Novel hydrolysis-probe based qPCR assay to detect saxitoxin transcripts of dinoflagellates in environmental samples

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ABSTRACT

Paralytic Shellfish Poisoning (PSP) is a serious human illness caused by ingestion of seafood enriched with paralytic shellfish toxins (PSTs). PSTs are neurotoxic compounds produced by marine dinoflagellates, specifically by Alexandrium spp., Gymnodinium catenatum and Pyrodinium bahamense. Every year, massive monitoring of PSTs and their producers is undertaken worldwide to avoid PSP incidences. Here we developed a sensitive, hydrolysis probe-based quantitative PCR (qPCR) assay to detect a gene essential for PST synthesis across different dinoflagellate species and genera and tested it on cDNA generated from environmental samples spiked with Alexandrium minutum or Alexandrium fundyense cells. The assay was then applied to two environmental sample series from Norway and Spain and the results were complemented with cell counts, LSU-based microarray data and toxin measurements (enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) biosensor method). The overall agreement between the results of the qPCR assay and the complementary data was good. The assay reliably detected sxtA transcripts from Alexandrium spp. and G. catenatum, even though Alexandrium spp. cell concentrations were mostly so low that they could not be quantified microscopically. Agreement between the novel assay and toxin measurements or cell counts was generally good; the few inconsistencies observed were most likely due to disparate residence times of sxtA transcripts and PSTs in seawater, or, in the case of cell counts, to dissimilar sxtA transcript numbers per cell in different dinoflagellate strains or species.

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1. Introduction

In humans, Paralytic Shellfish Poisoning (PSP) is a serious condition with symptoms of strong tingling sensations in the mouth, fingers, and toes, feelings of numbness, dizziness, headaches and nausea, and loss of motoric skills. In severe cases muscular paralysis and subsequent death may occur. The sickness is caused by Saxitoxin and its analogs, commonly known as Paralytic Shellfish Toxins (PSTs). PSTs are small molecular weight neurotoxic alkaloids that are synthesized by aquatic microorganisms (reviewed in Wiese et al., 2010). Filter feeders such as mussels and oysters that feed on these microorganisms may accumulate the toxins in their tissues. Consuming these animals can cause PSP.

The risk of contracting PSP from commercially farmed shellfish in industrialized countries is extremely low (Lawrence et al., 2011), but the efforts to minimize this risk are huge. To avoid PSP and other shellfish-poisoning incidences, countries with important shellfish and other coastal fisheries carry out regular surveillance programs. In Norway, for example, 25 sampling stations along the coast are sampled weekly throughout the year, plus 13 additional stations that are only sampled during the summer months. This generates >1600 samples per year (www.matportalen.no). Each sampling typically consists of water samples, which are screened for the presence of the causative microorganisms, as well as chemical analysis of shellfish extracts to detect and quantify PSTs directly.

The microorganisms responsible for PSTs in marine waters worldwide are dinoflagellates; specifically the species Gymnodinium catenatum and Pyrodinium bahamense as well as several species of the genus Alexandrium. Of these three, Alexandrium spp.
are the most abundant and widespread (Anderson et al., 2012), but P. bahamense is the most important PST-producing species in tropical and subtropical waters. Its motile cells have been reported from the Caribbean Sea and Central America, the Persian Gulf and the Red Sea, and the western Pacific (Usup et al., 2012). G. catenatum has been reported from coastal areas of every continent (Gárate-Lizárraga et al., 2005), but does not extend as far into temperate areas as Alexandrium spp.

Due to their ubiquity in coastal waters and their potentially devastating effects, much research has gone into detecting PST-producing dinoflagellates and understanding the relationship between their abundance and the actual occurrence of PSTs. Most of the methods developed rely on morphological identification and counting of potentially PST-producing species, on molecular tools targeting ribosomal RNA (rRNA) genes, or a combination of both (see Godhe et al., 2007 for a comparison of methods, and Anderson et al., 2012 for a recent overview of PCR assays). The problem with these methods is that neither morphology nor rRNA gene sequences are directly related to PST synthesis. For example, Alexandrium species may contain PST-producing and non-producing strains that are not separable morphologically or based on rRNA sequences (Touzet et al., 2007; Mccaulley et al., 2009).

Further, different PST-producing strains may produce dissimilar amounts and isomers of PSTs (e.g. Maranda et al., 1985; Ogata et al., 1987; Yoshida et al., 2001; Cembella et al., 2002; Alpermann et al., 2010).

The recent identification and characterization of the putative key genes for PST synthesis in dinoflagellates (Stuken et al., 2011; Orr et al., 2013) has opened the possibility to develop detection assays based on the genes directly involved in PST synthesis. One of these genes is sxtA, the putative starting gene of PST synthesis in dinoflagellates (Stuken et al., 2011). SxtA consists of four catalytic domains (sxtA1–sxtA4) in freshwater cyanobacteria (Kellmann et al., 2008), another group of organisms that can synthesize PSTs. Transcripts of Alexandrium fundyense have the same sxtA1–sxtA4 domain organization (Stuken et al., 2011). Studies on various dinoflagellate species and strains have shown that all PST-producing strains contain the domains sxtA1 and sxtA4 and neither of the domains have been detected in dinoflagellate species not known to synthesize the toxins (Murray et al., 2011; Stuken et al., 2011; Orr et al., 2013; Suikkonen et al., 2013). Thus, albeit its involvement in PST synthesis has not been functionally proven in dinoflagellates, sxtA appears a promising target to develop a genetic based assay to detect PST producing dinoflagellates in environmental samples. Indeed, a quantitative PCR (qPCR) assay targeting domain sxtA4 has been developed and successfully tested on Alexandrium catenella strains (Murray et al., 2011; Stuken et al., 2011) and on Australian bloom samples of A. catenella that led to PST uptake in oysters (Murray et al., 2011). However, while the theoretical detection limit of the assay when used with genomic DNA corresponded to 110 A. catenella cells per liter, it has not been tested on environmental samples with low Alexandrium cell numbers, nor has it been validated in other regions of the world or on field samples containing other PST-producing species than A. catenella. Here, we tested this assay with spiked- and field samples from Oslofjorden, Southern Norway, but found it not to be sufficiently specific to detect sxtA4 transcripts in samples where PST-producing algae were not dominant.

We therefore sought to develop a new, more sensitive sxtA-assay that could be an early warning system for dinoflagellate PSTs and PST producers across different genera. Our new assay was able to detect sxtA4 of different species at low concentrations and in mixed assemblies and was applied to the field samples from Oslofjorden, and another series from Ria de Pontevedra, Spain. Results were compared with immunochemical PST measurements, cell counts, and microarray data.

2. Materials and methods

2.1. Cultures

The dinoflagellate strains used in this study are listed in Table 1. They were grown in L1 medium (Guillard and Hargraves, 1993), at 30 PSU salinity, a 12:12 h light–dark photoperiod and a photon irradiance of 100 μmol photons m−2 s−1. Most strains were grown at 16°C, only Alexandrium insuetum was grown at 19°C and Polarella glacialis at 5°C. Cultures were xenic.

2.2. Field samples

Field samples were taken from two different sampling sites in Norway and Spain. Norwegian samples were collected at the sampling site OF2, Outer Oslofjorden, Skagerrak, Southern Norway (59°19′ N, 10°69′ E) in the course of the Microarrays for the Detection of Toxic Algae (MIDTAL) project (Dittami et al., 2013a). These samples were taken monthly from August 2009 until June 2010 (except in February 2010 due to ice cover in Oslofjorden) according to the standard MIDTAL protocol (Lewis et al., 2012). Briefly, 1 L water samples were collected from 1 m depth using a

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Niskin water sampler, pre-filtered through a 200 μm sieve, and concentrated on nine 25 mm nitrate cellulose filters (Sartorius AG, Göttingen, Germany; 3 μm pore size). Six replicate filters intended for RNA extraction were fixed with 1 mL of the RNA preservative and extraction reagent Tri-Reagent (Ambion – Applied Biosystems, Foster City, USA), and immediately frozen in liquid nitrogen until further processing. Three of these filters were used for microarray analyzes (Dittami et al., 2013a). Three additional filters to be used for toxin measurements were immediately frozen in liquid nitrogen without addition of a preservative.

In addition, two series of seawater samples were obtained at sampling station OF2. The first series was taken on October 19th, 2011. Duplicates of 1 L samples were spiked with 0, 30, 300, 3000 or 30,000 cells of Alexandrium minutum strain CCMP113. The second series was taken on December 20th, 2011 and duplicates of 1 L samples were spiked with 0, 50 or 500 cells of Alexandrium fundyense strain CCMP1719. The spiked seawater samples were treated in the same way as the regular seawater samples for RNA extraction as described above. Light- and electron microscopic identification of cells were performed in the course of the MIDTAL program for net haul samples (17–0 m, 20 μm mesh size) taken parallel to the RNA samples, and cell counts were performed on 10 mL aliquots of Lugol’s fixed seawater samples using Utermöhl’s standard technique (Utermöhl, 1958; Hasle, 1978) and a Nikon Eclipse TE200 inverted microscope (phase contrast and 100–400× magnification).

Spanish seawater samples were collected during a bloom of Gymnodinium catenatum from Ría de Pontevedra, Northwest Spain (station P2, 42°8’22’’N, 8°51’36’’W) on October 13th and 19th and November 9th and 16th 2009. Seawater from 0 to 10 m depth was collected with a submersible pump during 5–10 min and passed through a set of superimposed framed meshes (100-, 77- and 20-μm mesh size). The 20–77 μm size fraction was selected as a field concentrate and diluted with seawater into 5-L bottles so the plankton material was kept fresh and alive during transport (1 h) to the laboratory. Six times 500 mL samples of this concentrate, each of these representing about 69 L of the original seawater, were filtered as described above for RNA extraction and toxin analyzes, and immediately frozen in liquid nitrogen without further fixative. Light microscopic cell counts were performed as described above.

2.3. Toxin extraction and analyzes

The Norwegian and Spanish seawater filters for toxin analyzes were shipped frozen to Queen’s University, Belfast, where the PSTs were extracted according to McNamme et al. (2012). In brief, the frozen filter was defrosted, removed from the eppendorf tube and transferred to a 20 mL tube. The eppendorf tube was rinsed with deionised water (5× 1 mL), added to the 20 mL tube containing the filter, the sample vortexed for 20 s and rotated on a head over head mixer for 20 min. The filter was removed from the 20 mL tube ensuring that any algal cells had been washed off the filter. The supernatant was transferred to a 5 mL tube containing 0.5 mm glass beads (1 g) and shaken for 20 min on a merris minimix shaker (Merris Engineering Ltd, Galway, Ireland). Finally samples were centrifuged at 3000 × g for 10 min and the supernatant was filtered using a 0.45 μm nitrocellulose syringe filter (Millipore, Watford, UK). These extracts were analyzed for PSTs using a prototype multiplex surface plasmon resonance (SPR) biosensor (Campbell et al., 2011) and an enzyme-linked immunosorbent assay (ELISA; Dubois et al., 2010). Both methods are described in detail by McNamme et al. (2012). Samples were considered negative if the average of the three replicates was below the upper standard deviation of the IC20 to allow for the margin of error for the robustness of the method.

2.4. RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis of the cultured strains used for specificity testing (Table 1) were performed according to Orr et al. (Orr et al., 2012). Briefly, total RNA was isolated using bead beating and the ChargeSwitch® Total RNA Cell Kit (Invitrogen, Life Technologies, Carlsbad, USA). First strand cDNA was synthesized with 3’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) following the high GC protocol and utilizing the adapter primer provided with the kit.

RNA from field samples from Oslo was extracted using the standard Ambion TRI reagent protocol with two modifications (Lewis et al., 2012). First, ca. 300 μL of acid-washed glass beads (213–300 μm) were added to the cryovials and were homogenized (2× 15 s at 6000 rpm) using a Precellys 24 homogenizer (Bertin, Montigny le Bretonneux, France) prior to the standard protocol. Secondly, a final cleanup step was performed to remove possible traces of phenol, using the RNaseasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The final elution volume was 14 μL. This protocol was extensively tested in the course of the MIDTAL project and shown to efficiently lyse cells from a wide range of phytoplankton species including several strains of Alexandrium spp. (Lewis et al., 2012). Samples from Vigo were not frozen in TRI-Reagent, and RNA extraction could therefore be carried out using the NucleoSpin® RNA L Kit (Macherey Nagel, Düren, Germany) according to the manufacturer’s instructions. The final elution volume with this kit was 500 μL. Complementary DNA synthesis for field samples from Norway and Spain was carried out using the Fermentas First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, USA) according to the manufacturer’s instructions, but increasing the reaction temperature from 37 to 45 °C as recommended for GC-rich templates. Primers used were either random hexamers or 10 pmol of the sxt075 primer described below. 1.75 μL of RNA was used per 10 μL synthesis reaction, and the final cDNA was diluted 1:10 with nuclease-free water prior to use in qPCR reactions.

2.5. Quantitative PCR experiments

All quantitative PCRs (qPCRs) were performed on a Roche LightCycler®480 system in white 96-well-plates (Roche Diagnostics AG, Penzberg, Germany). For all qPCR experiments a standard curve was constructed from a five times ten-fold dilution series of a purified sxtA4 PCR product (ca. 160–1,600,000 copies) generated from DNA of strain CCMP113 with primers sxt007 and sxt008 according to Stüken et al. (2011). The PCR product was purified with the Wizard® SV Gel and PCR Clean-up System (Promega, Fitchburg, USA) and quantified with a NanoDrop 3300 (Thermo Scientific, Waltham, USA). The amplification efficiency of the qPCR assays was estimated from standard curves using the “Second Derivative Maximum Method” implemented in the LightCycler®480 software Version 1.5.0 (Roche Diagnostics AG). All qPCRs were run in 10 μL reactions with either LightCycler®480 SYBR Green I Master or Probes Master chemistries (Roche Diagnostics AG) using primer concentrations of 250 or 125 nM. All qPCR protocols started with a 10 min activation step at 95 °C, followed by 45 cycles amplification comprising 15 s at 95 °C, 15 s at annealing temperature and 30 s at 72 °C for extension. All SybrGreen qPCRs were followed by a melting curve analysis. The annealing temperature was gradually adapted during these experiments. Originally 60 °C was used as proposed by Murray et al. (2011), but this value was increased first to 62 and later to 64 °C to improve the specificity of the assay. The Oslofjorden samples were run in two biological replicates, of which each was run with two technical replicates. Signals were considered positive if at least three of the four individual reactions crossed the
Table 2
All primers used for generation of standard curves, qPCR, cDNA synthesis, as well as their estimated melting temperature (°C).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>T_melt °C</th>
<th>Use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sxtA4F</td>
<td>CTT ACC AAC GCC TTC TCT ATC</td>
<td>57.3</td>
<td>qPCR on domain sxtA4</td>
<td>Murray et al. (2011)</td>
</tr>
<tr>
<td>sxtA4R</td>
<td>TAC GAT MGC CCC TGT GAC C</td>
<td>59.4</td>
<td>qPCR on domain sxtA4</td>
<td>This publication</td>
</tr>
<tr>
<td>sxt072</td>
<td>CTT GCC CGC CAT ATG TGC T</td>
<td>59.4</td>
<td>cDNA synthesis</td>
<td>Fermentas kit</td>
</tr>
<tr>
<td>sxt073</td>
<td>GCC CGG CTT AGA TGA TGT TG</td>
<td>61.4</td>
<td>Fragment specific cDNA synthesis</td>
<td>This publication</td>
</tr>
<tr>
<td>random</td>
<td>Random hexamers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sxt075</td>
<td>TTG AAC GCC TTT CTC</td>
<td>47.8</td>
<td>To generate sxtA4 PCR product for standard curve</td>
<td>Stui&quot;ken et al. (2011)</td>
</tr>
<tr>
<td>sxt007</td>
<td>ATG CTC AAC ATG GGA GTC ATC C</td>
<td>60.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sxt008</td>
<td>GGG TCC AGT AGA TGT TGA CGA TG</td>
<td>62.4</td>
<td>Locked nucleic acid (LNA) based hydrolysis probe</td>
<td>Roche Diagnostics AG</td>
</tr>
<tr>
<td>UPL1#142</td>
<td>FAM-GCC AAC AA-quencher</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* According to MWG synthesis report.

2.6. Primer/probe design

New primers were designed on the consensus sequence of an alignment of all available sxtA4 sequences from dinoflagellates (NCBI accessions JF343259–JF343310, JF343311–JF343356). Primers sxt072, sxt073 and sxt075 (Table 2) were designed with the online version of Primer3 (Rozen and Skaletsky, 2000), checked in silico for specificity using the NCBI Primer-BLAST algorithm and for possible hair-pin or primer-dimer formation by the software AutoDimer Version 1.0 (Vallone and Butler, 2004). Primers were synthesized by MWG-Biotech AG, Ebersberg, Germany. The hydrolysis probe was selected by use of the Roche Universal Probe Library Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/). Primers and probes were checked for possible mismatches against the sxtA4 alignment.

3. Results

3.1. Testing of the original sxtA4 qPCR assay on culture material and spiked field samples

The original sxtA4 qPCR assay published by Murray et al. (2011) using the primers sxtA4F and sxtA4R (Table 2) on cDNA generated from culture material of Alexandrium minutum CCMP113 had an amplification efficiency of approximately 100% and resulted in a detection threshold in less than 40 cycles (Cp < 40; Cp is the crossing point (or fractional PCR cycle) at which the quantification threshold is reached), and if the amplified product had the correct melting temperature and size. Only single biological replicates were available for the Kia de Pontevedra samples, and samples were considered positive when both technical replicates had a Cp < 40. In order to further confirm product size and primer specificity, selected amplicons from SybrGreen or probe based qPCRs were analyzed on 2% agarose gels, purified with the Wizard® SV Gel and PCR Clean-up System (Promega) and Sanger sequenced on a Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Life Technologies, Carlsbad, USA). Specificity tests with cDNA from additional cultured dinoflagellate strains (Table 1) were run in triplicates with SybrGreen chemistry, primers sxt072 and sxt073, in 10 µL reactions and at an annealing temperature of 64 ºC.

Fig. 1. Comparison of sxtA4 qPCR melt curves obtained using SybrGreen chemistry and A: primers sxtA4F and sxtA4R (Murray et al., 2011) or B: primers sxt072 and sxt073 (developed during present study). The template was cDNA generated from environmental samples spiked with 0 to 30,000 A. minutum cells per L (strain CCMP113) and taken at OF2, Outer Oslofjorden, Norway on October 19th, 2011. Primers are listed in Table 2.
defined melting peak at 88.2 °C. However, some primer-dimer peaks with a median melting temperature of 77.2 °C were observed in the non-template (NTC) and no-reverse transcriptase (no-RT) controls (Fig. 1A). Close examination of the primer sequences confirmed the presence of several sites for potential self-annealing, especially of primer stxA4R, which may be responsible for these results. When the same assay was tested on field samples spiked with A. minutum CCMP133 cells, good melting curves were only consistently observed in samples with the highest concentration of A. minutum (30,000 cells L⁻¹). In all other samples, unspecific melting curves were observed (Fig. 1A). In the NTC and no-RT controls, primer dimers that amplified in the range of the lower cell concentration samples occurred. Thus, these results indicated only limited usefulness of the assay published by Murray and co-workers for the detection of low quantities of stxA cDNA in mixed plankton assemblies.

3.2. A novel hydrolysis probe-based stxA4 qPCR assay

To improve the sensitivity and specificity of qPCR-based stxA4 detection, a number of measures were taken. First, a new primer pair was designed (sxt072 and sxt073, Table 2) and used at an increased annealing temperature of 64 °C and a decreased primer concentration of 125 nM compared to the original assay of Murray et al. (2011). These primers amplify a 182 bp long fragment with a theoretical melting temperature of 87 °C based on CCMP113 transcript data and the basic algorithm (Kibbe, 2007). No primer-dimers were observed in the NTC and no-RT controls, but similar to stxA4F and stxA4R unspecific melting curves were observed in the lower cell concentration samples. Specificity was further increased by using a newly developed stxA4-specific primer for cDNA synthesis; sxt075 located 19 bp upstream of sxt073 (Table 1). This step drastically reduced the amount of non-target cDNA in the mixed field samples and thus increased sensitivity, at the cost of requiring dedicated cDNA synthesis for each application. This assay was subsequently validated with cDNA obtained from dinoflagellate cultures listed in Table 1 and furthermore tested with our Alexandrium minutum-spiked samples. It yielded comparable specificity to the original assay at 10-fold lower cell concentrations (Fig. 1B).

Finally, a hydrolysis-probe assay was developed based on the established SybrGreen assay. Probe #142 from the Universal Probe Library (UPL; Roche Diagnostics AG) was used, which binds to a conserved region between primers sxt072 and sxt073. The Universal Probe Library contains 165 short (8–9 mers), pre-validated, dual-labeled probes that target specific frequent sequence motifs. Several of the bases in the probes are substituted with locked-nucleic acid bases (LNA), a high-affinity DNA analog that increase both the specificity and the melting temperature of the probe (for further UPL assay description see Mouritsen et al., 2005 and www.roche-applied-science.com). The UPL probe was tested together with qPCR primers sxt072 and sxt073 on cDNA generated from Alexandrium strains CCMP113 and CCMP1719 and compared with our optimized SYBR green assay using both the aforementioned series of Alexandrium minutum CCMP113-spiked samples, and a second series of spiked field samples generated with Alexandrium fundyense (Fig. 2). Interestingly, both assays detected A. fundyense transcripts at six times lower cell concentrations, indicating that the number of stxA4 transcripts per cell differed between the two Alexandrium species. The amplification efficiency of the UPL-qPCR assay was lower compared to the SybrGreen assay (98% vs 100%), resulting in a delayed detection of the fluorescence in the UPL assay. Nevertheless, fluorescence was recorded in both cultures and in all spiked samples, including the lower spike concentrations, but was absent from the NTC and no-RT controls (Fig. 2). Surprisingly, fluorescence was also detected in the 0 cells L⁻¹ spiked sample taken on 19th October 2011. This could indicate non-specific amplification and binding of the UPL probe, contamination during sample processing, or the presence of very low concentrations of PST-producing dinoflagellates in the water column at the time of sampling. To distinguish between these different scenarios, direct sequencing of the 0 cells L⁻¹ and the 30,000 cells L⁻¹ treatment qPCR products was performed and resulted in two different sequences. The 142 bp sequence from the 30,000 cells L⁻¹ treatment was identical to known stxA4 sequences from CCMP113 and other A. minutum strains (e.g. CCMP13: JF343316; AMID16: JF343328; CCMP1888: JF343351). The sequence from the 0 cells L⁻¹ spiked sample (Supplementary Table 1) was of the same length, but contained five single nucleotide polymorphisms (SNPs) compared to the A. minutum sequence (i.e. 97% sequence identity). Using the NCBI BLASTn algorithm, it had the best hits to the same sequences as the 30,000 cells L⁻¹ spike sample sequence, but was not identical to any them.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhal.2013.06.003.

No putative PST-producing dinoflagellate species were detected during the light-microscopic analyzes of the seawater samples taken on 19th October 2011 in Oslofjorden. As the volume analyzed by cell counts was only 10 ml in the MIDTAL protocol compared to 1 L of sample for the molecular methods, we also examined qualitative net haul samples. No motile cells of Alexandrium or Gymnodinium catenatum were detected in these samples, but the presence of Alexandrium cysts could not be excluded.

3.3. Analysis of field samples with the optimized UPL qPCR assay

Finally, the MIDTAL sample series from Oslofjorden, Norway, and from Ría de Pontevedra, Spain, were analyzed with the optimized UPL-qPCR assay and the results compared with the immunochemical PST measurements, cell counts and, in the case of Oslofjorden, microarray analyzes (Figs. 3 and 4). The MIDTAL microarray did not contain probes for Gymnodinium catenatum.

For the Oslofjorden time series the UPL-qPCR assay yielded positive results for five dates: September and October 2009, and April, May and June 2010 (Fig. 3). The correspondence between the UPL-qPCR data and the other assays was good in November and December 2009 (all assays negative) and in April and May 2010 (all assays positive). On the remaining sampling dates at least some of the assays disagreed. Alexandrium cell concentrations were generally too low to be quantified microscopically with the MIDTAL protocol. The only exceptions were August 2009 and June 2010, when Alexandrium pseudogonyaulax was observed with cell concentrations of 1400 and 200 cells L⁻¹, respectively. Furthermore, some Alexandrium cells were detected in a net-haul sample at the sampling site in April 2010. Both SPR and ELISA analyzes indicated the presence of PST toxins in April and May 2010, but the ELISA was also positive at three additional dates. The microarray results indicated the presence of Alexandrium rRNA transcripts on the same dates as the UPL-qPCR in 2010, but no Alexandrium rRNA transcripts were detected in 2009 (Fig. 3).

The UPL results of the Spanish field samples were positive for the three samples containing Gymnodinium catenatum cells, and were negative for the last sample of the series (taken November 16th), in which no G. catenatum cells were found (Fig. 4). The 10-fold increase in cell numbers between October 13th and October 26th 2009 was not reflected in the sxtA4 transcript number estimated by the UPL-qPCR. However, the following 10-fold drop in cell numbers until November 30th was reflected. Both toxin assays were saturated for the first two sampling dates, and the toxin values gradually decreased for the remaining two sampling dates.
To confirm the results from the UPL-qPCR, the qPCR products from each of the positive samples were combined, purified and sequenced directly with primers sxt072 and sxt073 (Supplementary Table 1). The nucleotide sequences were blasted against GenBank using the BLASTN algorithm. The closest hits to all amplicons from the Oslofjorden samples were sxtA4 sequences from _Alexandrium_ species. The September 2009 sequence was most similar to _Alexandrium minutum_ sequences (97%), whereas the sequences from October 2009, April 2010 and June 2010 were closest to _Alexandrium fundyense_ sequences (99–100% identity). The sequences of the Ría of Pontevedra samples were all identical and 99% similar to the sxtA4 sequence of _Gymnodinium catenatum_ GCTRA02 (JF343268).

4. Discussion

A hydrolysis-probe based qPCR assay that can detect and quantify dinoflagellate sxtA4 transcripts in seawater samples was developed and tested with two independent series of field samples from Oslofjorden, Norway and from Ría de Pontevedra, Spain. The Oslofjorden samples was taken monthly over a one-year period and contained only low concentrations of PST-producing algae: At this site, _Alexandrium_ spp. is the only known PST producer and usually occurs at concentrations from 0 to 1500 cells per liter. In contrast, the Spanish samples covered the peak and die-down of a massive _Gymnodinium catenatum_ bloom that led to considerable impact in shellfish harvesting activities. These were banned during an average of ~30 days in all mussel raft areas inside the Ría of Pontevedra October–November 2009 (Blanco, 2011). Both time series were complemented with PST measurements and cell-counts. In addition, complementary microarray data from the MIDTAL-project was available for the Oslofjorden samples.

4.1. A new sxtA assay with improved sensitivity

For the analyzes of the Oslofjorden sample series, a qPCR assay was required that was specific and sensitive enough to detect sxtA4 transcripts in mixed phytoplankton assemblages containing low numbers of _Alexandrium_ spp. cells. PSTs in mussels have been reported from the entire coast of Norway, but the most toxic events usually occur in the North. _Alexandrium tamarense, Alexandrium minutum_ and _Alexandrium ostenfeldii_ are the most common PST producing species reported, but the relationship between the presence of these _Alexandrium_ species and mussel toxicity is not always consistent, indicating a variability in toxicity within and between the different _Alexandrium_ species present (Dahl et al., 2004). Tests of the existing sxtA4 qPCR assay (Murray et al., 2011) on cDNA generated from Oslofjorden seawater samples spiked with varying numbers of _Alexandrium_ cells did not give satisfactory results (Fig. 1A). This was most likely due to a combination of a low target:total transcripts-ratio and a reverse primer with self-annealing potential. By designing new PCR primers, reducing the
primer concentration, increasing annealing temperature, using target-specific primers for cDNA synthesis and a universal probe library (UPL) (probe) an increase in the sensitivity of the assay by nearly three orders of magnitude was achieved. This assay (hereafter UPL-qPCR) reliably detected sxtA4 transcripts from 50 Alexandrium fundyense cells L⁻¹ in environmental samples (Fig. 2). For A. minutum, the lower detection limit could not clearly be established because the 0 cells L⁻¹ spiked sample was positive (Fig. 2). The most likely explanation for this positive result is the presence of low numbers of Alexandrium cells or cysts in the water column at the time of sampling. The nucleotide sequence of the UPL-qPCR amplicon in this sample differed from that obtained for the 30,000 cells L⁻¹ spiked sample, but was still most similar to other Alexandrium sxtA sequences in the NCBI database. Thus, contamination or unspecific amplification was improbable. The presence of low numbers of PST-producing cells in the water column during the A. minutum spike experiment may also explain the lack of a linear relation between the number of cells added and the level of sxtA transcripts observed in the A. minutum treatments with low cell numbers (Fig. 2).
4.2. Assay application to environmental samples.

The UPL-qPCR yielded five positive results for the Oslofjorden sample series, even though cells of putatively PST producing species were only microscopically detected in three samples: *Alexandrium ostenfeldii* in April 2010 and *Alexandrium pseudogonyaulax* in August 2009 and June 2010 (Fig. 3). Nevertheless, direct sequencing of the UPL-qPCR amplicons resulted in nucleotide sequences identical or very similar to known sxtA4 sequences, providing a strong argument that sxtA4 transcripts were indeed present at the sampling site also on the other dates indicated by the UPL-qPCR assay. Further, *Alexandrium minutum, Alexandrium tamarense, A. ostenfeldii* and an unidentified *Alexandrium* sp. were registered at several dates throughout the sampling period at nearby sampling stations monitored by national authorities. The cell concentrations were generally low, only *A. tamarense* occurred in June 2010 at concentrations above the regulatory limit of 200 cells L⁻¹. However, no PST uptake in mussels was reported (Walday et al., 2011; http://algeinfo.imr.no/). *A. pseudogonyaulax* was the only *Alexandrium* species that occurred at bloom densities in Oslofjorden during this sampling series. The bloom started in June 2009 and peaked with 45,300 cells L⁻¹ in September 2009 in Inner Oslofjorden (Berge et al., 2009). At the sampling station in the Outer Oslofjorden, *A. pseudogonyaulax* was registered with 1400 cells L⁻¹ in August 2009 and 200 cells L⁻¹ in June 2010 (Fig. 3). *A. pseudogonyaulax* has not been reported to synthesize PST toxins. The observations that no PST uptake in mussels in Oslofjorden occurred as a consequence of the bloom, as well as negative ELISA and SPR analyzes for the August 2009 sample (Fig. 3) support the notion that this species does not produce PSTs. The negative UPL-qPCR results for August 2009 indicate that *A. pseudogonyaulax* also does not have or at least does not transcribe sxtA genes.

The microarray results were negative in August 2009 despite the presence of high numbers of *Alexandrium pseudogonyaulax* cells at the sampling station. This was proposed to be due to a central mismatch between the *Alexandrium* genus probe and the *A. pseudogonyaulax* LSU rDNA sequence (Dittami et al., 2013a). Similar issues might have also caused the discrepancies between UPL-qPCR and microarray results in September and October 2009. However, as no putatively PST-producing species have been found in the plankton samples at these dates, this issue could not be resolved. It is also not clear if the same species has been sampled twice or if different species have been present. Populations of *Alexandrium* species contain many genetically different strains (Alpermann et al., 2009, 2010; Erdner et al., 2011) and studies of an *Alexandrium fundyense* bloom in the Gulf of Maine have shown that changes in population composition may occur in the order of weeks (Erdner et al., 2011). Thus, it is likely that the Norwegian sampling scheme has not captured the same *Alexandrium* assemblage through time, but rather different populations and possibly even different species. Throughout 2010, the microarray and UPL-qPCR data corresponded well and indicated that sxtA4 mRNAs and *Alexandrium* rRNAs were present in April, May and June (Fig. 3).

The differences observed between the ELISA and SPR results for PST analyzes of the Oslofjorden samples reflect the higher sensitivity of the ELISA (McNamee et al., 2012). It is however not clear why there was a difference between the ELISA measurements and UPL-qPCR results in September 2009, and January, March and June 2010. A possible explanation is that immunochemical toxin testing and qPCR methods measure two different entities with different retention and degradation rates. PCR based methods detect specific nucleic acids, in our case mRNAs, whereas ELISA and SPR measure levels of saxitoxin and its analogs based on the cross-reactivity of the antibody employed. The mRNAs have a short turn-around time; they are rapidly produced and degraded. However, little is known about degradation and retention rates of PST analogs in marine waters. One in vitro study showed that several bacterial strains isolated from the digestive tract of blue mussels (*Mytilus edulis*) were able to fully degrade PSTs within 1–3 days (Donovan et al., 2008). Another study (Jones and Negri, 1997) demonstrated that PSTs could persist over 90 days in freshwater if no dilution occurred. Although these results are not directly applicable to natural marine environments, they do suggest that PSTs may remain in the water column for some time after the producing organisms, and with them the sxtA4 transcripts, have disappeared.

Results from the Ría de Pontevedra sample series support this hypothesis. The Spanish sample series had a much higher sampling density than the Norwegian sample series (one to two weeks vs. three to six weeks) and covered a distinct *Gymnodinium catenatum* bloom (Fig. 4, Blanco, 2011). Both toxin-testing methods were positive for all four sampling dates in October and November 2009, but *G. catenatum* cells and sxtA transcripts were only detected in the first three samples (Fig. 4). Since the two last samplings were only one week apart, it is likely that the PSTs detected in the last sample were remnants of the toxins produced by *G. catenatum* cells previously present in the water column. UPL-qPCR results and *Gymnodinium catenatum* cell counts corresponded well for the last three dates of the Spanish sample series; both showed an ~10 fold decrease between October 19th and November 9th and were below the detection limit on November 16th (Fig. 4). However, the 10-fold increase in cell-numbers between October 13th and 19th was not reflected in the

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**Fig. 4.** Comparison of A: cell counts, B: toxins, and C: sxtA4-transcript number determined by UPL-based qPCR during a *Gymnodinium catenatum* bloom at a sampling site close to Vigo, Spain. For the ELISA, only the 1:5 diluted replicate is shown (see Section 2) as all undiluted replicates were saturated.
UPL qPCR results. Instead, similar sxtA4 transcript levels were obtained for both dates. It is possible that the RNA extraction reaction or cDNA synthesis reactions were saturated by the large number of cells present in the sample, as previously observed in a qPCR assay of the silicoflagellate *Pseudoachatonella* spp. (Dittami et al., 2013b). An alternative hypothesis is that sxtA expression was elevated at the beginning of the sample series. Changes in per cell PST production have previously been reported depending on growth phase or life cycle (Taroncher-Oldenburg et al., 1997), but so far it is not known if and to what extent these changes correlate to changes in sxtA expression.

5. Conclusion

The UPL-qPCR assay developed in this study is a rapid and sensitive molecular method to detect sxtA4 transcripts from *Alexandrium* spp. and *Gymnodinium catenatum* in complex environmental samples. With the exception of samples taken shortly after a toxic bloom, where toxins seem to persist in the water column longer than cells or sxtA transcripts, a good correlation was detected between the presence of PSTs and sxtA4 mRNA transcripts even for low numbers of toxin-producing cells. Therefore, our qPCR assay is suitable for the early detection of toxic events, but similarly to cell counts or microarrays, cannot be used to determine when shellfish farms can be reopened after a toxic event.

One important advantage of this sxtA-specific assay over available ribosomal gene-based assays is that it detects a highly conserved region of a transcript essential for PST synthesis rather than genus-, species-, or ribotype-specific sequences. The ribosomal regions are more variable and the risk of missing a strain or species with an unknown sequence is higher. Besides, ribosomal sequences are not directly related to toxin synthesis. On the other hand, since this assay detects sxtA4 transcripts across species and genus borders, one limitation is that it does not allow us to determine, which species the sxtA4 transcripts came from, and whether they originated from the same species throughout the study period, from different species, or a mixture of species. This is also a constraint for the quantification of cell concentrations based on our sxtA4 assay, as different species, here *Alexandrium minutum* and *Alexandrium fundyense*, seem to have different sxtA4 expression levels per cell. Since these initial results need to be further investigated, it is crucial to expand our current understanding of PST genes and transcripts in dinoflagellates. Are PST genes actually transcriptionally regulated, or do they belong to the majority of dinoflagellate genes that are not? Does a higher number of genes or transcripts present in the environment actually translate into higher PST synthesis? Does the PST gene or transcript number per cell vary between species or even different strains of the same species? Or is the species-specific effect observed during this study only due to methodological artifacts such as different amplification efficiencies in the two species, possibly caused by the presence of different sequence variants? What is the PST gene and transcript sequence variation in different species and strains?

These questions need to be thoroughly explored before we can judge if PST gene- or transcript-based molecular assays can be used quantitatively, or if they are “only” a fast and reliable method to detect potentially PST-producing dinoflagellates in environmental samples. Nevertheless, using the sxtA4 qPCR in conjunction with group-specific assays, especially multiplex assays such as the ALEX chip (Gescher et al., 2008) or the MIDTAL microarray (Kegel et al., 2012) can greatly enhance our understanding of the ecology and distribution of PST-producing dinoflagellates and has a great potential to reduce the time, effort, and investment spent on coastal PST monitoring.

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