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## qPCR assays for *Alexandrium fundyense* and *A. ostenfeldii* (Dinophyceae) identified from Alaskan waters and a review of species-specific *Alexandrium* molecular assays

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### Abstract

Paralytic shellfish poisoning (PSP) poses a serious health threat in Alaska and prevents effective utilization of shellfish resources by subsistence and recreational harvesters. Substantial economic losses also affect shellfish growers during PSP events. The toxins responsible for PSP are produced by dinoflagellates in the genus *Alexandrium*. Despite the persistent threat posed by PSP and the long history of shellfish toxicity research, there is still confusion concerning the *Alexandrium* species that cause PSP in Alaska. The primary objective of this study was to identify the toxic *Alexandrium* species present in Alaska and to develop polymerase chain reaction (PCR)

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#### SUPPLEMENTARY DATA

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assays for use in screening phytoplankton and sediment samples. Before developing the PCR assays for this study, we evaluated published assays and many were not adequate because of primer dimer formation or because of cross-reactivity. Rather than continue to grapple with the uncertainty and inadequacy of published assays, we developed new assays for the *Alexandrium* species most likely to be present in Alaska. Only *Alexandrium fundyense* Group I and *A. ostenfeldii* were identified from four sampling regions from southeast Alaska to Kodiak Island, indicating that these two species are widely distributed. PCR assays for these two species were converted to quantitative (q)PCR format for use in monitoring programs. During the course of this study, we realized that a systematic evaluation of all published (~150) *Alexandrium* species-specific assays would be of benefit. Toward this objective, we collated published *Alexandrium* PCR, qPCR, and *in situ* hybridization assay primers and probes that targeted the small-subunit (SSU), internal transcribed spacer (ITS/5.8S), or D1-D3 large-subunit (LSU) (SSU/ITS/LSU) ribosomal DNA genes. Each individual primer or probe was screened against the GenBank database and *Alexandrium* gene sequence alignments constructed as part of this study. These data were used to identify a suite of species-specific *Alexandrium* assays that can be recommended for evaluation by the global harmful algal bloom community.

### Keywords

*Alexandrium catenella*; *Alexandrium minutum*; *Alexandrium monilatum*; *Alexandrium pacificum*; *Alexandrium tamarense*; *Alexandrium* monitoring; Gulf of Alaska; Paralytic shellfish poisoning (PSP); qPCR assay; Saxitoxin

## INTRODUCTION

Paralytic shellfish poisoning (PSP) is a persistent health threat in Alaska caused by consumption of bivalves and other shellfish containing saxitoxins (STXS) produced by dinoflagellate species in the genus *Alexandrium* Halim (Smith 2010). STXS interact with voltage-gated sodium, potassium, and calcium channels and modulate the flux of these ions into various cell types. This results in disruption of nervous system function (Cusick & Saylor 2013), which may be fatal in severe cases. Documented cases of PSP in Alaska date back centuries to Captain George Vancouver's survey of the Pacific coast in the early 1790s (Quayle 1969; Horner *et al.* 1997). PSP is sufficiently prevalent in Alaska that it has been designated as a public health emergency and all cases are required to be reported to the state's Division of Epidemiology (Castrodale 2015). The remote locations of Alaska's coastal communities and the high cost of shipping and testing make it impractical for the state to routinely monitor shellfish resources utilized by subsistence and recreational harvesters. Only commercially harvested shellfish are tested by Alaska's Division of Environmental Health for STXS before they are considered safe for sale or consumption. Despite the persistent PSP health threat, Alaskans harvest shellfish for personal use year round. Monitoring *Alexandrium* at key recreational shellfish harvesting beaches would help identify periods of elevated PSP risk that could be conveyed to the public before harvesting (R. RaLonde, personal observations).

The historical record of harmful algal bloom (HAB) literature for Alaska was described as “sparse” by Horner *et al.* (1997), with reports of possible *Alexandrium* species from Jack Bay in Prince William Sound, Tenakee Harbor, near Ketchikan, and other southeast Alaska sites. Hall (1982) collected samples containing *Alexandrium* (*Protogonyaulax* F.J.R.Taylor) from Dutch Harbor to Ketchikan and analyzed cellular toxin content. More recently, Trainer (2002) reported three species of *Alexandrium* in Alaska: *A. catenella* (Whedon & Kofoid) Balech, which was broadly distributed, *A. fundyense* (Balech) D.M.Anderson from Porpoise Island, and *A. tamarense* (M.Lebour) Balech (John *et al.* 2014a) from the Gulf of Alaska. Orlova *et al.* (2007) reported that seven species of *Alexandrium*, *A. insuetum* Balech, *A. margalefii* Balech, *A. ostensfeldii* (Paulsen) Balech & Tangen, *A. catenella*, *A. pseudogonyaulax* (Biecheler) Horiguchi ex Yuki & Fukuyo, *A. tamarense*, and *A. tamutum* Montresor, Beran & John, occur in the far eastern seas of Russia, including isolates collected from the Bering Sea in proximity to Alaskan waters. Most recently, Natsuike *et al.* (2013) assayed cysts collected from sediments at numerous stations in the Bering and Chukchi seas using species-specific polymerase chain reaction (PCR) assays for *A. affine* (H.Inoue & Y.Fukuyo) Balech, *A. fraterculus* Balech, *A. catenella* (Group IV, subsequently defined as *A. pacificum* by John *et al.* 2014a), *A. pseudogonyaulax*, *A. tamarense* Group III, and *A. tamiyavanichi* Balech. In their study, cyst densities ranged from  $10^2$  to  $10^4$  cm<sup>-3</sup> of sediment. Despite the high cyst densities, only *A. fundyense* Group I was detected. With the exception of results from the Natsuike *et al.* (2013) study, the other *Alexandrium* species identifications previously reported were based solely on morphology. Unfortunately, morphological identification without molecular confirmation is unreliable for many *Alexandrium* species (John *et al.* 2014a; Kremp *et al.* 2014). This is particularly true for the *A. tamarense* species complex, which originally contained three broadly distributed, morphologically defined species, *A. catenella*, *A. fundyense*, and *A. tamarense* (John *et al.* 2014a). Molecular phylogenies based on ribosomal (r)DNA genes have consistently shown that the *A. tamarense* species complex is actually comprised of five distinct ribotype clades (Lilly *et al.* 2007; John *et al.* 2014a). Recently, John *et al.* (2014a) formally assigned species designations to *A. fundyense* (Group I), *A. mediterraneum* (Group II), *A. tamarense* (Group III), *A. pacificum* (Group IV), and *A. australiense* (Group V) and proposed formal rejection of the name *Gonyaulax catenella* (*A. catenella*) in John *et al.* (2014b). The John *et al.* (2014a) nomenclature is used throughout this study and references to the *A. tamarense* species complex are accompanied by a Groups I–V designation. This enables unambiguous species identification no matter the species name used in previous studies.

Taking into account recent taxonomic revisions within *Alexandrium* (John *et al.* 2014a; Kremp *et al.* 2014), the goals of this study were to develop and use quantitative, species-specific PCR (qPCR) assays that targeted the ribosomal gene complex to detect and quantify cells of *Alexandrium* collected from Alaska coastal sites. Six *Alexandrium* species were selected as subjects for assay development on the basis of the distributional data presented in Balech (1995). Initially, these assays were used qualitatively to detect *Alexandrium* species in surface water and sediment samples. After the presence of *Alexandrium* species was confirmed, species-specific assays were converted into a quantitative (qPCR) format. *Alexandrium* cysts from four geographically dispersed regions including Ketchikan, Juneau, Kachemak Bay, and Kodiak Island were isolated from sediment samples and propagated to

establish cultures. This material was used to validate qPCR assays and to investigate the possible existence of other *Alexandrium* species.

Before developing the new species-specific assays we tested several published qPCR assays (data not shown). The results obtained in our laboratory revealed varying levels of primer dimer formation, occasional cross-reactivity with other *Alexandrium* species, and variable dynamic ranges. Consequently, we elected to develop our own species-specific qPCR assays. This approach has been used in the past by numerous researchers because of uncertainty surrounding the specificity and efficacy of published assays. Having developed molecular assays for other HAB projects (Litaker *et al.* 2002, 2003; Vandersea *et al.* 2006, 2012), we were conscious of the time and effort required to validate molecular assays. Therefore to aid other researchers in selecting creditable *Alexandrium* molecular assays, we opted to systematically evaluate existing *Alexandrium* assays and make recommendations to the scientific community. As a first step in this evaluation process, we collated published *Alexandrium* PCR, qPCR, and *in situ* hybridization assay primers and probes that target the small-subunit/internal transcribed spacer/large-subunit (SSU/ITS/LSU) rDNA genes. These data facilitated identification of validated, species-specific *Alexandrium* assays for monitoring and ecological studies worldwide.

## MATERIAL AND METHODS

This study used two methods to identify the *Alexandrium* species present in Alaska. The first was to isolate cells from environmental samples and confirm their identity by molecular cloning and sequencing of diagnostic rDNA regions. The second was to develop PCR assays for species that were likely to be found in Alaska on the basis of previous reports and for which sufficient sequence data were available. Once species from these samples were identified using species-specific PCR assays, the assays were converted into a quantitative (qPCR) format. The PCR assays were used to confirm the identity of the isolates established from field material as well as to screen for species that were not easily cultured, or those present in such low numbers that a representative cell isolate could not be established.

### Collection of phytoplankton and sediment samples

Live phytoplankton from nearshore locations in Ketchikan, Juneau, Kachemak Bay, and Kodiak Island (Figs 1–5) were collected via surface-water net tows using a 20- $\mu$ m-mesh plankton net (model 9000, 1-litre cod end, Sea-Gear Corp., Melbourne, Florida, USA). The cod end from each tow was gently shaken to homogenize the sample and a 100-ml aliquot was preserved in neutral Lugol's solution for screening using species-specific molecular assays. In this study sediment samples were collected from embayments and beaches possessing fine sediments and salinities ranging from 20 to 25. Surface sediment samples (upper 1–2 cm) were collected at low tide or by scuba divers using 50-ml screw-cap tubes. Samples were stored at 4°C in the dark until they could be processed for cyst germination or DNA extraction and PCR assay. Unpreserved whole-water and sediment samples were shipped with ice packs to the National Oceanic and Atmospheric Administration (NOAA) Center for Coastal Fisheries and Habitat Research in Beaufort, North Carolina, USA for single-cell isolations. Upon arrival the water samples were filtered sequentially through 500-

$\mu\text{m}$  and 200- $\mu\text{m}$  mesh to remove larger fauna and debris. One-hundred-millilitre aliquots of each sieved sample were placed in vented, polystyrene tissue culture flasks and supplemented with ~20 ml of modified K medium (no Tris, no Cu, no Si) (Keller *et al.* 1987) and incubated for several days at 14°C under ~50  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  of light (light:dark = 12:12 h). *Alexandrium* cultures were then established from this incubated material via single-cell isolations (see methods below).

### Sediment processing, cyst germination, and single-cell isolates

Sediment samples proved to be the best source of *Alexandrium* cells for single-cell isolation. Sediment samples were removed from storage at 4°C and rinsed through a 53- $\mu\text{m}$  mesh screen with filtered seawater to remove large particulates. The 53- $\mu\text{m}$  mesh screens were cleaned in 5% bleach between samples and were rinsed thoroughly with deionized (d)H<sub>2</sub>O to eliminate cross-contamination of *Alexandrium* DNA and other remaining debris. Approximately 0.5–3 ml of the resulting < 53- $\mu\text{m}$  sediment slurry was added to three replicate 100 × 15 mm polystyrene Petri dishes containing 30 ml of filtered seawater at a salinity of 25. Next, 60  $\mu\text{l}$  of NaNO<sub>3</sub> stock ( $1.76 \times 10^{-3}$  M), 30  $\mu\text{l}$  of Na<sub>2</sub>  $\beta$ -glycerophosphate stock ( $1.00 \times 10^{-5}$  M), and 60  $\mu\text{l}$  of germanium dioxide stock ( $1.23 \times 10^{-4}$  M) were added to each dish. The dishes were then gently swirled to mix the contents and were incubated using the same conditions as above. The plates were checked for *Alexandrium* cells over the next 7 days using a stereoscope at ×40 magnification. Parallel 0.5-ml aliquots of sediment slurry were also filtered onto 47-mm, 8- $\mu\text{m}$  pore size polycarbonate filters and subjected to DNA extraction as described below. Once motile *Alexandrium* cells were observed in the dishes they were collected with a pipette and transferred to a 55-mm Petri dish for isolation. A single cell was picked from the Petri dish using a micropipette and sequentially transferred through three to four drops of sterile medium. After each transfer, the drop was examined to ensure that only a single cell was present. After the final transfer, each isolated cell was placed into a separate well of a sterile 6- or 24-well tissue culture plate with a small volume of modified K medium (salinity 25). Isolates were then incubated as described above. When sufficient cell density was achieved, the clonal cultures were transferred to 50-ml glass Erlenmeyer flasks containing modified K medium and were eventually transferred to 250-ml glass Erlenmeyer flasks. Additional cultures were obtained from the National Center for Marine Algae and Microbiota (NCMA), reisolated, and maintained as described above to supplement the material isolated from Alaska. The genetic identities of clonal cultures established from sediment samples and from isolates established from NCMA were confirmed by sequencing the D1-D3 region of the LSU ribosomal gene as described below to ensure that they matched published sequences in GenBank. The *Alexandrium* cultures used in this study are listed in Table 1.

### DNA extraction and sequencing protocols

DNA extraction, PCR amplification of the 3' SSU through the 5' LSU rDNA region, subcloning, and sequencing were performed using reagents and methods adapted from Vandersea *et al.* (2012). Approximately 10 ml of each *Alexandrium* culture (Table 1) at  $1 \times 10^3$  cells·ml<sup>-1</sup> were concentrated by filtration onto a 47-mm, 8- $\mu\text{m}$  pore size polycarbonate filter (Whatman Nucleopore™, GE Healthcare Bio-sciences, Pittsburgh, Pennsylvania, USA). DNA was extracted from each filter using the Mo Bio Laboratories Power® Soil

DNA isolation kit (Mo Bio Laboratories, Solana Beach, California, USA) following the manufacturer's protocol except that 350  $\mu$ l of cell lysate rather than the prescribed 450  $\mu$ l was processed. The DNA extracts were eluted from the minicolumns using 50  $\mu$ l of elution buffer. Spectrophotometric analysis (260 nm/280 nm) of the DNA was conducted to assess DNA concentration and purity. The DNA extracts were stored at 4°C. A ~1700-base pair (bp) product containing the 3' SSU, ITS, and 5' LSU rDNA domains was PCR amplified using the Alex1600F and LSUB primers (Table 2). The PCR amplification reaction mixtures contained 20 mM Tris-HCl, pH 8.4, 3 mM MgCl<sub>2</sub>, 50 mM KCl, 25 pmol of each primer, 2.5 mM of each deoxynucleoside triphosphate, 0.2 units of Platinum *Taq* DNA polymerase (Invitrogen™ Life Technologies, Carlsbad, California, USA), and 10 ng of genomic DNA in a total reaction volume of 50  $\mu$ l. A Bio Rad PTC-100 Peltier thermal cycler (Bio Rad, Hercules, California, USA) was used to conduct the PCR with the following cycling conditions: 2 min at 95°C, 35 $\times$  (30-s denaturation at 95°C, 30-s annealing temperature at 60°C, and an extension of 1.5 min at 72°C), and a final 5-min extension at 72°C. A 5- $\mu$ l aliquot of each PCR reaction was checked for the presence of a specific amplification product by agarose gel electrophoresis (2% Tris-acetate/EDTA, 100 V) and ethidium bromide staining. The PCR products were cloned into the plasmid vector pCR2.1® (Invitrogen Life Technologies) using the TOPO TA cloning® kit following the manufacturer's protocol. Plasmids were isolated and purified using the Promega Wizard® Plus Minipreps DNA purification system (Promega, Madison, Wisconsin, USA) and sequenced using an ABI3730xl DNA sequencer using the Deoxy™ Terminator Cycle sequencing kit (Applied Biosystems - ABI, Foster City, California, USA). DNA templates were sequenced completely in both directions using the primers listed in Table 2. The 3' SSU to 5' LSU sequence was assembled using the Vector NTI Advance™ 11 program (Invitrogen Life Technologies) and the resulting sequences compared with those in GenBank using basic local alignment search tool (BLAST) searches (Altschul *et al.* 1997) to establish the species identity of each isolate. The gene sequences obtained in this study were deposited in GenBank. (GenBank accession numbers: *A. affine* KX599339; KX599340, *A. andersoni* KX599343, *A. fundyense* KX599344; KX599345; KJ127879-KJ127911, *A. minutum* KX599341; KX599348, *A. pacificum* KX599346; KX599347, *A. tamarensis* KX599342).

### Screening of environmental samples using the species-specific PCR assays

Water samples, sediment, and cell isolates from Ketchikan, Juneau, Kachemak Bay, and Kodiak Island were processed as described above. Genomic DNA was extracted from the phytoplankton or sediment samples collected at all four sampling locations (Figs 1–5) according to the methods described above. The resulting DNA extracts were used as the template for the species-specific PCR assays.

### Design and testing of species-specific *Alexandrium* PCR assays

The SSU/ITS/LSU rDNA sequences of *Alexandrium* species obtained in this study were aligned with corresponding SSU, ITS, and D1-D3 LSU rDNA sequences from other *Alexandrium* species available from GenBank using the ClustalX program (Thompson *et al.* 1997) and sorted into species groups using the phylogenetic analyses methods described in John *et al.* (2014a). The species included in the alignments were *A. affine* (Inoue & Fukuyo)

Balech, *A. andersoni* Balech, *A. hiranoi* Kita & Fukuyo, *A. insuetum*, *A. minutum* Halim, *A. monilatum* (J.F. Howell) Balech, *A. cohorticula* (Balech) Balech, *A. tamiyavanichi* Balech, *A. fraterculus* (Balech) Balech, *A. leeii* Balech, *A. margalefii*, *A. ostenfeldii*, *A. pseudogonyaulax*, *A. satoanum* Yuki & Fukuyo, *A. tamutum* Montresor, *A. taylori* Balech, *A. tropicale* Balech, *A. fundyense* Group I, *A. tamarensis* Group III, *A. pacificum* Group IV, and *A. mediterraneum* Group II John (John *et al.* 2014a). The D1-D3 LSU alignments were used for developing species-specific assays for *A. fundyense* Group I, *A. minutum*, *A. monilatum*, *A. ostenfeldii*, *A. pacificum* Group IV, and *A. tamarensis* Group III. Unique forward and reverse species-specific primer sites were visually identified within the alignments for each species. The primer pairs were selected with the object of limiting the PCR amplicons to less than 200 bp in length. Each primer was also subjected to a BLAST search using the National Center for Biotechnology Information tool to determine potential cross-reactivity with non-*Alexandrium* species (Altschul *et al.* 1997). Primers that exhibited cross-reactivity with other *Alexandrium* species or with other dinoflagellates were eliminated from consideration. The primers were then tested systematically for secondary PCR products using genomic DNA extracted from the intended target species using the amplification conditions described below. Successful candidate primer pairs were analyzed for species specificity and cross-reactivity using genomic DNA extracted from other *Alexandrium* species (Table 1). The primer pairs chosen for final PCR assay validation are listed in Tables 3 and S1 (Vandersea *et al.* this study).

To further confirm species specificity, a mixture of DNA from seven *Alexandrium* species (*A. affine*, *A. andersoni*, *A. fundyense* Group I, *A. minutum*, *A. ostenfeldii*, *A. pacificum* Group IV, *A. tamarensis* Group III) was PCR amplified using each of the PCR assay primer sets. The PCR products were purified using the Invitrogen Pure Link™ PCR purification kit following the manufacturer's protocol, cloned using the TOPO TA cloning kit, and sequenced using an ABI3730xl DNA sequencer. The sequences were aligned and checked to ensure that they corresponded to the intended target species.

### PCR assay cycling conditions

PCR assays were performed using an Eppendorf Master-cycler® ep realplex 4 system with white Eppendorf real-time tube strips (Eppendorf North America, Inc., Westbury, New York, USA) and a total reaction volume of 10.5 µl per tube. Each PCR reaction mixture contained 4.5 µl of 5 Prime RealMasterMix SYBR ROX 2.5× [0.05 units µl<sup>-1</sup> *Taq* DNA polymerase, 10 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 1.0 mM deoxynucleotide triphosphates, 20× SYBR® Green solution], each primer at a concentration of 0.15 µM, 4.7 µl of sterile deionized water, and 1 µl of template DNA. Thermal cycling conditions included denaturation at 95°C for 2 min followed by 40 cycles at 95°C for 10 s and annealing for 15 s at 60°C, with a subsequent extension at 68°C for 20 s. The fluorescence threshold was determined by the Eppendorf realplex 4 analytical software, and the PCR cycle during which fluorescence crossed the threshold was designated the quantification cycle (C<sub>q</sub>) as defined by Bustin *et al.* (2009). A melting-curve analysis was performed after thermal cycling to check the specificity of the PCR reactions. The melting-curve profile consisted of denaturation at 95°C for 15 s followed by an annealing step for 15 s at 60°C. The fluorescence was continuously monitored during a steady 20-min temperature ramp from 60 to 95°C that was held at 95°C

for 15 s. The melting-curve analysis was conducted by comparing the melting-temperature peak of positive control DNA to other experimental DNA samples. A limit of  $\pm 0.5^{\circ}\text{C}$  for melting-temperature peak shift was set as the cutoff for species-specific amplifications.

### **Conversion of PCR assays into qPCR assays, qPCR standard curves, amplification efficiency, and limits of detection**

The PCR assays were converted into qPCR assays using methods adapted from Vandersea *et al.* (2012). To construct cell-based standard curves, multiple strains ( $n = 5$ ) of *A. fundyense* Group I cultures in log-phase growth were sieved through 50- $\mu\text{m}$  mesh to remove cell debris and multicell aggregates. The sieved cells were gently mixed to ensure homogenization and then cell concentrations were measured in triplicate using a Beckman Coulter Multisizer™ 3 fitted with a 280- $\mu\text{m}$  aperture tube (Beckman Coulter Inc., Brea, California, USA).

Individual cell dilutions ranging from 100 to 150,000 cells were prepared in triplicate using sterile filtered seawater at a salinity of 25. For each replicate sample 100 ml were concentrated by filtration onto 47-mm, 8- $\mu\text{m}$  pore size polycarbonate filters as above. To obtain accurate results for low-range cell concentrations (10–100 cells), the cells were manually pipetted onto each polycarbonate filter and counted using a stereomicroscope. Using a range of cell concentrations to construct standard curves provides a more accurate calculation of qPCR cell concentration than amplifying DNA diluted from a single known cell concentration, as the latter method can greatly overestimate or underestimate actual cell concentrations from field samples. Genomic DNA extraction and qPCR amplification were carried out as described above. Each assay incorporated 1  $\mu\text{l}$  of the 50- $\mu\text{l}$  DNA extract in the qPCR reaction mix. Standard curves were constructed by plotting the triplicate Cq values against the log-transformed cell concentrations. A regression analysis of the Cq vs log-transformed cell concentrations was performed to calculate the slope values and confirm the linearity of the standard curves. The qPCR assay limit of detection (LOD) for the cell-based standard curves was the lowest cell concentration, ranging over four orders of magnitude, that amplified and yielded linear Cq values.

PCR amplicons were serially diluted and used to construct standard curves after the PCR amplicon copy numbers were determined. First, rDNA fragments for each species were PCR amplified using the Alex1600F and LSUB primers that flanked the qPCR assay target sites. The rDNA fragments were then cloned into the plasmid vector pCR2.1 using the TOPO TA cloning kit. Plasmid DNAs were isolated and purified using the Promega Wizard Plus Minipreps DNA purification system and stored at  $-20^{\circ}\text{C}$ . The plasmid DNAs served as templates for producing the PCR amplicons used to construct the standard curves. When PCR amplicons were needed to construct standard curves, stock plasmids were thawed, diluted 1:3000 in sterile deionized water, and PCR amplified using the M13F and M13R primers. The PCR amplification reaction mixtures contained 20 mM Tris-HCl, pH 8.4, 3 mM MgCl<sub>2</sub>, 50 mM KCl, 25 pmol of each primer, 2.5 mM each deoxynucleoside triphosphate, 0.2 units of Platinum *Taq* DNA polymerase, and 10 ng of genomic DNA in a total reaction volume of 50  $\mu\text{l}$ . The PCR amplification profile used to amplify ~ 1700-bp insert was as follows: 2 min at  $95^{\circ}\text{C}$ , 35 $\times$  (30 s at  $95^{\circ}\text{C}$ , 30 s at  $50^{\circ}\text{C}$ , and 1.5 min at  $72^{\circ}\text{C}$ ), and a final extension of 5 min at  $72^{\circ}\text{C}$ . The PCR reactions were checked for the presence of



a specific amplification product by agarose gel electrophoresis (2% Tris-acetate/EDTA, 100 V) and ethidium bromide staining. PCR reactions containing specific amplicons were purified using the Invitrogen Pure Link PCR purification kit (Invitrogen Life Technologies) following the manufacturer's protocol. The DNA concentration of the purified amplicons was estimated spectrophotometrically by measuring ultraviolet absorbance at 260 nm. The number of target copies per microlitre of stock solution for each species-specific PCR amplicon was determined as described in Vandersea *et al.* (2012).

To construct standard curves, the purified amplicons were serially diluted 1:10 in dH<sub>2</sub>O and encompassed six orders of magnitude. Each serial dilution was qPCR amplified for 40 cycles using the qPCR assay primers designed to amplify each species, followed by a melting-curve analysis to check for secondary qPCR products and primer dimers. Only C<sub>q</sub> values from qPCR reactions that produced a specific product within 35 amplification cycles were included in the standard curves. For each standard curve, the C<sub>q</sub> values generated from the 10-fold serial dilutions were plotted against the log-transformed copy numbers to obtain regression equations. A regression analysis was performed to calculate the slope values and confirm the linearity of the standard curves. The qPCR assay LOD for the diluted PCR amplicon-based standard curves was the lowest copy number concentration ranging over six orders of magnitude that amplified yielding linear C<sub>q</sub> values. Regression equations derived from the standard curves were used to estimate the number of PCR amplicons per cell as described below.

### Estimating cell numbers using qPCR standard curves

To obtain qPCR cell number estimates, the ratio of extractable PCR amplicons per cell was determined. The number of extractable PCR amplicons per cell was calculated by solving the regression equations derived from the diluted PCR amplicon-based standard curves using C<sub>q</sub> values acquired from qPCR amplification of known numbers of *Alexandrium* cells. These values represented a functional ratio of the numbers of copies extracted per cell and cannot be used to calculate the number of rDNA copies per cell. An essential criterion for using this approach is that the slope values of the diluted PCR amplicon standard curves and cell-based standard curves must be almost equivalent (i.e. differ by  $\pm 0.1$ ). Adhering to this criterion ensures that the qPCR efficiency of both standard curves is nearly equivalent, facilitating a valid conversion of extractable PCR amplicons per cell to cell numbers (Bustin *et al.* 2009).

### qPCR assay controls

To assess potential DNA contamination and PCR inhibitors in the extracted field samples, each qPCR assay included a positive control, a negative DNA control, a blank extraction control, and two supplemented DNA controls. The positive control contained a known amount of target DNA in the qPCR reaction mixture and ensured that the qPCR reagents were properly assembled and the DNA *Taq* polymerase was functional. The negative control included the addition of 1  $\mu$ l of reaction buffer to a subset of reaction mixes to test for contaminated reagents or cross-contamination between samples. The blank controls were incorporated during the DNA extractions of the field samples to test for potential DNA

contamination during the extraction process. Supplemented controls consisted of adding target DNA to a subset of the field samples to determine if PCR inhibitors were present.

### Experimental analysis of qPCR accuracy

To check the accuracy and sensitivity of the qPCR assays, known concentrations of multiple *Alexandrium* isolates were mixed together and the live cells were enumerated using a Beckman Coulter Multisizer 3 fitted with a 280- $\mu\text{m}$  aperture tube. Dilution series containing multiple strains of either *A. fundyense* Group I or *A. ostenfeldii* at concentrations ranging from  $\sim 0.10$  to 9800 cells  $\text{ml}^{-1}$  were made in triplicate in natural seawater collected from the Center for Coastal Fisheries and Habitat Research's dock in Beaufort, North Carolina. The diluted cells were concentrated onto 47-mm polycarbonate filters and the DNAs were extracted and qPCR assayed. Standard curves constructed from diluted PCR amplicons were run at the same time. The accuracy of the assays was determined by correlating qPCR cell estimates to the cell dilutions that were based on the multisizer cell counts.

### Literature review of species-specific assays for *Alexandrium* species

To facilitate future molecular studies of *Alexandrium* species, we surveyed the literature for species-specific molecular assays that targeted the SSU through D1-D3 LSU rDNA region. A comprehensive table was assembled that included the author citation, the targeted ribosomal gene, the target species as published, the type of assay, the sequences of the primers or probes, and their species specificities (Tables 3 and S1). Each of the species-specific primer and probe sequences was manually mapped onto consensus SSU, ITS/5.8S, and D1-D3 alignments. The alignments were prepared as described in John *et al.* (2014a) and used to analyze the gene regions with the greatest concentration of species-specific sequences, and the relative intra- vs interspecific sequence variation. Analogous alignments were constructed for the ITS and SSU regions.

## RESULTS

### PCR assay of environmental samples

The species-specific PCR assays for *Alexandrium fundyense* Group I, *A. pacificum* Group IV, *A. tamarense* Group III, *A. ostenfeldii*, (Kremp *et al.* 2014) *A. minutum*, and *A. monilatum* [= *Gonyaulax monilatum* (Howell) Loeblich III] developed in this study did not cross-react with nontarget species as determined by melting-curve and sequencing analysis of PCR products (Figs S1–S6). They were successfully used to screen 108 water and sediment samples as well as 17 cell isolates collected from 10 stations at four coastal sites in Ketchikan, Juneau, Kachemak Bay, and Kodiak Island from September 2009 to November 2011 (Figs 1–5). Of the six species assayed, only *A. fundyense* Group I and *A. ostenfeldii* were detected in the four geographically isolated regions (Figs 6, 7). All 17 of the cell isolates were identified genetically as *A. fundyense* Group I (Table 1). Fifty-three percent of the environmental samples were positive for *A. fundyense* Group I, whereas 47% were positive for *A. ostenfeldii*. *Alexandrium fundyense* Group I was present in the majority of the samples from Ketchikan, Juneau, and Kodiak Island, but in only 31% of the samples from Kachemak Bay (Fig. 6). *Alexandrium ostenfeldii* was present in 58% of the samples from Ketchikan and Kodiak Island, in 37% of the Kachemak Bay samples, and in 30% of

the Juneau samples (Fig. 7). *Alexandrium fundyense* Group I and *A. ostenfeldii* occurred together in 57% of the positive samples. Attempts to establish isolates of *A. ostenfeldii* from either phytoplankton or sediment samples were not successful. Whether this was due to low numbers of cells or unfavourable culture conditions was not determined.

### Assay sensitivity and amplification efficiency

The *A. fundyense* Group I qPCR assay sensitivity was tested using cell concentrations ranging from ~0.01 to 14,800 cells·mL<sup>-1</sup> diluted in local seawater. The *A. ostenfeldii* qPCR assay sensitivity was tested using cell concentrations ranging from 0.01 to 18,967 cells·mL<sup>-1</sup>. Both assays were capable of detecting less than 10 cells in a 1-litre sample, but the *A. fundyense* Group I Cq values were inconsistent below 0.09 cells·mL<sup>-1</sup>, whereas the *A. ostenfeldii* Cq values were inconsistent below 0.01 cells·mL<sup>-1</sup> and fell outside the linear ranges of the standard curves. The assay sensitivities were also tested using serial dilutions of PCR amplicons that contained appropriate target sequence. The LOD for *A. fundyense* Group I assay was ~2440 copies and the LOD for *A. ostenfeldii* assay was ~241 copies. The standard curves were linear over at least six orders of magnitude. Amplification efficiencies were calculated using typical slope values of the regression lines for Cq vs log-transformed cell numbers and Cq vs log-transformed copy numbers using the equation  $E = 10^{(-1/\text{slope})} - 1$ . For the *A. fundyense* Group I assay, typical slope values approached the theoretical optimum of -3.3 and the amplification efficiencies ranged from 0.996 to 1.005. For the *A. ostenfeldii* assay typical slope values of the regression for Cq vs log-transformed copy number also approached -3.3, with an amplification efficiency of 0.979. In contrast, the slope of Cq vs log-transformed cell numbers was -2.91, with an amplification efficiency of 1.206. The differences in the slopes of the cell-based standard curves and diluted PCR amplicon standard curves were compared. The *A. fundyense* Group I assay varied by less than 0.1, whereas the *A. ostenfeldii* assay varied by 0.39. The linear relationships and representative data from the standard curves that were used to calculate the regressions are shown (Figs 8–11). The empirical relationship between PCR amplicons and cell numbers for *A. fundyense* Group I and *A. ostenfeldii* was derived by solving the regression equations for the PCR amplicon and cell-based standard curves using the methods described in Vandersea *et al.* (2012). The relationship between PCR amplicon number and cell number for *A. fundyense* Group I was determined to be 11,107 amplicons per cell and 12 amplicons per cell for *A. ostenfeldii*.

### Assay accuracy

Five experiments using known concentrations of cells that were enumerated using a Beckman Coulter Multisizer were conducted to assess the accuracy of the *A. fundyense* Group I and *A. ostenfeldii* qPCR assays. Known numbers of cells were mixed with 100 ml of seawater from Beaufort, North Carolina. Each experiment contained three replicates of multiple strains of each species ranging from 10 to 9800 cells·mL<sup>-1</sup> as well as standard curves constructed using serially diluted PCR amplicons. The qPCR assay cell concentration estimates for *A. fundyense* Group I exhibited a strong correlation with the multisizer cell counts (Fig. 12). In contrast, the qPCR assay cell concentration estimates for *A. ostenfeldii* did not correlate well with the multisizer cell counts (Fig. 13).

## Evaluation of the specificity of published *Alexandrium* molecular assays as a basis for developing standard assays

*Alexandrium* molecular assays were listed alphabetically by first author in Tables 3, 4 and S1. Each of the primers or probes was checked for cross-reactivity with other species using the National Center for Biotechnology Information (NCBI) nucleotide BLAST tool (Altschul *et al.* 1997). To convey a visual presentation of the BLAST results, the molecular probe and primer locations were manually mapped onto *Alexandrium* species consensus sequence alignments of the SSU/ITS/LSU rDNA genes (Figs 14 and S7–S9).

## DISCUSSION

Two toxic *Alexandrium* species, *A. fundyense* Group I and *A. ostentfeldii*, were found in sediment and water samples obtained from coastal sites in Ketchikan, Juneau, Kachemak Bay, and Kodiak (Figs 1–5). *Alexandrium pacificum* Group IV, *A. tamarense* Group III, *A. minutum*, and *A. monilatum* were not detected. These findings are significant because they are the first unambiguous report of *Alexandrium* species distribution for Alaskan coastal waters. They are consistent with the molecular results of Natsuike *et al.* (2013), who detected *A. fundyense* Group I cysts in sediments collected from the Bering and Chukchi Seas, often at extremely high densities. Even though our sampling efforts and those of Natsuike *et al.* (2013) suggest low *Alexandrium* diversity in this region, it is possible that with more intense sampling efforts other *Alexandrium* species will be identified in Alaskan waters using molecular methods.

The original reports of *A. catenella* occurring in Alaskan coastal waters (Horner *et al.* 1997; Trainer 2002) were based primarily on the observation that cells of *A. catenella* formed chains, which was previously considered a morphologically diagnostic feature for the species (Balech 1995). However, the review conducted by John *et al.* (2014a) demonstrated that chain formation is not a reliable species characteristic. Indeed, the only *A. tamarense* complex species identified from Alaska in our study was *A. fundyense* Group I, which is also known to form chains. Similarly, *A. fundyense* Group I and *A. tamarense* Group III have been shown to be morphologically indistinguishable (John *et al.* 2014a), indicating that the report of *A. tamarense* in Alaskan waters by Trainer (2002) may have been due to misidentification of *A. fundyense* Group I cells, as the molecular screening conducted in this study found no *A. tamarense* Group III cells.

Interestingly, *A. fundyense* Group I is the dominant species found in eastern Canada and the Gulf of Maine, where it co-occurs with *A. ostentfeldii* (McGillicuddy *et al.* 2014). Though not conclusive, the similarity in the *Alexandrium* species composition between Alaska and eastern Canada and the Gulf of Maine is consistent with the hypothesis of Scholin *et al.* (1995), that eastern and western populations of *Alexandrium* may have originated from the same parental stocks. Given that *A. fundyense* Group I is the principal source of STXs in the Bay of Fundy and Gulf of Maine (Martin & Richard 1996; Anderson *et al.* 2005a, 2014; Martin *et al.* 2008; McGillicuddy *et al.* 2014), the presence of this species in the Gulf of Alaska likely accounts for the persistent occurrences of PSP in Alaska. This conclusion is supported by the identification of *A. fundyense* Group I in samples collected in this study

(Thomas Basin, Mountain Point; Fig. 2) during a significant PSP event near Ketchikan, Alaska where 21 cases of PSP occurred in May-June 2011 (Porter *et al.* 2011).

The extent to which *A. ostenfeldii* contributes to the toxicity of shellfish in Alaska is unknown. Some strains of *A. ostenfeldii* have been reported to exhibit low toxicity, whereas others are known to be very toxic (Hansen *et al.* 1992; Mackenzie *et al.* 1996). Borkman *et al.* (2012) found STX production in *A. ostenfeldii* isolates obtained from Narragansett Bay, Rhode Island, as did Brown *et al.* (2010) from Scottish isolates. Toxicity testing of *A. ostenfeldii* isolates from Alaska is needed to address this question.

Historically, shellfish toxicity testing has been used to extrapolate *Alexandrium* species distributions and cell concentrations in Alaska (Hall 1982; Horner *et al.* 1997). Though indicative of where PSP problems are most severe, this type of monitoring does not provide crucial, real-time warning capability to identify the early stages of bloom development that can precede the onset of shellfish toxicity by days to weeks (Neale 1967; Matweyou 2003; Lefebvre *et al.* 2008). Initially, the *A. fundyense* Group I and *A. ostenfeldii* assays were used qualitatively in this study for species detection. After the presence of these species was confirmed in the environmental samples, the species-specific assays were converted into a qPCR format that could be used in near real time to determine *Alexandrium* distributions and relative abundances of cells. The feasibility of using the assays for these purposes was tested by adding known concentrations of *A. fundyense* and *A. ostenfeldii* into local filtered seawater and then comparing qPCR cell estimations to cell counts. We used this approach because the environmental samples collected in Alaska for this study were not collected quantitatively. Unfortunately, this negated our ability to make quantitative estimates using qPCR methods on the samples. Figs 12 and 13 show the qPCR assay cell estimates of supplemented seawater plotted against the cell numbers included in the samples. The *A. fundyense* qPCR cell estimates exhibited a strong positive correlation with Coulter counter-based cell counts and confirmed that the qPCR assay was quantitative ( $R^2 = 0.987$ , Fig. 12). In contrast, the *A. ostenfeldii* qPCR cell estimates did not correlate with Coulter counter-based cell counts and was not quantitative ( $R^2 = 0.437$ , Fig. 13), leading to the obvious question of why the *A. fundyense* qPCR assay was quantitative and the *A. ostenfeldii* was nonquantitative. One possibility we explored was qPCR inhibition. To test this, *A. ostenfeldii* DNA extracts were diluted 1:10 and 1:100 and PCR amplified. The results did not indicate qPCR inhibition. Supplemented control assays were also conducted in which concentrated genomic *A. ostenfeldii* DNA was added into replicate *A. fundyense* qPCR reactions and the Cq values of nonsupplemented *A. fundyense* qPCR reactions were compared with supplemented *A. fundyense* Cq values. On the basis of the equivalent Cq values of the supplemented and nonsupplemented *A. fundyense* assays, there was no sign of qPCR inhibition.

Inconsistent cell lysis could also account for the results, but microscopic inspection of the experimental extracts revealed no intact cells, indicating that this is not likely the primary cause of qPCR variability. It is more probable that an unidentified compound contained in *A. ostenfeldii* cells affected DNA extraction efficiency. It is known from molecular studies of plants and kelps that polyphenols, mucilaginous polysaccharides, and carbohydrates can interfere with the recovery of nucleic acids from certain species (Fox & Swanson 2007;

Souza *et al.* 2012; Maeda *et al.* 2013; Ramosa *et al.* 2014). *Alexandrium ostenfeldii* may contain a compound that interfered with DNA purification. During our experiments DNA was purified from *A. ostenfeldii* cell concentrations that scaled over two orders of magnitude. Spectrophotometric determination of DNA concentrations revealed only a twofold difference between the highest and lowest cell concentrations. In comparison, DNA purified from *A. fundyense* cell concentrations, which also scaled over two orders of magnitude, yielded DNA concentrations that differed by 15-fold between the highest and lowest cell concentrations. This was a strong indication of interference in the DNA purification of the *A. ostenfeldii* cells. This premise is further supported by the limited dynamic range of the *A. ostenfeldii* cell-based standard curves (Fig. 10). The arrows in Fig. 10 indicate that the C<sub>q</sub> values obtained from cell concentrations ranging from ~5600 to ~151,000 cells plateaued at roughly 18 cycles. These results signaled the assay's upper limit of detection and that the DNA extraction method was saturated. The *A. ostenfeldii* cell-based standard curves exhibited more variability than the *A. fundyense* cell-based standard curves ( $R^2 = 0.878$  vs  $R^2 = 0.928$ , respectively; Fig. 10). In contrast, typical diluted PCR amplicon standard curves for *A. ostenfeldii* were almost always linear over seven orders of magnitude ( $R^2 = 0.996$ ), demonstrating that the qPCR thermocycling, reagents, and reaction mixes were appropriately administered (Fig. 10). In conclusion, the *A. fundyense* Group I qPCR assay was robust and can be used to accurately assess a wide range of cell concentrations from field samples. More research, however, is needed to establish a DNA purification method for *A. ostenfeldii* before the assay can be used to quantify concentrations of this species in natural populations.

### Review of species-specific molecular assays for identifying *Alexandrium* species

The earliest *Alexandrium* molecular studies were conducted by Scholin *et al.* (1993) and Scholin & Anderson (1994), who sequenced the *A. fundyense* SSU and the LSU D1-D2 rDNA genes and by Adachi *et al.* (1994), who conducted restricted fragment length polymorphism analysis of the ITS-5.8S regions of *A. catenella*, *A. tamarensis*, *A. insuetum*, and *A. pseudogonyaulax*. During the intervening two decades many other investigators have conducted molecular studies and designed species-specific assays for numerous *Alexandrium* species. The majority of the assays are redundant and target identical species, particularly those known to produce STXs. One factor driving the development of seemingly duplicative assays is that no systematic compilation and evaluation of existing molecular assays has been carried out. In an effort to reduce the development of redundant species-specific assays we have collated and mapped published primer and probe locations shown in Figures 14 and S7–S9. These primer and probe locations targeted SSU/ITS/LSU rDNA gene regions. Analysis of the locations revealed that a hypervariable gene region in the LSU D1-D2 domain, from bp 420 and extending to bp 740, was most commonly targeted. Assays developed in our study also targeted this variable region. Further scrutiny revealed that probes and primers developed by different investigators for identical species often overlapped and were shifted several bps up or down from each other (Fig. S7). Similarly, the ITS/5.8S region contained numerous species-specific polymorphisms that were targeted for assay development and that tended to be evenly distributed over the entire region (Fig. S8). Molecular studies of *Alexandrium* have targeted the SSU rDNA gene regions less often because the SSU genes are more conserved than either the LSU D1-D2 or ITS1–5.8S-ITS2

gene regions and contain proportionately fewer polymorphic sites that can be targeted for molecular assay development. The most commonly targeted SSU gene region was located in a hypervariable region at the 5' end of the gene between bps 140 and 260 (Fig. S9).

A systematic evaluation of published *Alexandrium* molecular assays is presented in Tables 3 and S1. The authors are listed in alphabetical order followed by the targeted rDNA domain, the intended target species, type of assay, probe and primer sequences, and species specificity as determined by NCBI BLAST search and by visual confirmation within the alignments (Figs 14 and S7–S9). The molecular analyses indicated that the majority of the assays were well designed and incorporated species-specific gene sequences as their targets (unshaded sections of Table S1). However, the analyses also revealed that some of the assays exhibited potential flaws due to a variety of reasons (shaded sections of Table S1). For example, numerous assays were not specific for their intended target species. The most apparent reason for this was that early investigations did not have access to the quantity and diversity of ribosomal gene sequence data that are available today. Consequently, determining cross-reactivity of molecular assays, which can be readily accomplished now, was not possible previously. Other sources of assay inaccuracies included mismatched gene sequence where the primer or probe sequence did not match the intended target sequence and assays that targeted polymorphic loci without incorporating degenerate bases in the probe design. Other assays were species specific, but only for a subset of the total sequence variants contained in GenBank. This could have the detrimental effect of reducing assay signal strength and efficiency as well as yielding false-negative results. Thorough assessment of within-species sequence variation is critical for optimal assay design and requires alignment of numerous gene sequences to identify unique sites that are conserved among alleles. This is particularly true for some *Alexandrium* species because sequence variation among ribosomal gene copies is high in some species but quite low in others (Scholin *et al.* 1993; Kim *et al.* 2004b; Miranda *et al.* 2012).

## Recommendations

It is clear that a majority of the species-specific assays listed in Tables 3 and S1 (unshaded) are useful. However, at least for the toxic species, it would be beneficial if standard assays were adopted for worldwide monitoring. An immediate positive benefit of such an approach is that it would make implementation of the assays easier and more affordable. A logical starting point would be to evaluate and promote assays that have been used successfully in established monitoring programs and that have large linear dynamic ranges in the number of cells that can be detected in environmental samples. Examples include the qPCR assays used by the Cawthron Institute (Nelson, New Zealand) for several years to monitor *A. australiense* Group V, *A. fundyense* Group I, *A. minutum*, and *A. pacificum* Group IV (Harlow *et al.* 2005) and the one validated for *A. fundyense* Group I in this study. Other assays include those for *A. andersoni* and *A. taylori* developed by Penna *et al.* (2007). Similarly, the microarray sites identified in Galluzzi *et al.* (2011) for monitoring population densities of *A. andersoni*, *A. mediterraneum* Group II, *A. minutum*, *A. pacificum* Group IV, *A. pseudogonyaulax*, *A. tamarensis* Group III, and *A. taylori* have been well vetted and may also prove useful as *in situ* probes. Representative *in situ* and sandwich hybridization assays include those for *A. affine* (Kim *et al.* 2005), *A. andersoni* (Touzet & Raine 2007), *A.*

*fraterculus* (Kim *et al.* 2005), *A. fundyense* Group I (Scholin *et al.* 1996; Anderson *et al.* 1999; 2005b; John *et al.* 2003; Sako *et al.* 2004), *A. mediterraneum* Group II (John *et al.* 2005), *A. minutum* (Diercks *et al.* 2008; Touzet *et al.* 2008), *A. ostenfeldii* (Metfies *et al.* 2005), *A. pacificum* Group IV (Adachi *et al.* 1996a; Sako *et al.* 2004), *A. tamarensis* Group III (John *et al.* 2005; Touzet *et al.* 2008), and *A. tamiyavanichi* (Kim *et al.* 2004a). Vetting and selecting representative assays from this group or others listed in Tables 3 and S1 will reduce future duplication of effort and facilitate investigation of this ecologically and toxicologically important genus. It will also widen the appeal of molecular identification methods to resource managers, public health officials, and commercial shellfish operators as well as improve public health protection and utilization of valuable shellfish resources.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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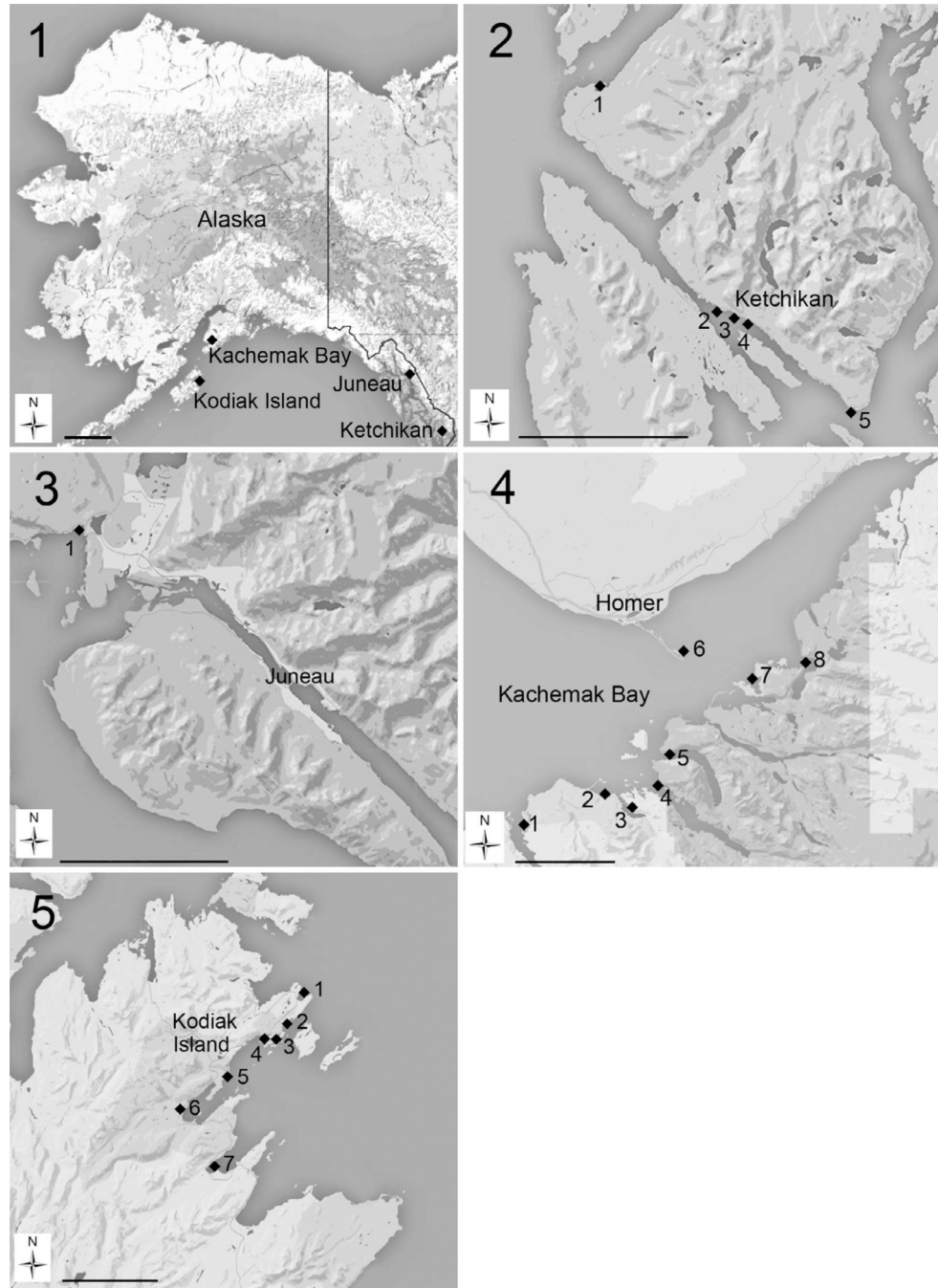
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**Figs 1–5. Locations of sampling sites.**

**Fig. 1:** Location of coastal Alaska sampling sites. Scale bar = 250 km.

**Fig. 2:** Location of sampling sites in Ketchikan, Alaska. (1) Knudson Cove, (2) Bar Harbor Ramp, (3) City Float, (4) Thomas Basin, (5) Mountain Point. Scale bar = 10 km.

**Fig. 3:** Location of the Auke Bay sampling site in Juneau, Alaska. Scale bar = 10 km.

**Fig. 4:** Location of sampling sites in Kachemak Bay, Alaska. (1) Seldovia Harbor, (2) Kasitsna Bay NOAA Laboratory, (3) Jakolof Bay, (4) Tutka Bay, (5) Sadie Cove, (6) Homer Harbor, (7) Peterson Bay, (8) Halibut Cove. Scale bar = 10 km.

**Fig. 5:** Location of sampling sites in Kodiak Island, Alaska. (1) Mill Bay, (2) Mission Beach, (3) Trident Basin, (4) U.S. Coast Guard Station, (5) Buskin River, (6) Sargent Creek, (7) Womens Bay.

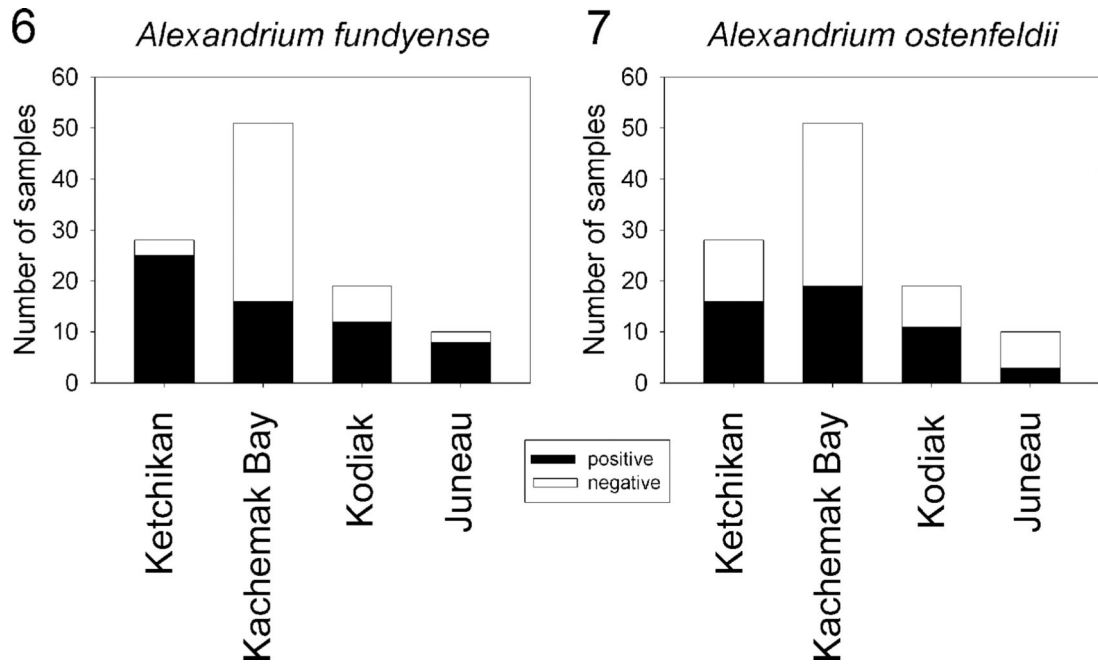
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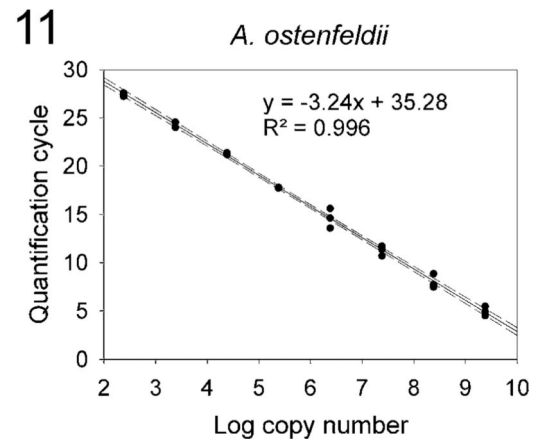
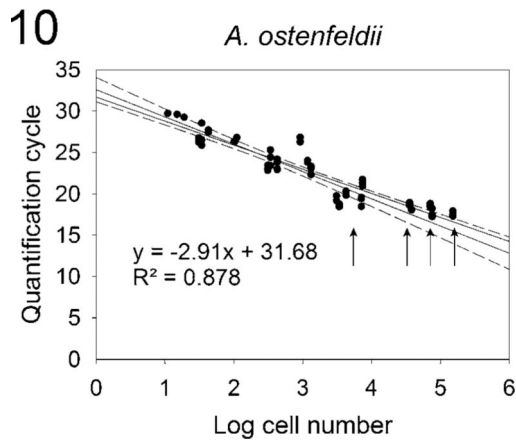
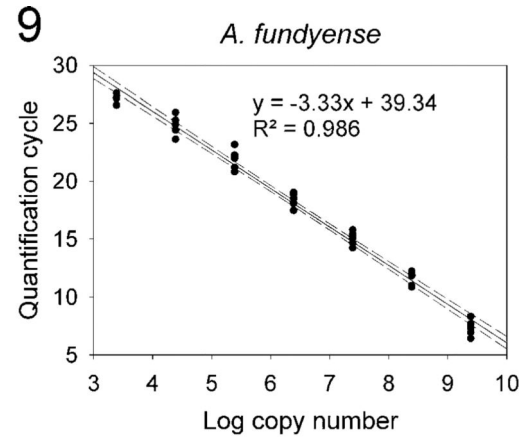
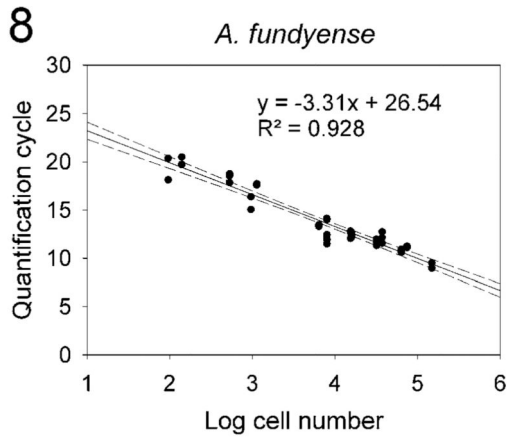
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**Figs 6, 7.**

Six PCR assays were developed in this study and used to screen 108 water and sediment samples collected from coastal sites in Ketchikan, Kachemak Bay, Juneau, and Kodiak Island during September 2009–November 2011. The graphs show the proportion of PCR-positive and -negative samples for each sampling area. The analysis combined data obtained from isolates, sediment, and water samples. *Alexandrium fundyense* Group I (Fig. 6) and *A. ostenfeldii* (Fig. 7) were the only species detected.



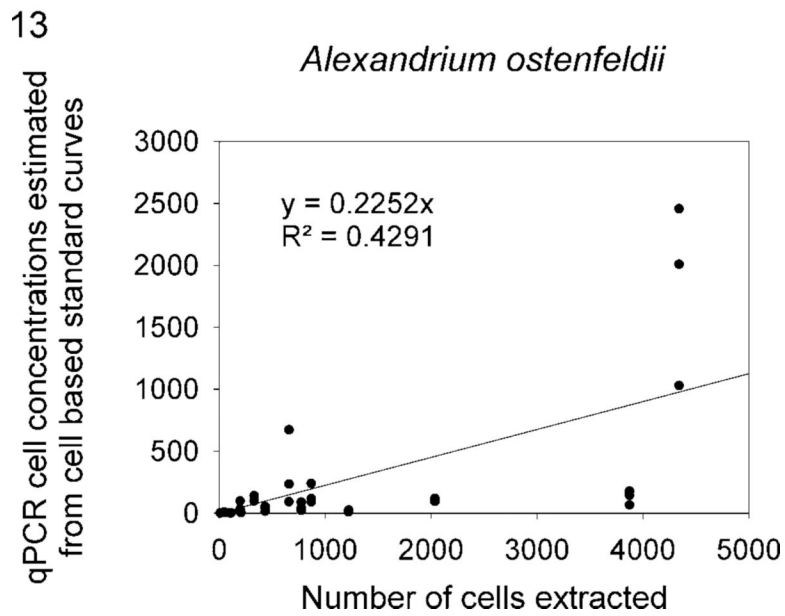
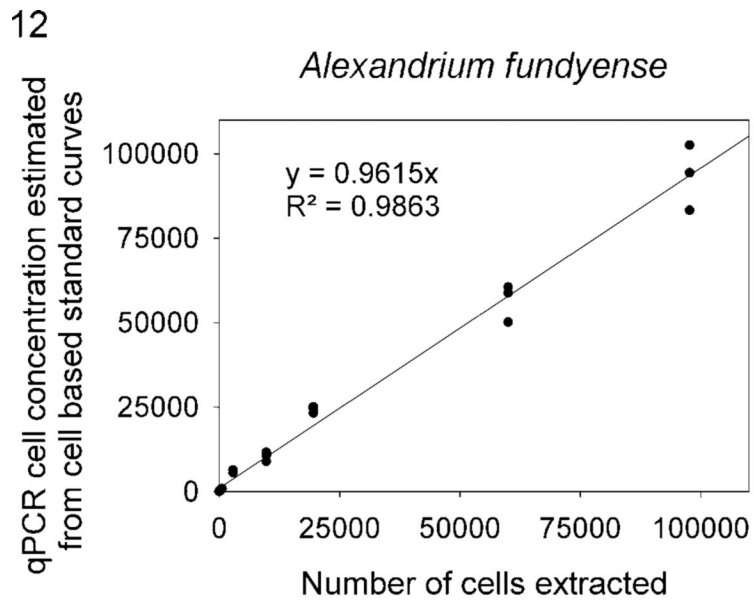
**Figs 8–11. *Alexandrium fundyense* and *Alexandrium ostenfeldii* qPCR standard curves.**

**Fig. 8.** Representative *Alexandrium fundyense* Group I qPCR assay standard curve constructed using a range of known cell concentrations. The solid line indicates the regression of log cell numbers vs quantification cycle (Cq). The 95% confidence interval is represented by the dashed lines.

**Fig. 9.** An *Alexandrium fundyense* qPCR assay standard curve constructed using 10-fold serial dilutions of a purified PCR amplicon containing the *A. fundyense* qPCR assay target domain. The regression of log copy numbers vs quantification cycle was plotted and dashed lines show the 95% confidence interval.

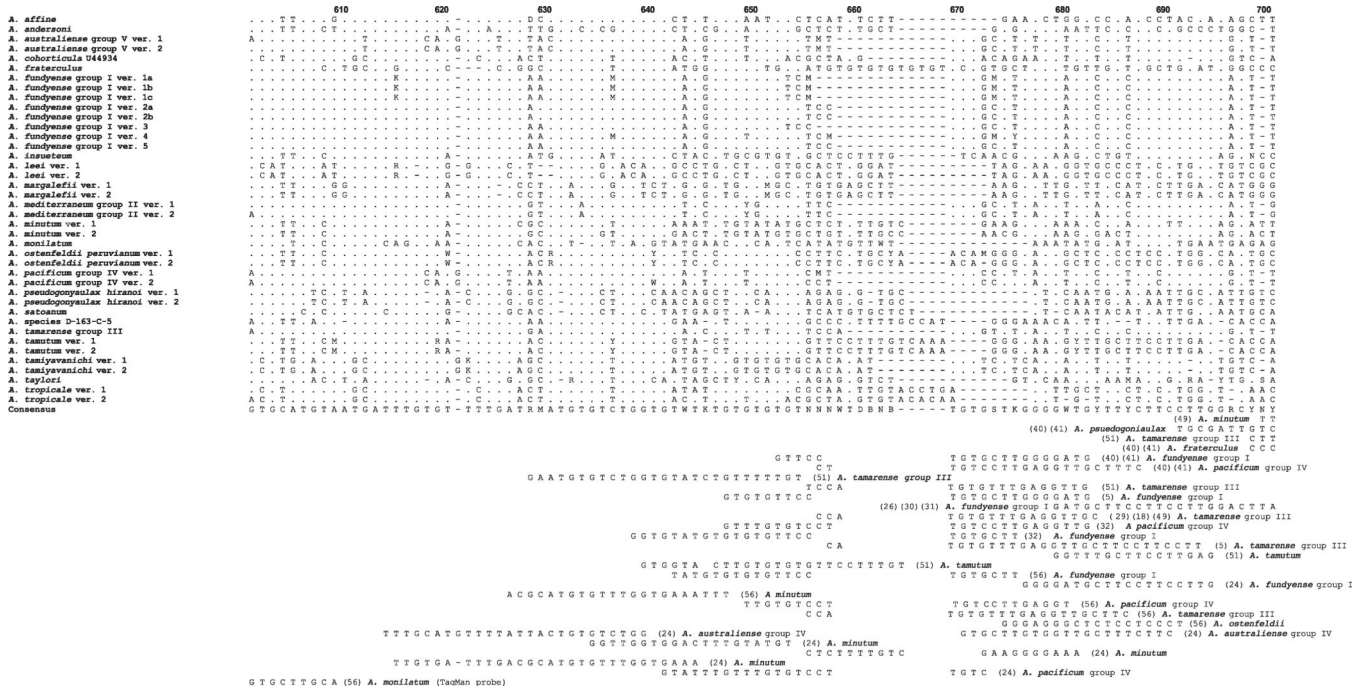
**Fig. 10.** Same as Fig. 8 except that *Alexandrium ostenfeldii* cells were used. The arrows indicate where Cq values obtained from cell concentrations ranging from ~5600 to ~151,000 cells plateaued at roughly 18 cycles and signaled the assay's upper limit of detection.

**Fig. 11.** Same as Fig. 9 except the PCR amplicon contained the *A. ostenfeldii* qPCR assay target domain.



**Figs 12, 13.**

The relationship between known concentrations of cells of *Alexandrium fundyense* or *A. ostenfeldii* spiked into seawater samples and cell numbers estimated using qPCR.



**Fig. 14.** Consensus sequences for *Alexandrium* species were aligned and used to map the location of hybridization sites in the LSU D1-D2 rDNA region. The figure shows one subsection of the D1-D2 region. The entire data set is presented in Fig. S7. Sequences from many species clustered into distinct subgroups. Identification of the subgroups including GenBank accession numbers is described in the Material and Methods section and the supplementary data in John *et al.* (2014a). To better illustrate differences among species, only variations from the consensus sequence are shown. A space in a position above the consensus sequence indicates that the base pair in that position is identical to the consensus sequence. A dash in a given position indicates that there is no corresponding base pair in the consensus sequence (i.e. missing nucleotide). Note: To simplify the figure, only coding strand sequences are displayed, including the reverse PCR primers and probes. All primers and probe locations are identified on the coding strand in 5'–3' orientation. The sequences for the noncoding strand primers and probes are listed in Tables 3 and S1. The numbers in parentheses next to the primers and probe sequences correspond to authors listed in Tables 3 and S1.

Table 1.

*Alexandrium* cultures used in this study.

Strain	Species	Location
CCMP 112	<i>A. affine</i>	Ria de Vigo, Spain
CCMP 113	<i>A. minutum</i>	Ria de Vigo, Spain
CCMP 115	<i>A. tamarense</i> Group III	Tamar Estuary, Plymouth, UK
CCMP 116	<i>A. affine</i>	Ria de Vigo, Spain
CCMP 1598	<i>A. pacificum</i> Group IV	Da-ya Bay Guan-dong (Canton) Province, bay west of Hong Kong Island, China
CCMP 1718	<i>A. andersoni</i>	Town Cove, Eastham, Massachusetts, USA
CCMP 1719	<i>A. fundyense</i> Group I	Portsmouth, New Hampshire, USA
CCMP 1771	<i>A. tamarense</i> Group III	Tamar Estuary, Plymouth, UK
CCMP 1773	<i>A. ostenfeldtii</i>	Lim Fjorden, Denmark
CCMP 1888	<i>A. minutum</i>	Laguna Obidos, Portugal
CCMP 1908	<i>A. fundyense</i> Group I	East Sound, Washington, USA
CCMP 2082	<i>A. insuetum</i>	Uchiumi Bay, Kagawa, Japan
CCMP 2215	<i>A. hiranoi</i>	Jogashima, Misaki, Kanagawa, Japan
CCMP 2955	<i>A. leei</i>	Singapore Strait, Singapore
Amon Miss VIMS	<i>A. monilatum</i>	Gulfport, Mississippi, USA
Amon YK VIMS	<i>A. monilatum</i>	York River, Virginia, USA
AoF0933	<i>A. ostenfeldtii</i>	Baltic Sea, Åland, Finland
AoNOR4	<i>A. ostenfeldtii</i>	Oslofjorden, Norway
AoTVA4	<i>A. ostenfeldtii</i>	Baltic Sea, Åland Finland
Ap002/B2NR	<i>A. ostenfeldtii</i>	New River, North Carolina, USA
Ap003/A4NR	<i>A. ostenfeldtii</i>	New River, North Carolina, USA
Ap004/A2NR	<i>A. ostenfeldtii</i>	New River, North Carolina, USA
Ap006/A3NR	<i>A. ostenfeldtii</i>	New River, North Carolina, USA
Auke Bay 1	<i>A. fundyense</i> Group I	Juneau, Alaska, USA
Auke Bay 4	<i>A. fundyense</i> Group I	Juneau, Alaska, USA
Auke Bay 9	<i>A. fundyense</i> Group I	Juneau, Alaska, USA
Dentist Dock 1	<i>A. fundyense</i> Group I	Ketchikan, Alaska, USA
Dentist Dock 2	<i>A. fundyense</i> Group I	Ketchikan, Alaska, USA

Strain	Species	Location
Knudson Cove	<i>A. fundyense</i> Group I	Ketchikan, Alaska, USA
Mill Bay 3	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA
Mission Beach	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA
Sargent Creek 1	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA
Sargent Creek 5	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA
Sargent Creek 6	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA
Sargent Creek 8	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA
Thomas Basin 1	<i>A. fundyense</i> Group I	Ketchikan, Alaska, USA
Thomas Basin 2	<i>A. fundyense</i> Group I	Ketchikan, Alaska, USA
Trident 1	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA
USCG 2	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA
Womens Bay 1	<i>A. fundyense</i> Group I	Kodiak Island, Alaska, USA

**Table 2.**

Oligonucleotide primers used in this study to amplify and sequence *Alexandrium* SSU, ITS1, 5.8S, ITS2, and the D1–D3 domain of the LSU.

Primer name	Sequence (5′–3′)	Melting temperature, °C
PCR amplification primers		
Alex1600F	GCYTGAGTCATCAGCTTGTGC	64
LSUB	ACGAACGATTTGCACGTCAG	60
Sequencing primers		
5.8SF	CATTGTGAATTGCAGAATTCC	58
Alex5.8SR	GCTCACGGAATTCTGCAATTC	62
AlexLSURI	CCACCCACTTTGCATTC	52
DIR	ACCCGCTGAATTTAAGCATA (Scholin et al. 1994)	56
M13F	GTAAAACGACGGCCAG	50
M13R	CAGGAAACAGCTATGAC	50

Table 3.

Excerpt from a literature survey completed in 2015 of the various probes available for detecting *Alexandrium* species. The complete literature survey is presented as supplemental material in Table S1. The literature citation, rDNA target domain, the original species the probe was designed to target, the probe sequences, and the actual species the probes will detect on the basis of current taxonomy are listed. The reverse primers and *in situ* hybridization probes target the noncoding strand. The probes are all listed in 5'–3' orientation. The ribosomal gene locations of the primers and probes listed in this table are also identified in Figs 14 and S7–S9 on the species coding strand in 5'–3' orientation.

Reference	Domain	Target species	Assay type	PCR primers			Reporter probe/Detects		Forward probe		Reverse probe	
				Forward primer coding (sense) strand	Reverse primer/ <i>in situ</i> probe noncoding (antisense) strand	Coding strand	BLAST search results	BLAST search results	BLAST search results			
56	LSU D1-D2	<i>A. fundyense</i>	qPCR SYBR green	GATAAGTCTCTCTGT GGGGGG	AAGCACAGGAACACA CACATA		<i>A. fundyense</i>	<i>A. fundyense</i>	<i>A. fundyense</i>		<i>A. fundyense</i>	
	LSU D1-D2	<i>A. pacificum</i>	PCR	CTTGCCTTTGTGTGCCAGTTTT	ACCTCAAGGACAAGGACACAAA		<i>A. pacificum</i>	<i>A. pacificum</i>	<i>A. pacificum</i>		<i>A. pacificum</i>	
	LSU D1-D2	<i>A. minutum</i>	PCR	TGTGCTTACTCTATCATTTATAT	AAATTTACACAAAACACATGCGT		<i>A. minutum</i>	<i>A. minutum</i>	<i>A. minutum</i>		<i>A. minutum</i>	
	LSU D1-D2	<i>A. monilatum</i>	PCR	TGTTTGCAITTAITGTGTGAACA	CACAGGCACCTTACCTTTTCAT		<i>A. monilatum</i>	<i>A. monilatum</i>	<i>A. monilatum</i>		<i>A. monilatum</i>	
	LSU D1-D2	<i>A. monilatum</i>	qPCR TaqMan	TGAAAGGTAAAGTGCCTGTG	GCAGAAACATGTTGCCAAAAG	TGCAAGCACAAAGCAA CCCAGC A. monilatum	<i>A. monilatum</i>	<i>A. monilatum</i>	<i>A. monilatum</i>		<i>A. monilatum</i>	
	LSU D1-D2	<i>A. ostenfeldii</i>	qPCR SYBR green	TGAGATTGTGTGCGTCCACTTGT	AGGAGGAGAGGCCCTCCC		<i>A. ostenfeldii</i>	<i>A. ostenfeldii</i>	<i>A. ostenfeldii</i>		<i>A. ostenfeldii</i>	
	LSU D1-D2	<i>A. tamarense</i>	PCR	TGAGATTGTGTGCGTCCACTTGT	AGGAGGAGAGGCCCTCCC		<i>A. tamarense</i>	<i>A. tamarense</i>	<i>A. tamarense</i>		<i>A. tamarense</i>	
57	5.8S	<i>Alexandrium</i> genus	qPCR SYBR green	GATGAAGAATGCAGCAAAAATG	CAAAACCTTCAAGAATATCC		<i>Alexandrium</i> genus	<i>Alexandrium</i> genus	<i>Alexandrium</i> genus		<i>Alexandrium</i> genus	
58	SSU	<i>A. catenella</i>	nuclease protection - sandwich hybridization		AITTTGGCACAGCCTGAGCAITTAATC capture probe		<i>A. fundyense</i> <i>A. pacificum</i> <i>A. tamarense</i>	<i>A. fundyense</i> <i>A. pacificum</i> <i>A. tamarense</i>	<i>A. fundyense</i> <i>A. pacificum</i> <i>A. tamarense</i>		<i>A. fundyense</i> <i>A. pacificum</i> <i>A. tamarense</i>	
	SSU				CACACCACACAGTCAAGTGCAGTTGTGCTTTCAAGATAAAATGCTCAGGCTGTGCCAAAAT nuclease protection probe		<i>A. affine</i> <i>A. cohoritcula</i> <i>A. fundyense</i> <i>A. pacificum</i> <i>A. tamarense</i>	<i>A. affine</i> <i>A. cohoritcula</i> <i>A. fundyense</i> <i>A. pacificum</i> <i>A. tamarense</i>	<i>A. affine</i> <i>A. cohoritcula</i> <i>A. fundyense</i> <i>A. pacificum</i> <i>A. tamarense</i>		<i>A. affine</i> <i>A. cohoritcula</i> <i>A. fundyense</i> <i>A. pacificum</i> <i>A. tamarense</i>	
	SSU				ACAACCTGCACCTTGACTGTGTGGTGTG signal probe		<i>A. tamarywanichii</i> <i>Alexandrium</i> genus	<i>A. tamarywanichii</i> <i>Alexandrium</i> genus	<i>A. tamarywanichii</i> <i>Alexandrium</i> genus		<i>A. tamarywanichii</i> <i>Alexandrium</i> genus	



Additional references cited in a literature survey of *Alexandrium* molecular assays presented in supplemental materials Table S1 and Figs S7–S9.

**Table 4.**

Adachi et al. (1996b)	Diercks-Horn et al. (2011)	Dyhrman et al. (2006)
Dyhrman et al. (2010)	Erdner et al. (2010)	Galluzzi et al. (2004)
Galluzzi et al. (2005)	Galluzzi et al. (2006)	Galluzzi et al. (2010)
Gameau et al. (2011)	Genovesi et al. (2011)	Gescher et al. (2008)
Giacobbe et al. (2007)	Godhe et al. (2001)	Gribble et al. (2005)
Guillou et al. (2002)	Haley et al. (1999)	Hosoi-Tanabe & Sako (2005a)
Hosoi-Tanabe & Sako (2005b)	Hosoi-Tanabe & Sako (2006)	Kamikawa et al. (2005)
Kamikawa et al. (2007)	Ki & Han (2006)	Kim et al. (2005)
Lazerges et al. (2006)	Leonardi et al. (2006)	Nagai (2011)
Penna & Magnani (1999)	Penna & Magnani (2000)	Penna et al. (2010)
Scholin et al. (1994)	Tang et al. (2012)	Taylor et al. (2014)
Toebe et al. (2013)	Touzet et al. (2007)	Touzet et al. (2009)
Touzet et al. (2010)		