell numbers are substantial, use of total *Alexandrium* cell concentrations for toxin risk assessment and early warning of toxicity may be seriously biased. In North America and Europe, where populations of members of the *Alexandrium tamarense* species complex are usually assumed to produce only PSP toxins, a monitoring programme based exclusively on enumeration of cells of *Alexandrium* spp. and/or determination of PSP toxins could miss recurring threats of spirolide toxicity resulting from blooms of *A. ostenfeldii.*

In Europe, the constraints on designing effective taxon-specific probes for species discrimination within the genus *Alexandrium* may be particularly acute. Populations belonging to different clades of the *A. tamarense* species complex are found in European waters (Medlin *et al*., 1998; John *et al*., submitted). In addition to the two toxic *Alexandrium* clades described from North American and temperate Asian regions (Medlin *et al*., 1998), populations belonging to the non-toxic Western European and Mediterranean clades (John *et al*., 2002 submitted) have been identified in Europe. Given this complexity, it was critical to design alternative rDNA probes with different hybridization specificities for application to European field populations of *A. tamarense*. Similarly, in the absence of evidence regarding possible different clades of *A. ostenfeldii* in Europe, it was prudent to design and apply two rRNA probes, one specific for 18S rRNA and the other for the D1/D2 region of the 28S rRNA, to northern European populations.

The DNA dot-blot test is a powerful tool for probe development, offering the opportunity to test probe specificity over a broad range of control strains. In addition, dot-blots are a sensitive technique well suited for detection of target species in mixed phytoplankton assemblages. Dot-blots can be used in field studies to analyse large sample series simultaneously, especially when PCR products of the target gene are used, as was recently demonstrated for bacteria (Raskin *et al*., 1994). All these rRNA probes designed for *Alexandrium* work at the DNA level with the dot-blot technique. PCR products were used in this study to avoid false positive hybridization signals with short oligonucleotides ($\sim$20 bases) as parts of the genomic DNA.

The fluorescence *in situ* hybridisation (FISH) arrays (Figs 5, 6) clearly show that the *A. ostenfeldii* probes AOST01 and AOST02 are effective as species-specific probes in whole-cell hybridization experiments. Although both probes proved to be specific in these experiments, probe AOST01 is recommended for FISH, because AOST02 provides only a single base mismatch with the *A. minutum*/*lusitanicum* species complex. A single nucleotide is sufficient to distinguish target from non-target species under stringent laboratory conditions, but discrimination might be more difficult in field studies where the rRNA population is reduced. Since two probes are available, the application of the less problematic AOST01 probe is suggested for field studies.

The FITC-labelled probes showed that in addition to their usefulness in discriminating among *Alexandrium* species in culture, they have the capability to distinguish among taxa in complex field plankton assemblages. The application of molecular probes to field plankton studies has been shown previously for diatoms (Miller & Scholin, 1998). In the study reported here, the cell concentrations determined by FISH for each *Alexandrium* species were positively correlated with those enumerated by the classic Utermöhl technique, although the total cell counts by the latter method were an order of magnitude higher. In exceptional cases, such as at stations 10 and 33, *A. ostenfeldii* cells were detected by the FISH technique with the AOST1 probe, whereas no cells were counted by the Utermöhl method. These quantitative discrepancies cannot be fully explained, but are unlikely to be related to probe failure, since adequate controls were used. Fluorescence labelling of *Alexandrium* cells from unialgal cultures with the appropriate probe generally achieved almost complete hybridization ($>95\%$ of viable cells labelled). It is conceivable that cells from field samples could be in diverse physiological states, whereby reduced metabolic activity in some cells might result in fewer ribosomes and hence in weaker or fewer hybridization signals (Anderson *et al*., 1999). Nevertheless, the high percentage of DAPI-positive and well-pigmented *Alexandrium* cells (indicating intact nuclei and apparently 'healthy' cells) suggests that this was not a major factor. A more likely scenario is that even among healthy cells, the recognition of the minimum degree of fluorescence labelling required to be considered 'positive' is highly subjective to the human eye – a conservative approach could lead to serious underestimates of the numbers of the targeted taxon.

There are other possible methodological explanations for the difference in counts registered by the FISH technique versus the Utermöhl method. Particulate material, such as organic and inorganic debris, can bind probe constituents, and this non-specifically bound probe is no longer available for labelling target cells (Miller & Scholin, 1998). Excess debris may also hide cells that are then overlooked, but the efforts made to avoid overloading the filters would minimize this problem. Gross inaccuracies in the respective species counts by optical microscopy are unlikely, given the experience of the taxonomist responsible for this aspect. Furthermore, since Utermöhl counts of *A. ostenfeldii* were always much lower (about ten-fold) than those of *A. tamarense* at each station, simple misidentification of *A. ostenfeldii* cells as *A. tamarense* would not greatly affect the total count of the latter species. It is reasonable to attribute the apparent discrepancy between counting methods to sampling inconsistencies and differences in the volumes of water sampled. For example, the Utermöhl counts were based upon a settled 25 ml sample, whereas the cells filtered for FISH probing represented a 1 l volume of seawater. Uneven distribution of the cells on the filter is also a potential source of sampling error; this effect is compounded by the fact that only a portion of the filter was scanned for labelled cells. Observed variability among cell counts with FISH probes at several sample dilutions led Miller & Scholin (1998) to propose counting at least three entire filters. In any case, it is clear that further refinements must be made to the application of the FISH probes for the technique to be considered fully quantitative.

The biogeographical relationship between the distribution of *A. ostenfeldii* and *A. tamarense* and their respective toxins is also noteworthy. For example, by LC/MS, spirolides were detected at two stations where *A. ostenfeldii* was not recorded by the Utermöhl method but was detected by the FISH probe. To date, *A. ostenfeldii* has been considered to be the unique spirolide producer (Cembella *et al*., 2000); thus there is circumstantial evidence that Utermöhl counts performed on small

volumes may not detect rare cells. Of course, the possibility that other protistan taxa or bacteria may also produce spirolides, or that spirolides might be bound in zooplankton faecal pellets and other detritus, cannot be completely excluded. Nevertheless, these results illustrate an advantage of the FISH technique, especially when the cell concentration of a target species in field samples is low, because even events as rare as a single cell generate a clear fluorescence signal. The probability of encountering a rare event is further increased if an instrument, such as a solid phase cytometer (Chemunex), is used to scan the filter and to record all positive signals.

The relatively weak correlations between toxin concentrations and *Alexandrium* cell counts, in contrast to the results of Cembella *et al.* (2001), are best explained as artefacts of the procedures used to sample the surface waters for these different parameters (i.e. net tows versus pumping from discrete depths). Again, in addition to intact recognizable *Alexandrium* cells there may have been some contribution to the toxin pool from other sources, including bacteria, detritus, and grazers upon *Alexandrium*. Cells of *A. minutum* and *A. ostenfeldii* were found at these stations, but they were not specifically isolated and tested for PSP toxicity. In any case, potentially toxic *A. minutum* cells were observed only at a few sampling sites as minor components (maximum 400 cells l^{-1} at station 14) and were not quantitatively important enough to have substantially biased the relationship between *A. tamarense* cell abundance and PSP toxin concentration. Interestingly, the highest PSP toxin concentration was found at station 14, whereas the concentration of *A. tamarense* cells was highest at station 7. Wide variability in PSP toxin content among field and cultured populations of cells of the *A. tamarense*/*fundyense*/*catenella* species complex has been attributed to both genetic differences and variability in localized environmental factors (Cembella, 1998). These results clearly show that rRNA probes are a powerful tool for taxonomic discrimination in field studies. Such probes can be used for monitoring species composition even in the absence of advanced taxonomic expertise. In combination with DNA dot-blots, rRNA probes permit a taxonomically precise identification of microalgal composition, with selectivity adjustable according to probe design. The application of hierarchical probes, as carried out during this study, allows computation of proportions of each algal group even if the complete taxonomic spectrum is unknown. By using rRNA probes with *in situ* hybridization, large numbers of field samples can be simultaneously analysed. If coupled with flow cytometry, *in situ* hybridization offers the potential to measure microalgal diversity on a larger scale, even in real time on board research vessels. In addition, algal cells can be sorted automatically and can then be used for further studies or verification, for example by gene sequencing.

In the study reported here, the validity of rRNA probes for qualitative and semi-quantitative discrimination of *A. tamarense* and *A. ostenfeldii* cells in field samples from northern Europe was demonstrated for the first time. Further work will be directed towards development of more accurate and precise quantitative application of such probes to field populations.

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