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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 *sxtA4* 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.

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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; Mccauley et al., 2009). Further, different PST-producing strains may produce dissimilar amounts and isoforms 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 (Stüken 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 (Stüken 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 (Stüken 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; Stüken et al., 2011; Orr et al., 2013; Suikkanen 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; Stüken 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 *stxA4* transcripts in samples where PST-producing algae were not dominant.

We therefore sought to develop a new, more sensitive *stxA*-assay that could be an early warning system for dinoflagellate PSTs and PST producers across different genera. Our new assay was able to detect *stxA4* of different species at low concentrations and in mixed assemblies and was applied to the field samples from Oslofjorden, and another series from Ría 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

Table 1
Cultured strains used to test the specificity of the new *sxtA4* qPCR primers on cDNA. + (presence) and − (absence) indicate if *sxtA4* has been detected with qPCR primers sxt072 and 073 (this study) or with *sxtA4* primers sxt007 and sxt008 (previous studies).

Species	Strain	stx4A qPCR (this study)	sxtA4 PCR (prev. studies)	Reference
Adenoides eludens	CCMP1819	−	−	Orr et al. (2012)
Alexandrium fundyense	CCMP1719	+	+	Stüken et al. (2011)
Alexandrium insuetum	CCMP2082	−	−	Orr et al. (2013)
Alexandrium minutum	CCMP113	+	+	Stüken et al. (2011)
Azadinium spinosum	RCC2538	−	−	Orr et al. (2012)
Ceratium longipes	CCMP1770	−	−	Orr et al. (2012)
Gymnodinium catenatum[a]	CCMP1937	+	+	Orr et al. (2013)
Heterocapsa triquetra[b]	RCC2540	−	−	Orr et al. (2012)
Lepidodinium chlorophorum	RCC2537	−	−	Orr et al. (2012)
Pentapharsodinium dalei	SCCAP K-1100	−	−	Orr et al. (2012)
Polarella glacialis	CCMP2088	−	−	Orr et al. (2012)
Scrippsiella trochoideae	BS-46	−	−	Orr et al. (2012)
Thecadinium kofoidii	SCCAP K-1504	−	−	Orr et al. (2012)

−, not detected; + detected.
[a] Total RNA kindly provided by Johannes A. Hagström, Linnaeus University, Sweden.
[b] GDNA used instead of cDNA, because the strain died before RNA was extracted. GDNA isolated with the ChargeSwitch® gDNA Plant Kit (Invitrogen) according to (Orr et al., 2012).

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