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Molecular discrimination of toxic and non-toxic *Alexandrium* species (Dinophyta) in natural phytoplankton assemblages from the Scottish coast of the North Sea

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Molecular methods provide promising tools for routine detection and quantification of toxic microalgae in plankton samples. To this end, novel TaqMan minor groove binding probes and primers targeting the small (SSU) or large (LSU) ribosomal subunit (rRNA) were developed for two species of the marine dinoflagellate genus *Alexandrium* (*A. minutum*, *A. tamutum*) and for three groups/ribotypes of the *A. tamarense* species complex: Group I/North American (NA), Group II/Mediterranean (ME) and Group III/Western European (WE). Primers and probes for real-time quantitative PCR (qPCR) were species-specific and highly efficient when tested in qPCR assays for cross-validation with pure DNA from cultured *Alexandrium* strains. Suitability of the qPCR assays as molecular tools for the detection and estimation of relative cell abundances of *Alexandrium* species and groups was evaluated from samples of natural plankton assemblages along the Scottish east coast. The results were compared with inverted microscope cell counts (Utermöhl technique) of *Alexandrium* spp. and associated paralytic shellfish poisoning (PSP) toxin concentrations. The qPCR assays indicated that *A. tamarense* (Group I) and *A. tamutum* were the most abundant *Alexandrium* taxa and both were highly positively correlated with PSP toxin content of plankton samples. Cells of *A. tamarense* (Group III) were present at nearly all stations but in low abundance. *Alexandrium minutum* and *A. tamarense* (Group II) cells were not detected in any of the samples, thereby arguing for their absence from the specific North Sea region, at least at the time of the survey. The sympatric occurrence of *A. tamarense* Group I and Group III gives further support to the hypothesis that the groups/ribotypes of the *A. tamarense* species complex are cryptic species rather than variants belonging to the same species.

Key words: *Alexandrium*, dinoflagellates, field survey, fluorescence *in situ* hybridization, harmful algal blooms, molecular probes, phycotoxin, real-time quantitative PCR

Abbreviations: HAB, harmful algal bloom; MGB, minor groove binding; LSU, large subunit; SSU, small subunit; PSP, paralytic shellfish poisoning; qPCR, real-time quantitative polymerase chain reaction

Introduction

Harmful algal blooms (HABs) caused by toxic species of the genus *Alexandrium* have had a severe impact on aquaculture, fisheries and human health on a worldwide basis (Anderson *et al.*, 2012). Bloom phenomena associated with *Alexandrium* spp. have apparently been increasing globally in frequency, geographical distribution and magnitude of effect over the last few decades (Hallegraeff, 2010). About a dozen species of this genus produce highly potent neurotoxins (Cembella, 1998), most notably the tetrahydropurine saxitoxin and its derivatives, which are associated with paralytic shellfish poisoning (PSP) in humans after consumption of contaminated seafood. In northern and western European waters, *A. minutum* and *A. tamarense* are the most widely distributed PSP-toxin producers (Persson *et al.*, 2000; John *et al.*, 2003*a*; Touzet *et al.*, 2008). The morphospecies *A. tamarense*, however, has been reported to occur as either a toxigenic North American ribotype referred to as *A. tamarense* Group I, or a non-toxic Western European ribotype known as *A. tamarense* Group III. These most likely represent two of five cryptic species within the '*A. tamarense* species complex'

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(Scholin *et al*., 1994, 1995; John *et al*., 2003*b*; Lilly *et al*., 2007). Several toxic members of the genus *Alexandrium* are difficult to distinguish by traditional methods, such as light microscopy (LM), from morphologically similar but non-toxic relatives. Even within a morphospecies toxigenesis may be inconsistent, ranging from highly toxic to having undetectable toxin content. Efficient methods for rapid and reliable detection and discrimination of various HAB species, including among members of the *A. tamarense* species complex, are therefore desirable for routine monitoring, as well as for field ecological studies.

Various molecular methods based upon oligonucleotide probes have been developed to detect and discriminate among harmful algal taxa. Among these molecular methods, fluorescence *in situ* hybridization (FISH), targeting ribosomal RNA (rRNA), has been often used for identification of harmful species in field samples (e.g. Miller & Scholin, 1998; Anderson *et al*., 2005; Touzet *et al*., 2009). The FISH method is quite straightforward and target cells are directly visible by fluorescence microscopy. However, weak probe penetration, loss of cells during preparatory steps, and autofluorescence of the targeted cells, which can mask the fluorescence signal, have been reported (Simon *et al*., 2000). Furthermore, extensive analysis of field samples with FISH is very laborious and therefore of limited use for routine monitoring of HAB species (Kudela *et al*., 2010).

Real-time quantitative polymerase chain reaction (qPCR) represents an alternative for HAB taxon discrimination, with lower detection limits than for FISH, and with a higher sample throughput and the potential for automation. There has been extensive application of various qPCR methods to determine changes in phytoplankton communities (Handy *et al*., 2006, 2008) and they have also been used successfully for the detection of harmful microalgae (e.g. Galluzzi *et al*., 2004; Dyhrman *et al*., 2006; Touzet *et al*., 2009; Garneau *et al*., 2011), including cysts of *Alexandrium* spp. (Kamikawa *et al*., 2005, 2007), in laboratory cultures and field samples. With a reported detection limit of less than one cell equivalent per millilitre water sample (Kamikawa *et al*., 2006; Lin *et al*., 2006; Zhang *et al*., 2008), even rare species can be detected reliably in field samples. Furthermore, the high specificity of qPCR – in contrast to FISH – enables discrimination among target sequences that differ by only one or a few base pairs. Therefore, even closely related species or populations can be distinguished if specific sequence motifs are known. The qPCR technique can yield relative or, under optimal circumstances, quantitative detection of the target molecules, because amplicon saturation during qualitative PCR can be avoided by monitoring amplification efficiency for each cycle. One common qPCR approach employs the fluorescent dye SYBR Green, which binds to the minor groove of double-stranded DNA (dsDNA), so that increases in fluorescence emission are proportional to PCR amplicon formation. Other more sensitive and specific qPCR approaches, such as TaqMan, molecular beacon, and hybridization probe assays, involve specific or non-specific primers together with a specific fluorigenic oligonucleotide probe. In the present study a TaqMan minor groove binding (MGB) probe qPCR assay was applied after testing the primers with SYBR Green for their specificity. These TaqMan MGB probes possess a 5′ reporter dye and a 3′ non-fluorescent quencher, which diminishes background fluorescence. The MGB moiety stabilizes the hybridized probe and raises the melting temperature. The MGB probes can therefore be considerably shorter than traditional TaqMan probes and thereby they can hybridize to shorter taxon-specific sequence stretches than traditional probes by simultaneously maintaining the requisite annealing temperature (Kutyavin *et al*., 2000).

Sets of primers and numerous probes are available for identification of different *Alexandrium* species by qPCR. Galluzzi *et al*. (2004) first introduced primers specific for the genus *Alexandrium*, and also developed a primer specific for *A. minutum* for application, together with genus-specific primers, in a SYBR Green qPCR assay. Hosoi-Tanabe & Sako (2005) developed a TaqMan-based qPCR assay for toxigenic *A. tamarense* Group I and *A. catenella* Group IV. Dyhrman *et al*. (2006) designed a genus-specific forward primer for *Alexandrium* and employed an oligonucleotide (NA1, Anderson *et al*., 1999) specific for the *A. tamarense* Group I as a reverse primer in a SYBR Green qPCR assay. Touzet *et al*. (2009) designed a qPCR assay based on a hybridization probe specific for *A. minutum* (Global clade), whereas Garneau *et al*. (2011) developed a qPCR assay with a molecular beacon to specifically detect *A. catenella* Group I. Very recently, Murray *et al*. (2011) developed a SYBR green qPCR assay to detect and quantify a gene (*sxtA*) purportedly involved in the synthesis of saxitoxin, one of the key PSP toxins. However, even though this qPCR approach is promising, because it enables the detection of various possible saxitoxin-producing taxa in a single assay, it does not allow for discrimination among toxigenic taxa and therefore is of limited use in ecological studies.

In any case, in spite of numerous alternative qPCR assays, the rather inconsistent development of phylogenetic and taxonomic probes and their

Table 1. *Alexandrium* species and phylogenetic ribotype groups used in this study, including information on toxicity and toxin type (PSP = paralytic shellfish poisoning toxins) and technical application for each strain. PD = probe design, rDNA = determination of DNA yield per cell.

Alexandrium taxon	Strain	Geographical origin	Toxicity	Application
Alexandrium tamarense Group I/North American ribotype	NA	Orkney Islands, UK	toxic (PSP)	PD
	S6_P8_D7	North Sea, UK	toxic (PSP)	rDNA
Alexandrium tamarense Group II/Mediterranean ribotype	SZN1	Gulf of Naples, Italy	non-toxic	PD
Alexandrium tamarense Group III/Western European ribotype	WE 31/9	Cork Harbour, Ireland	non-toxic	PD
Alexandrium tamutum	S6_P4_D7	North Sea, UK	non-toxic	PD, rDNA
Alexandrium minutum	AMP4	Galicia, Spain	toxic (PSP)	PD
Alexandrium minutum	AL1T	Gulf of Trieste, Italy	non-toxic	rDNA

biogeographical application to *Alexandrium* populations has led to the situation that no comprehensive set of primers and probes is available for toxic versus non-toxic, morphologically similar *Alexandrium* taxa. Discrimination of actually or potentially toxic species and their congeners is critical for monitoring programmes because populations of toxigenic and non-toxigenic taxa may co-occur temporally as well as spatially (John *et al*., 2003*b*; Brosnahan *et al*., 2010; Touzet *et al*., 2010). Given the limitations of traditional LM approaches for *Alexandrium* taxon discrimination, including the problem of cryptic speciation, it is desirable to develop additional qPCR assays for all potentially co-occurring *Alexandrium* species for monitoring and field ecological studies.

The aim of the present study was to reliably distinguish and quantify various representatives of the genus *Alexandrium* in order to provide a better understanding of the biogeographical distribution and co-occurrence of selected, potentially toxigenic *Alexandrium* species along the North Sea coast of Scotland, from the Firth of Forth to the Pentland Firth. For this purpose, a qPCR assay was developed and evaluated for the toxic *A. tamarense* Group I, and also for two non-PSP toxin-producing members within the *A. tamarense* species complex, namely Group II (formerly designated as the Mediterranean [ME] ribotype) and Group III (formerly called the Western European [WE] ribotype), based on previously developed probes (John *et al*., 2005). In addition, primers and probes were newly designed and established for the non-toxic species *A. tamutum* (Montresor *et al*., 2004). Finally, a qPCR assay for *A. minutum* was developed based on an earlier probe (Diercks *et al*., 2008); *A. minutum* is typically toxic, but non-toxic representatives have also been found (Touzet *et al*., 2008; Yang *et al*., 2010). The relative abundance and distribution patterns of *Alexandrium* species and ribotype groups were determined by application of the novel qPCR assays to field samples and the results compared with enumerations by traditional LM methods and toxin data. The specificity of the qPCR assay was also validated by fluorescence *in situ* hybridization (FISH) experiments.

Materials and methods

Design of primer and probes for qPCR assays

Oligonucleotide qPCR-primers and TaqMan minor groove binding (MGB) probes targeting the small sub-unit (SSU or 18 S) or large sub-unit (LSU or 28 S) rDNA were designed for the different *A. tamarense* ribotype groups, and also *A. tamutum* and *A. minutum*, using Primer Express software Version 2.0.0 (Applied Biosystems, Darmstadt, Germany) and the ARB software package with its sub-function 'probe design' (Ludwig *et al*., 2004). For this purpose, two alignments for LSU and SSU rDNA, each containing > 800 sequences, were generated in ARB. In order to determine exclusively specific motifs for the target species, the alignments included all sequences of *Alexandrium* species and closely related dinoflagellates available from GenBank, in addition to sequences from cultured strains of the target species and some of our own unpublished sequences. The species-specific primers and those probe sequences characterized by the highest number of mismatches to non-target sequences were tested *in silico* for their specificity by a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) against the GenBank nucleotide collection, following the design protocol of Groben *et al*. (2004).

Laboratory cultures and extraction of genomic DNA

Monoclonal strains of *A. tamarense* (Groups I–III), *A. tamutum* and *A. minutum* (Table 1) were grown in enriched seawater K medium (Keller *et al*., 1987) to provide target DNA for the establishment of qPCR assays. Cultures were grown on a 14:10 h light:dark cycle at 15°C and at a photon flux density of 150 $\mu mol\, m^{-2}\, s^{-1}$. Cultures in exponential growth phase were harvested by centrifugation ($3220 \times g$ for 15 min) and pellets were stored at −80°C until extraction of DNA. Genomic DNA was extracted from the frozen pellets with the DNeasy 96 Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After addition of 400 µl lysis buffer (pre-warmed to 65°C), a 0.5 mm stainless steel ball, and about 50 mg

of acid-rinsed glass beads, algal cells were disrupted in a MM200 mixer mill (Retsch, Haan, Germany) by running each sample twice for 1 min at 20 Hz. The purity and quantity of the DNA was analysed by UV-spectroscopy with a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany) and the integrity of DNA fragments of a molecular weight of about 20 kb was verified on 0.8% agarose gel.

The amount of DNA determined in an experimental sample can be converted into the equivalent number of cells if the DNA yield per cell under the standardized DNA extraction protocol has been determined. For this purpose, DNA yield per cell was determined for *A. minutum*, *A. tamarense* Group I and *A. tamutum* (Table 1) from genomic DNA extracted from batch cultures in exponential growth phase (grown as described above). Directly before harvesting, cell concentrations were determined from triplicate subsamples by LM counting. Samples (100 µl) diluted in 900 µl sterile-filtered (0.2 µm) seawater were fixed with Lugol's iodine solution in a settling chamber and the cell content of the whole chamber was counted under an inverted microscope (Utermöhl, 1958). In order to investigate the dependence of the resulting yield of genomic DNA on the initial cell concentration of a sample, different volumes of cell suspension were harvested for all strains, for total cell numbers of 8×10^5, 1.6×10^5, 3.2×10^4 and 6.4×10^3 cells. Each sample was prepared in duplicate to assess the methodological variability of the DNA extraction protocol. Extraction of genomic DNA and tests for DNA purity and quantity were as described above.

Development and optimization of the qPCR assays

Genes encoding the SSU or LSU rDNA were amplified from genomic DNA of all cultured *Alexandrium* strains by a standard PCR protocol. Amplification of the SSU rRNA gene of *A. minutum* was performed with the universal eukaryotic primers 1 F (5′-AACCTGGTTGATCCTGCCAGT-3′) and 1528R (5′-GATCCTTCTGCAGGTTCACCTAC-3′) as described by Medlin *et al.* (1988) without the polylinkers. Each 50 µl PCR reaction contained 5 µl of 10×, HotMaster Taq buffer with 25 mM Mg^{2+} (5 Prime, Hamburg, Germany), 0.5 units of HotMaster-Taq DNA polymerase (5 Prime, Hamburg, Germany) and 1 µl of DNA (100 ng μl^{-1}). After the initial denaturation (94°C, 5 min), 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 2 min and elongation at 70°C for 2 min were carried out, followed by a final extension at 72°C for 7 min in a Gradient Mastercycler (Eppendorf, Hamburg, Germany).

The variable D1–D2 region of the LSU rRNA of the different members of the *A. tamarense* species complex and *A. tamutum* was amplified using the forward primer D1R and the reverse primer D2C (Scholin *et al.*, 1994) with the same PCR reagents as described above for amplification of the SSU rDNA gene. Initial denaturation (94°C, 5 min) was followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 54°C for 20 s, elongation at 70°C for 1 min and a final extension at 70°C for 10 min. Subsequently, amplification products were checked for appropriate length and purity by agarose gel electrophoresis as described above. Positive controls with *A. tamarense* DNA and negative controls with no DNA added were performed in each PCR reaction.

In order to evaluate the efficiency and specificity (no primer dimers, no non-specific amplicons on non-target DNA) of the primers designed for the TaqMan MGB qPCR assays, each primer pair was first tested with SYBR Green qPCR assays on DNA of the respective *Alexandrium* target DNA and on non-target DNA of closely related species (data not shown). For this approach, the PCR amplicons of the different *Alexandrium* taxa generated from laboratory cultures were adjusted to 100 ng ml^{-1} and further subjected to 10-fold serial dilutions to yield the samples for generating the standard curve for the primer tests. The final standard curve contained six concentrations and ranged from 1000 pg ml^{-1} down to 0.1 pg ml^{-1}. All experiments were performed in triplicate for each standard curve point and a non-template control was present in each qPCR experiment. The SYBR Green assays for qPCR were performed on an ABI PRISM 7000 SDS real time PCR thermocycler (Applied Biosystems, Darmstadt, Germany) in a final volume of 20 µl containing 10 µl of a 2× Power SYBR Green PCR Master Mix (including SYBR Green 1 dye, AmpliTaqGoldDNA polymerase, dNTPs) (Applied Biosystems, Darmstadt, Germany) and the passive reference dye ROX, and primers at a final concentration of 300 nM each.

The qPCR conditions for the SYBR Green approach were: hold 95°C for 10 min, followed by 40 cycles 95°C for 15 s and 59°C for 1 min. At the end of the programme a melt curve protocol was added to confirm that only the specific PCR amplicon was present and to reveal any primer dimer formation. All of the newly developed qPCR primer pairs performed perfectly well in the SYBR Green set-up, with highly efficient primer binding and absence of dimer formation, allowing the development and implementation of the final qPCR assays with TaqMan MGB probes. The qPCR generated amplicons were always additionally checked by agarose gel electrophoresis to monitor for unspecific amplicon formation.

Following their success in the above tests, the primer pairs were tested in combination with species-specific TaqMan MGB probes. The standard dilution series of the qualitative PCR amplicons from cultured target and non-target *Alexandrium* taxa were applied in the same concentration range as in the SYBR green assay. TaqMan MGB-probe qPCR assays were performed in a final volume of 20 µl containing 10 µl of a 2× TaqMan Universal PCR Master Mix, containing AmpliTaq Gold DNA polymerase and dNTPs and the passive reference dye ROX (Applied Biosystems, Darmstadt, Germany), primers at a final concentration of 900 nM each and the TaqMan MGB-probe. The MGB probes were labelled with the fluorescence dye Vic (excitation wavelength 523 nm, emission wavelength 568 nm) or 6FAM (6-carboxyfluorescein: excitation wavelength 483 nm, emission wavelength 533 nm) at a final concentration of 200 nM. Each primer and probe combination was run individually, but could be multiplexed in future studies.

The cycle conditions for the TaqMan MGB-probe qPCR assays were hold 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles 95°C for 15 s and 59°C for 1 min. The efficiency (E) of the qPCR reactions was calculated by the following equation: $E = 100 \times 10^{-1/m} - 1$, where m is the slope of the standard curve for the respective qPCR assay. The slope was determined from the linear regression of log versus values of the template concentration of the 10-fold standard dilution series cycle threshold.

Calibration of the qPCR assays

The ability to determine accurately the number of target cells in an 'environmental matrix' was tested with the TaqMan MGB-probe qPCR assays in complex field samples. The results obtained by qPCR were compared with those obtained by fluorescence *in situ* hybridization (FISH). To this end, plankton samples for generating a natural background matrix consisting mainly of non-target phytoplankton and detritus were collected in the German Bight at Helgoland Roads (long term sampling station 'Kabeltonne', 54°11.3′N; 7°54.0′ E) with a plankton net haul (20 µm mesh) from on board ship. The plankton sample was directly transferred to the laboratory and immediately filtered over nylon gauze (200 µm mesh) to remove metazoans such as copepods. The sieved fraction was further concentrated by filtration over 20 µm nylon gauze and used as background matrix for the experiment.

Laboratory cultures of the qPCR target species *A. tamarense* (Group I) and *A. tamutum* were grown as described above to serve as defined standards for cell enumeration by qPCR and FISH. Cell concentrations of both species were determined by the Utermöhl counting technique. Simultaneously, two 500 ml aliquots of the concentrated field plankton sample were spiked with 10, 100 or 1000 cells of *A. tamarense* (Group I) and *A. tamutum*, respectively, for subsequent qPCR and FISH assays. The spiked 500 ml plankton samples were mixed and filtered onto polycarbonate membranes (10 µm pore-size, 47 mm diameter, Millipore, Billerica, USA). Filters for the qPCR assay were stored at −80°C whereas filters for FISH analysis were fixed with modified saline–ethanol fixative (22 ml 96% ethanol, 5 ml deionized water, 3 ml 25 × SET [3.75 M NaCl, 25 mM EDTA, 0.5 M Tris–HCl, pH 7.8]), as described in Miller & Scholin (2000), and stored at −20°C until assay preparation. Additionally, negative control filters for FISH and qPCR analysis were prepared by applying the same amount of concentrated plankton sample without addition of *Alexandrium* cells to determine any unspecific amplification of non-target DNA. The DNA extraction and qPCR experiments with TaqMan MGB-probes with 1 ng and 10 ng DNA per reaction from each dilution of both *Alexandrium* species were performed in triplicate as described above. Standard dilution series were made from 1 to 1000 pg genomic DNA from each *Alexandrium* species; DNA was amplified with the respective qPCR primers and probes. The fluorescence threshold was set by the analytical software for the ABI PRISM cycler. The PCR cycle during which this threshold was crossed for each sample, designated as C_T, was reported as the average of triplicate experimental samples, standards or internal controls. Based on the regression formula resulting from the C_T values of the standard dilution series, the sample C_T was converted to taxon-specific DNA quantity in the samples and finally to the number of cells per *Alexandrium* taxon according to the values of taxon-specific DNA yield as determined above.

The spiked plankton samples were analysed by FISH according to John *et al.* (2005), with species-specific oligonucleotide probes targeting the 28 S rRNA of *A. tamarense* Group I (probe ATNA02: John *et al.*, 2005) and *A. tamutum* (probe ATMU2199: 5′-TGGTGCTCAAGGAAGCAA-3′), labelled with fluorescein-5-isothiocyanate (FITC; Thermo Scientific, Ebersberg, Germany) and including 40% formamide in the hybridization buffer. After hybridization, the whole filter surfaces were scanned by epifluorescence light microscopy (LM) for identification and counting of positive hybridization signals to recover the spiked *Alexandrium* spp. cells and to compare the cell numbers with those originally added and as estimated by qPCR assay.

Collection and processing of field samples

Plankton communities were sampled during an oceanographic cruise (*R.V. Heincke*) along the Scottish North Sea coast from the Firth of Forth to the Pentland Firth region on 26 May and 9 to 10 June 2004. On 26 May, a south to north transect of five stations (S2, S4, S5, S6, S7) was followed, whereas on 9–10 June a second transect of five stations was sampled from north to south (S10, S11, S12, S13, S15) (Fig. 1). At each station plankton were sampled from discrete water depths (1, 3, 5, 10, 20 m) by Niskin bottles mounted on a Rosette sampler. Surface water (0 m) was also collected by a bucket cast. Each sample was filtered through 20 µm nylon gauze and the retained material carefully rinsed with 0.2 µm-filtered seawater. After fixation of two small aliquots with (18 ml) Lugol's iodine solution (Utermöhl, 1958) and 2% paraformaldehyde (1.8 ml) for later LM examination, the remaining plankton samples were partitioned into 50-ml centrifugation tubes, and centrifuged (3220 × *g*) for 5 min. Each pelleted sample corresponded to the > 20 µm particulate fraction of 7.6 to 10.9 l of seawater taken from discrete water depths. Each pellet was transferred into a 2 ml cryovial (APEX SC, Alpha Laboratories, Eastleigh, UK) and again centrifuged (13 000 × *g*) for 5 min. Immediately after centrifugation the supernatant was carefully removed and pellets were frozen in liquid nitrogen and stored at −80°C until further processing for toxin content and composition, as well as molecular diversity analyses.

Genomic DNA of plankton samples was extracted by the standard protocol as detailed above and DNA quantity and integrity were checked afterwards by UV-spectrophotometry and agarose gel electrophoresis. Samples S2-0 m, S2-3 m, S12-0 m and S15-0 m were lost during the DNA clean-up procedure and therefore excluded from analysis. To mimic field conditions in each qPCR

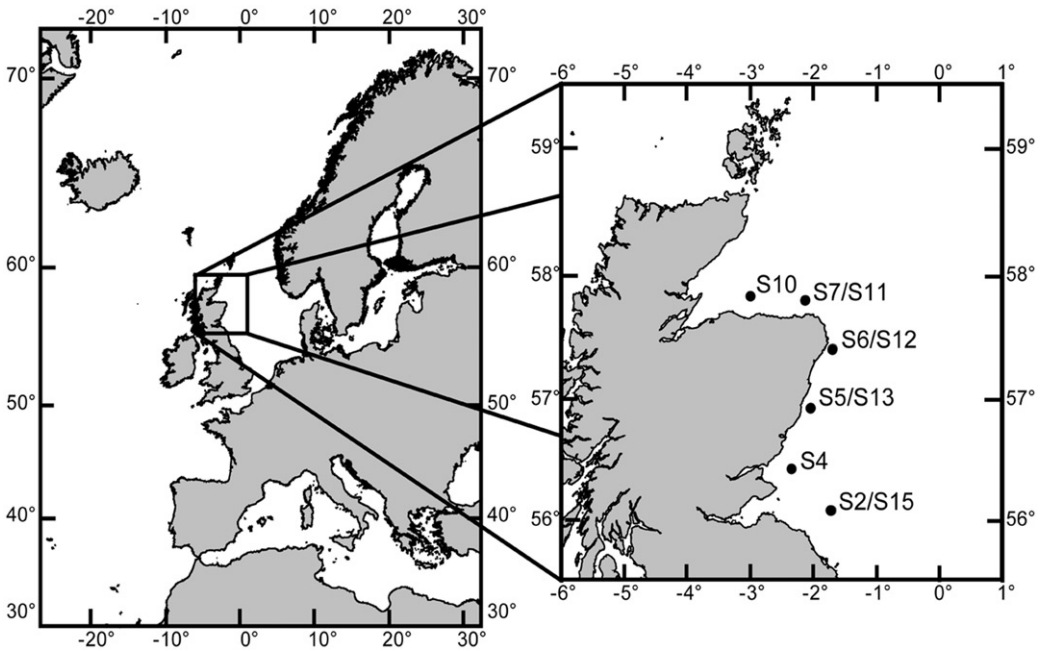

Fig. 1. Sampling stations during the oceanographic cruise (*R.V. Heincke*) along the Scottish North Sea coast from the Firth of Forth to the Pentland Firth region in 2004.

field sample analysis, the standard dilution series for determination of genomic DNA of target species per sample consisted of an equally mixed matrix of genomic DNA from all different *Alexandrium* species tested (Table 1). This standard dilution series contained 10-fold serial dilutions in a range from 1 to 1000 pg genomic DNA of the target and non-target *Alexandrium* taxa. Two variants of non-template controls were also present, containing all the qPCR ingredients for each primer and probe combination except DNA, or containing mixtures of non-target DNA, respectively. Standard dilution series and all field samples were analysed together in the same microtitre plates and triplicate analyses were separated over different microtitre plates in order to account for potential plate and run differences. Dilutions of 1:100 to reduce PCR-inhibiting factors of the isolated DNA from field samples were amplified by qPCR to determine the occurrence of the different *Alexandrium* taxa in the respective sample. The appropriate dilution factors for the field samples were established in preliminary qPCR amplification experiments. Concentrations of *Alexandrium* targets (as cells l^{-1} seawater) were calculated after estimation of cell numbers in the diluted DNA sample as detailed in section 'Calibration of the qPCR assays' and by converting the volume of diluted DNA template to the respective volume of seawater.

Validation of qPCR results with reference to Utermöhl cell counts and FISH analysis

One millilitre of Lugol's iodine-fixed concentrated field samples from Niskin bottles was placed in each microscope settling chamber. After settling of plankton, *Alexandrium* cells were identified at the genus level and collectively counted in the whole chamber with an inverted microscope (Axiovert 40, Zeiss, Jena, Germany) according to the Utermöhl (1958) method.

Field samples from Stations 2, 4, 5, 6, 7, 10, 12, 13 and 15 at depths of 0 m and 10 m were analysed by FISH to confirm the presence of qPCR-detected taxa of each *Alexandrium* species by LM. For each sample 1 ml of the same Lugol's iodine-fixed concentrate from Niskin bottles as used in the Utermöhl counts was filtered, fixed and analysed by FISH as described above. The species- and ribotype-specific FITC-labelled oligonucleotide probes comprised the 28S rRNA probes ATNA02 (John *et al*., 2005) for *A. tamarense* Group I, ATME04 for *A. tamarense* Group II, ATWE03 for *A. tamarense* Group III (John *et al*., 2005), ATMU2199 (sequence above) for *A. tamutum* and the 18 S rRNA probe AMINC for *A. minutum* (Diercks *et al*., 2008). The FISH analyses were conducted qualitatively for species- and ribotype-discrimination and to confirm the presence or absence of individual *Alexandrium* taxa by scanning the whole hybridized filter.

Determination of PSP toxin derivatives in plankton samples

For PSP toxin analysis, frozen plankton pellets were extracted in 1.0 ml 0.03 N acetic acid by ultrasonication for 10 min with a Sonopuls HM 70 ultrasonicator (Bandelin, Germany). The sonicate was centrifuged for 10 min ($2980 \times g$), and the supernatant was passed through a 10 mm diameter 0.45 µm nylon filter (Rotilab, Carl Roth, Karlsruhe, Germany) into an autosampler vial for analysis.

Paralytic shellfish poisoning (PSP) toxins were analysed by liquid chromatography with fluorescence detection (LC-FD) by the method of Thielert *et al*. (1991), with modifications described in Hummert *et al*. (1997)

and Yu *et al.* (1998). This post-column oxidation method is based on ion-pair chromatography with octane sulfonic acid followed by oxidation with periodic acid to fluorescent derivatives. Liquid chromatography was performed with an SIL-10A intelligent autosampler, an LC-10ATvp intelligent pump, an SCL-10Avp system controller, a 1 ml CRX400 post-column reaction unit (Pickering Laboratories, Mountain View, California, USA), two LC-9A pumps for delivery of post-column reaction solutions, and an RF-10Axl fluorescence detector (all Shimadzu, Germany). Data were analysed with Class-vp 5.3 Shimadzu software.

Saxitoxin (STX), neosaxitoxin (NEO) and gonyautoxins (GTXs) were purchased as certified analytical PSP toxin standards from the Certified Reference Materials Program, Institute for Marine Biosciences, National Research Council, Halifax, Canada. The standard solutions of GTX2 and GTX3 contained dcGTX2 and dcGTX3 as minor components, but the exact content of these toxins was not given; dcSTX was provided by the European Commission from BCR, the Community Bureau of Reference, Brussels, Belgium.

For indirect determination of *N*-sulfocarbamoyl toxins, 150 µl of acetic acid extract were mixed with 37 µl of 1.0 N hydrochloric acid and heated for 15 min at 90°C. After cooling to room temperature, the mixture was neutralized with 75 µl 1.0 N sodium acetate. Concentrations of *N*-sulfocarbamoyl toxins C1 and C2 were calculated by the difference (increase of GTX2 and GTX3) of the peak areas of the corresponding toxins in the acetic acid extract. After determination of the concentration of individual PSP toxin derivatives, toxin concentrations were converted to molar quantities using the known molecular weights of each toxin. The molar concentrations of PSP toxins were calculated for samples from discrete water depths and compared with qPCR estimates of cell abundance of *A. tamarense* (Group I) and other *Alexandrium* taxa, as well as counts of *Alexandrium* spp. by the Utermöhl (1958) method. The Pearson product–moment correlation coefficients (r) for comparisons of cell and toxins concentrations were calculated in STATISTICA (StatSoft, Tulsa, OK, USA).

Results

Calibration, efficiency and specificity of the qPCR assay

The primers and probes targeting 18 S and 28 S rDNA (Table 2) were found to be appropriately specific via *in silico* analysis by a BLAST search and by *in vitro* qPCR assays on DNA from laboratory cultures of target and non-target *Alexandrium* species and groups. No unspecific amplicons were detected by agarose gel electrophoresis from culture or field samples. All qPCR assays were highly species-specific, as well as group-specific, and no cross-reaction with other *Alexandrium* species or groups occurred as tested with non-target DNA templates from different *A1exandrium* targets. The experiments showed high efficiency of the PCR reactions, with efficiency of amplification always > 90%, and a high r^2 in the assays (> 0.997: $n = 3$) indicated a near perfect correlation between C_T and the log of the starting copy number (Table 2). An estimate of the abundance of *Alexandrium* cells in the qPCR assay was calculated for each sample, based upon the C_T values of the standard dilution series obtained from one representative strain of the respective *Alexandrium* species. The mean concentration (± SD) of extracted DNA from the highest initial cell number was 4.6 ± 1.15 pg DNA cell^{-1} ($n = 3$) for *A. minutum*, 10.5 ± 0.52 pg DNA cell^{-1} ($n = 3$) for *A. tamarense* (Group I) and 13.2 ± 1.32 pg DNA cell^{-1} ($n = 3$) for *A. tamutum*.

Field samples were spiked with known numbers of *Alexandrium* cells to calibrate the developed qPCR assay and analysed by qPCR and FISH. The target species *Alexandrium tamarense* (Group I) and *A. tamutum* have not been documented so far at the Helgoland Roads site, even over several decades of observations (Wiltshire *et al.*, 2010; Löder *et al.*, 2012). As expected, therefore, no *Alexandrium* cells were detected by qPCR, nor by FISH, in the negative control assays (environmental background matrix alone) (Supplementary Table S1). The qPCR results showed no detectable inhibition by matrix effects, in terms of reduction in sensitivity or accuracy. Nevertheless, when a higher amount of DNA (10 ng) was applied, partial inhibition or reduced sensitivity of the reaction by an order of magnitude was observed (data not shown).

The number of *Alexandrium* cells calculated from the C_T values of the amplified target DNA increased with the number of inoculated cells and calculated estimates yielded approximately 80–85% of the spiked cell numbers counted by the Utermöhl technique. The technical replicates (among DNA extraction trials, qPCR runs, etc.) showed low variability (< 10%).

There were substantial discrepancies between *Alexandrium* cell numbers as calculated from the qPCR assay of spiked field samples and estimates made by counting fluorescently labelled cells from the FISH method. The numbers of target cells recovered on whole scanned filters were relatively low in comparison to qPCR results in that only up to 66% from both low and high inoculated cell numbers were detected (Supplementary Table S1).

Alexandrium *abundances and toxin concentration on the Scottish coast*

The estimated cell numbers obtained by qPCR assays for the different *Alexandrium* taxa varied widely among the field samples (Fig. 2a–f, Supplementary Table S2). The most prominent

Table 2. Species-specific primer and TaqMan MGB-probes applied in this study for different taxa of the genus *Alexandrium* and their efficiencies.

Species/Group	Target sequence	TaqMan MGB-probe [5′–3′]	Primer forward [5′–3′]	Primer reverse [5′–3′]	Amplicon size in bp	Coefficient of determination (r^2)	Efficiency
A. minutum	18S rDNA	*Amin*156 6FAM-catccaaacctgacttc-MGB	119F acatggataactgtggtaattctatagctaa	207R gttggttctgtaactaatgaccacaac	89	0.999	91.5%
A. tamutum	28S rDNA	*amut*65 6FAM-ctcaaggaagcaaacc-MGB	27F gaacattttgcagcaacattgtg	116R gtggtacttgtgtgttccttgt	90	0.998	95.1%
A. tamarense (Group I)	28S rDNA	*ATNA*279 6FAM-aacactcccaccaagca-MGB	248F ccacagcccaaagctcttgt	378R ccatgaggggaaatatgaaaagga	131	0.998	100.4%
A. tamarense (Group II)	28S rDNA	*ATME*275 VIC-agcacaacaacctcacca-MGB	224F gcatgccaagaaagaatcattgta	331R tgtatttgctgaacaaaaagtaaacagact	108	0.997	97.6%
A. tamarense (Group III)	28S rDNA	*ATWE*85 VIC-caacctcaaacacatgga-MGB	48F ttgtgtgtcagggcttgtaag	129R gaatgtgtctggtgtatctgttttgt	82	0.999	95.5%

Alexandrium taxa in the depth profile samples were *A. tamarense* (Group I) and *A. tamutum*, which ranged from 9 to 4269 cells l^{-1} (mean $\pm$ SD $= 701 \pm 764$) and from 19 to 4575 cells l^{-1} (664 ± 960), respectively (Fig. 2d and 2f, Supplementary Table S2). With the exception of stations S13 and S15, where it was not detected, *A. tamarense* Group III was found frequently in at least one sample from different depths at all stations (Fig. 2e, Supplementary Table S2). However, very low cell abundances were recorded, ranging from 0 to 343 cells l^{-1} (65 ± 84). *Alexandrium minutum* and the *A. tamarense* Group II were not detected in any field samples and detailed examination of the raw qPCR data and results showed no traces of these two taxa, with no amplicon formation observed. Absence of these taxa was supported by additional analyses in agarose gel electrophoresis of the qPCR generated amplicons. In general, there were only minor differences in cell numbers between the PSP-producing species *Alexandrium tamarense* Group I and the non-toxic species *Alexandrium tamutum*, and indeed their numbers were significantly correlated (Pearson correlation coefficient $r = 0.35$, $n = 56$; $P < 0.01$). The estimates of *Alexandrium* species by qPCR (summed from all qPCR assays for single species) ranged from 45 to 7802 cells l^{-1} (1433 ± 1540) and were also significantly correlated with cell estimates of *A. tamarense* (Group I) ($r = 0.85$; $n = 56$; $P < 0.0001$) and *A. tamutum* ($r = 0.9$; $n = 56$; $P < 0.0001$) as obtained by qPCR.

FISH assays agreed with the qPCR results in showing the presence of *A. tamarense* Groups I and III and *A. tamutum* in the field samples. Absence of *A. tamarense* Group II and *A minutum* was affirmed by the lack of positive signals on filters hybridized with probes specific for these species (Supplementary Table S3). *Alexandrium* cells were present in all field samples, at concentrations from 14 to 1854 cells l^{-1} (684 ± 543 cells l^{-1}, $n = 61$).

The qPCR-generated *Alexandrium* cell numbers were often close to the Utermöhl counts. However, in a few cases extreme deviations between the two methods were observed (Fig. 3, Supplementary Table S2). The two methods for quantification of *Alexandrium*, applied to water samples from discrete depths from stations along the Scottish North Sea coast, showed a significant correlation ($r = 0.69$; $n = 56$; $P < 0.0001$).

PSP toxins were detectable at most stations, with saxitoxin derivatives reaching the highest concentrations at station S5 at 10 m (770 pmol l^{-1}), whereas only low concentrations (0 to $\sim$20 pmol l^{-1}) were present at station S7. No PSP toxins were detected at S13 and S15 (Fig. 2f, Supplementary Table S2). The qPCR results

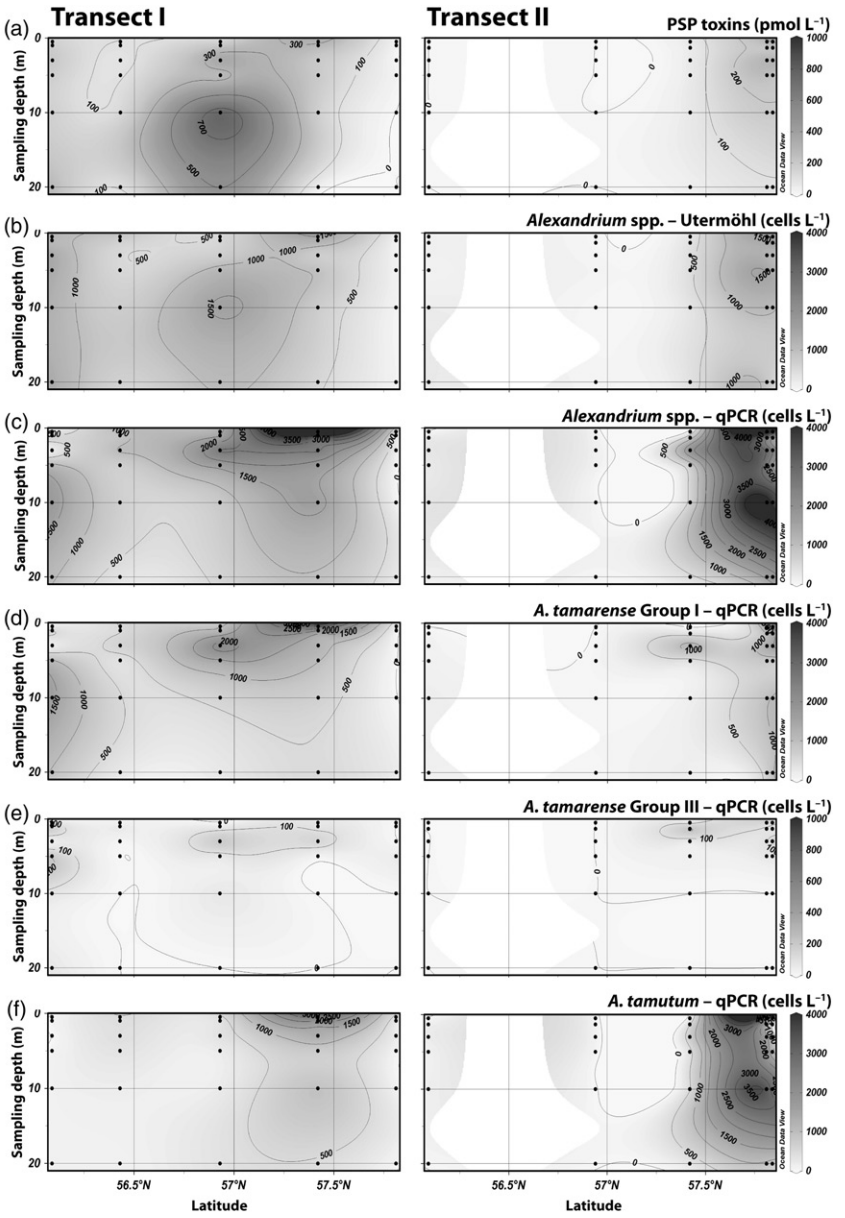

Fig. 2. Vertical profiles of transect I (2.1a–f) and transect II (2.2a–f). Concentrations (cells l^{-1}) are represented by differences in shading intensity in the contour plots, always showing the most dynamic differences from non-normalized data. 2.1a and 2.2a: particulate PSP toxin content; 2.1b and 2.2b: *Alexandrium* spp. Utermöhl microscopic counts; 2.1c–2.2c: *Alexandrium* spp. calculated from qPCR analyses 2.1d and 2.2d: *A. tamarense* Group I calculated from qPCR analyses; 2.14 and 2.2 e: *A. tamarense* Group III calculated from qPCR analyses; 2.1f and 2.2f: *A. tamutum* calculated from qPCR analyses. Cell calculations were transformed into graphics with Ocean Data View (ODV) software Version 3.4.1 (http://odv.awi.de)

correlated well with the abundance of PSP toxins. The best correlations were found between Utermöhl counts and PSP toxin concentrations, although toxic and non-toxic species or groups were not distinguished in the Utermöhl counts. Pearson correlations of concentrations of PSP toxins and *Alexandrium* cell concentrations, as obtained from qPCR assays and Utermöhl counts (ranked from highest to lowest) were: *Alexandrium* spp. (Utermöhl): $r = 0.77$ ($n = 60$), *A. tamarense* Group I (qPCR): $r = 0.57$ ($n = 56$), *Alexandrium* spp. (qPCR): $r = 0.52$ ($n = 56$). All correlations were significant at a level of $P < 0.0001$ and scatter plots display the goodness of fit of the correlations (Fig. 4). However, noteworthy exceptions, for example where extremely high PSP toxin concentrations did not fit well with qPCR cell estimates, such as at station S5 at 10 m depth, significantly influence the shape of the contour plots.

Discussion

Tests of the new qPCR assay

In this study we present novel highly sensitive quantitative PCR assays for five *Alexandrium* species and ribotype groups, comprising the toxic

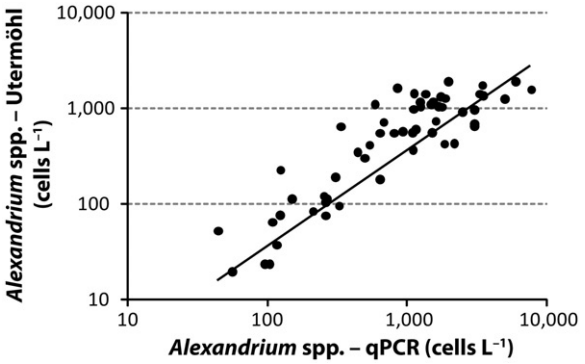

Fig. 3. Correlation between *Alexandrium* spp. cell concentrations determined by qPCR assays versus those determined by the Utermöhl method (Pearson correlation $r = 0.69$; $P < 0.0001$; $n = 56$), where *Alexandrium* spp. by qPCR corresponds to the sum of cell concentrations determined for *A. tamarense* Group I, *A. tamarense* Group III and *A. tamutum* by species specific qPCR (Supplementary Table S2). Cell concentrations from both methods are displayed on a logarithmic scale; the solid line represents the linear regression curve forced through the origin.

Group I and the non-toxic Group II and Group III of the *A. tamarense* species complex, the usually toxic species *A. minutum*, and the non-toxic species *A. tamutum*. The new established assays were successfully evaluated for their target specificity and for their capability to detect and discriminate among *Alexandrium* species in natural samples from the marine environment. *In vitro* tests showed that all qPCR assays are highly sensitive and efficient.

The specificity of the TaqMan MGB-probes applied here has been extensively tested against different *Alexandrium* strains and related taxa in previous studies (John *et al.*, 2003*a*, 2005; Diercks *et al.*, 2008), whereas the current study is the first to test the qPCR assay and the FISH probe for *A. tamutum* on target and non-target species (Table 1). The application of these specific probes in combination with specific primer pairs in the TaqMan MGB-probe qPCR assays markedly increases the specificity and reduces potential background signals. The primers and probes given for the qPCR assays are therefore reliable for detection and quantification of the target taxa even at low cell numbers or when they co-occur with closely related species with similar sequence characteristics.

In the field sample validation, the qPCR assays efficiently recovered the spiked cells. However, there were some losses (on average < 20%), which might be explained by sample handling. Moreover, laboratory conditions and unialgal cultures do not mimic all potential pitfalls of the application of qPCR to field samples. Substances that inhibit qPCR, leading to false negative results, are a

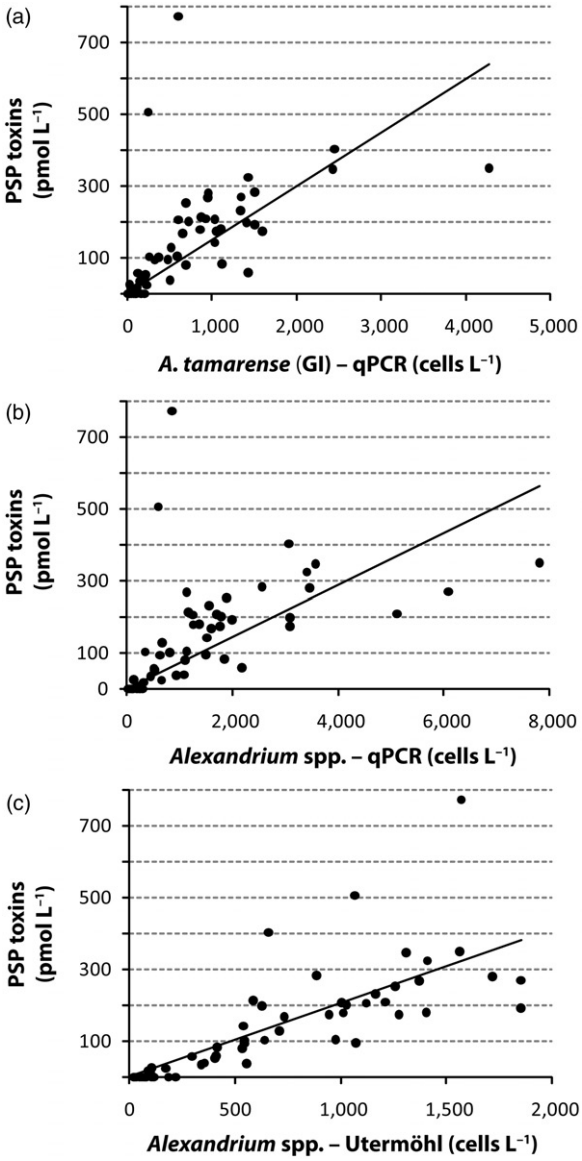

Fig. 4. Correlation comparison of *Alexandrium* cell concentrations determined by alternative quantification methods and of paralytic shellfish poisoning (PSP) toxin content in water samples from discrete depths along two transects off the Scottish North Sea coast. **a**, *A. tamarense* Group I by qPCR assay versus particulate PSP toxin content ($r = 0.57$; $P < 0.0001$; $n = 56$); **b**, *Alexandrium* spp. by qPCR assay (sum of cell concentrations determined for *A. tamarense* Group I, *A. tamarense* Group III and *A. tamutum* by species-specific qPCR (Supplementary Table S2) versus particulate PSP toxin content ($r = 0.52$; $P < 0.0001$; $n = 56$); **c**, *Alexandrium* spp. by the Utermöhl method versus particulate PSP toxin content ($r = 0.77$; $P < 0.0001$; $n = 60$). Solid lines represent the linear regression curve forced through the origin.

commonly reported problem with field samples (Park *et al.*, 2007; Kudela *et al.*, 2010). Several attempts have been made to reduce these inhibiting factors, including addition of chemicals to the qPCR reaction or preparation of dilution series of the field sample DNA (Park *et al.*, 2007; Dyhrman *et al.*, 2010). The low variability in the

qPCR results of the performed spiked field samples indicate that application of a correction factor for systematic variability (DNA extraction yield and quality) may assist in explaining the discrepancy between qPCR calculated cell numbers and the spiked cell number. Spike controls, with a known number of added algal cells as an extraction method control and pure DNA added as a PCR-inhibition control, will allow for correction of preparation biases in future studies with field samples. The FISH counted cells were considerably lower than the inoculated cell numbers counted by the Utermöhl technique, which might be related to methodological constraints of FISH, where several washing steps are involved during the hybridization procedure and may lead to losses of cells. In any case, major discrepancies are commonly reported between phytoplankton cell counts (including of *Alexandrium* taxa) by FISH and those made by classical microscopical methods (e.g. Utermöhl counts) (John *et al*., 2003a; Toebe *et al*., 2006; Touzet *et al*., 2008; Chen *et al*., 2011). The qPCR method is superior to the FISH technique in detecting low cell numbers in water samples due to its higher sensitivity and the possibility to filter larger volumes of water than is practical for FISH experiments. In the FISH method often only very small sample volumes can be filtered if a high background matrix is to be avoided, which makes counting of target cells more difficult (Kudela *et al*., 2010).

Differences in DNA yield among the analysed culture strains indicated that DNA content is strongly species- and/or strain-specific. In cultures, the DNA yield is unlikely to be greatly influenced by the culture regime, at least for non-synchronized cells harvested during G1 phase in the light period ('day time') in exponential growth. Cellular DNA concentration, however, also varies during the cell cycle (Taroncher-Oldenburg *et al*., 1997; John *et al*., 2001; Eschbach *et al*., 2005). Moreover, DNA yield is dependent upon the DNA extraction method (Valentin *et al*., 2005). In the current study, therefore, the same DNA extraction method was applied consistently to all field- and laboratory-derived samples, to reduce variability in the subsequent estimates of cell numbers. However, the number of ribosomal genes may differ among clonal isolates, even of the same species or group (ribotype) (Galluzzi *et al*., 2010; Medlin & Kooistra, 2010), thus potentially affecting the estimation of cell numbers by qPCR assays.

In spite of the methodological constraints outlined above, the qPCR assay applied to the field samples yielded a valid semi-quantitative species diversity and distribution dataset for the Scottish east coast from the Firth of Forth to the Pentland Firth region. Application of the FISH method confirmed the presence or absence of *Alexandrium* taxa and relative composition in the subset of concentrated plankton samples from the Scottish east coast. These experiments unambiguously confirmed the qPCR data on the specificity and composition of *Alexandrium* taxa. The FISH analysis confirmed the presence of *A. tamarense* Groups I and III and *A. tamutum* and supported the presumed absence of *A. minutum* and *A. tamarense* Group II. The presence of *A. tamarense* Groups I and III and *A. tamutum*, and the absence of *A. tamarense* Group II and *A. minutum*, were also confirmed by sequencing the LSU D1–D2 region of >100 isolates of *Alexandrium* spp. that were established from the sample stations during the same cruise (Alpermann *et al*., 2008, 2010).

However, judging by previous studies, some discrepancy between qPCR cell estimates and planktonic PSP toxin concentration, and between qPCR and Utermöhl counts can perhaps be expected. Garneau *et al*. (2011) reported a rather weak correlation ($r_s = 0.54$, $P < 0.0001$) in a study on PSP-toxigenic *A. catenella* (Group I) from California, based upon a similar number of samples ($n = 91$). In Garneau *et al*.'s study, the highest peak in cell numbers did not match the highest amount of measured PSP toxin in the samples and sometimes the *Alexandrium* cells detected and the toxin concentration did not match at all. Some of the high values observed for particulate PSP toxin content in our samples from the Scottish coast (e.g. S5 at 10 m depth) might be explained by sampling of accumulated PSP toxins in grazers of toxigenic microalgae or their faeces. For example, Krock *et al*. (2009) reported the occurrence of azaspiracid from larger-size fractions of filtered seawater than was expected by the cell size of *Azadinium spinosum*, the producer of the toxin. Another explanation for discrepancies between qPCR and Utermöhl counts for *Alexandrium* spp. and between qPCR estimates for toxigenic *A. tamarense* Group I cells and PSP toxin concentrations is that other PSP toxin-producing *Alexandrium* species might have been present but not accounted for. Thus, while cells of additional *Alexandrium* species in the diverse plankton assemblage would have been counted by the Utermöhl method applied at the generic level, they would not have been included in the summed *Alexandrium* estimate by qPCR. A study by Brown *et al*. (2010) reports on the occurrence of potentially PSP-toxigenic *A. ostenfeldii* in the region covered by our study and this species was also found along the Scottish east coast, albeit in low abundance, in an earlier field application of FISH probes (John *et al*., 2003*a*). If present in sufficient numbers, *A. ostenfeldii* could conceivably bias the fit between qPCR and toxin

data. As *A. ostenfeldii* from the North Sea typically produces spirolides rather than PSP toxins, the qPCR assay developed by Murray *et al.* (2011) could be used in parallel in future to resolve such discrepancies and to detect distantly related, putatively saxitoxin-producing species and other toxigenic species not previously known from a study area.

The present study confirms the usefulness of qPCR for detection and quantification of cells of *Alexandrium tamarense* Groups I–III, *A. tamutum* and *A. minutum* in culture, as well as in field samples from the North Sea. Nevertheless, accurate quantitative application to field samples instead of laboratory cultures may remain problematic due to the presence of undetermined contaminants in the extracted DNA that affect PCR efficiency and thereby reproducibility (Dyhrman *et al.*, 2010). In addition, the possible variability of rDNA content in target cells could affect the efficiency of qPCR approaches. Galluzzi *et al.* (2010) showed general rDNA variability in two Mediterranean *Alexandrium* representatives. Therefore, baseline data on the rDNA variability of populations of *Alexandrium* taxa at the local or regional scale should be considered if qPCR is to be applied accurately to environmental samples (Galluzzi *et al.*, 2010). There are alternative ways to solve qPCR interference problems that are not related to cellular or strain variability, e.g. DNA can be extracted from smaller samples with a higher volume of lysis buffer. In our experiments, therefore, the assays were performed with diluted DNA samples. As the sensitivity of the qPCR approach is generally decreased when the applied DNA is near the detection limit of the qPCR reaction, a proposed solution for controlling the efficiency and purity of DNA extractions in future qPCR applications is the introduction of 'alien DNA' or foreign algal cells with which to spike the samples, to have a measure of DNA extraction efficiency and purity (Coyne *et al.*, 2005).

Biogeography and ecology of North Sea Alexandrium

The distribution and abundance patterns of the different *Alexandrium* species and ribotype groups obtained from qPCR and FISH analyses of field samples from the Scottish North Sea coast allows for several interpretations with respect to the biogeography and ecology of the species investigated. In general, samples from this region contained a mixture of *Alexandrium tamarense* Group I, *A. tamarense* Group III and *A. tamutum* in varying proportions. *Alexandrium tamarense* Group I and *A. tamutum* were consistently found in higher cell numbers at each sampled station than the other *Alexandrium* species (Fig. 2). The fact that *A. minutum* was not found in any field plankton samples may be significant because John *et al.* (2003*a*) reported the presence of *A. minutum* from the same waters sampled four years earlier. However, *A. tamutum* was not described until after this earlier cruise and so it cannot be ruled out that John *et al.* (2003*a*) misidentified *A. tamutum* as *A. minutum*. Another important finding of our study was the detection of low but significant numbers of *A. tamarense* Group III, but apparently no cells from Group II, which to date is known only from the Mediterranean Sea (John *et al.*, 2003*b*; Anderson *et al.*, 2012).

The co-occurrence of Group I and III *A. tamarense* along the Scottish coast, also found by Collins *et al.* (2009) and Touzet *et al.* (2010), has major evolutionary implications, as a sympatric distribution is a strong indication that the two are separate but cryptic species. A biological barrier to interbreeding is indicated by mating experiments between isolates of Group I and Group III (Brosnahan *et al.*, 2010), as zygotes formed after mixing gametes of these groups were not viable. The absence of hybrid genotypes among a large number ($n = 88$) of clonal isolates of *A. tamarense* established from station S2 of the transect along the Scottish east coast also supports the existence of a reproductive barrier between the two groups (Alpermann *et al.*, 2010). Brosnahan *et al.* (2010) hypothesized that the maintenance of compatibility between gametes, leading to zygote formation, might represent a mechanism by which the expansion of one *A. tamarense* group into an area already densely populated by another group can be suppressed, because the majority of gametes of the minority group will form non-viable hybrid zygotes with the gametes of the numerically dominant group.

An analysis of cysts derived from the studied region would provide further insights into the distribution of the different groups and if groups exist exclusively in the extensive cyst beds found along the north-east coast of the UK (Joint *et al.*, 1997). The qPCR assays developed in this study could be a valuable tool for such investigations.

The initial division of the *A. tamarense* species complex into four ribotypes (Scholin *et al.*, 1994, 1995) was supplemented with the description of a fifth ribotype (John *et al.*, 2003*b*). In this latter study the authors argued that the molecular data support speciation in progress and represent allopatric vicariant populations. However, at this time the sympatric occurrence of the different ribotypes in several regions of the world was not known. Lilly *et al.* (2007) altered the terminology for the *A. tamarense* species complex from geographically distinguished ribotypes to numbered groups in

order to comprise the phylogenetic clades within the species complex. Thereby inconsistencies in basing ribotype descriptors on biogeographical location are avoided. In any case, recent evidence of mating incompatibilities, thereby supporting species barriers according to the biological species concept, strongly argues for the separation of the *A. tamarense* species complex into at least five different species.

Future prospects

One of the major contributions of this study is the successful development and validation of molecular detection and counting methods for harmful dinoflagellate species. These qPCR assays enable the detection of diverse *Alexandrium* species and groups within complexes of poorly defined species, and also the estimation of cell abundances of *Alexandrium* taxa in field samples. The distribution of cryptic *Alexandrium* species and groups cannot be determined by routine LM analysis. Furthermore, the qPCR assay enables the detection of target species, even when the cell numbers are below the typical detection limit for optical microscopy. For a statistically acceptable estimate of cell numbers using the Utermöhl technique, a minimum of at least 50 cells or count units for each taxon is recommended (Rott *et al.*, 2007). This relatively high number does not allow an early detection of harmful species before a bloom develops. Hence, in pre-bloom periods when cell numbers are low, values obtained through LM counts are often highly unreliable. The discrepancy between LM and qPCR cell estimates in environmental samples containing low cell numbers may be explained if the threshold limits of microscopical counts are taken into account (Perini *et al.*, 2011).

The development and application of additional qPCR assays for a greater variety of harmful algal species, including other *Alexandrium* species, will assist in the rapid determination of changes in species composition. Molecular probe techniques offer the possibility to identify and quantify cryptic species but can also alleviate two other major problems of classical LM methods, namely the high level of taxonomic expertise needed and high labour costs for routine analysis. Hence, targeted qPCR primers and probes provide practical tools for HAB monitoring programmes throughout the world.

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Supplementary information

The following supplementary material is available for this article, accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org/10.1080/09670262.2012.752870

Table S1. Recovery analysis of quantitative PCR and whole-cell hybridization experiments of environmental samples from the German bight, Helgoland Roads (Germany) spiked with known numbers of *Alexandrium* representatives.

Table S2. Cell concentrations of individual and combined *Alexandrium* spp. and phylogenetic groups as obtained by qPCR assay and Utermöhl counts, as well as particulate PSP toxin concentrations from discrete water depths.

Table S3. Presence or absence of *Alexandrium* representatives at the time of the field survey analysed by qPCR and FISH experiments.

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