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Sandwich hybridization probes for the detection of *Pseudo-nitzschia* (Bacillariophyceae) species: An update to existing probes and a description of new probes

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ABSTRACT

New sandwich hybridization assay (SHA) probes for detecting *Pseudo-nitzschia* species (*P. arenysensis*, *P. fraudulenta*, *P. hasleana*, *P. pungens*) are presented, along with updated cross-reactivity information on historical probes (SHA and FISH; fluorescence in situ hybridization) targeting *P. australis* and *P. multiseries*. *Pseudo-nitzschia* species are a cosmopolitan group of diatoms that produce varying levels of domoic acid (DA), a neurotoxin that can accumulate in finfish and shellfish and transfer throughout the food web. Consumption of infected food sources can lead to illness in humans (amnesic shellfish poisoning; ASP) and marine wildlife (domoic acid poisoning; DAP). The threat of human illness, along with economic loss from fishery closures has resulted in the implementation of monitoring protocols and intensive ecological studies. SHA probes have been instrumental in some of these efforts, as the technique performs well in complex heterogeneous sample matrices and has been adapted to benchtop and deployable (Environmental Sample Processor) platforms. The expanded probe set will enhance future efforts towards understanding spatial, temporal and successional patterns in species during bloom and non-bloom periods.

1. Introduction

A majority of the world's coastlines and inland water sources experience periodic proliferations of phytoplankton, some of which lead to harmful or noxious algal bloom (HAB) events (e.g. Anderson et al., 2012; Trainer et al., 2012). Blooms can vary dramatically in time and space, from relatively small innocuous events with minor ecosystem disruptions (e.g. Gárate-Lizárraga et al., 2007; García-Mendoza et al.,2009), to large, recurring episodes in which wildlife, local economies and human health can be dramatically threatened (e.g. Scholin et al., 2000; Trainer and Suddleson, 2005). Periods of bloom initiation, persistence and decline are complicated and subject to anthropogenic influences (e.g. eutrophication, global changes in water temperatures, shifts in pH) and natural forcings (e.g. advection, upwelling, stratification, swimming behavior, grazing, parasitism). Given this complexity, it is desirable to develop rapid and inexpensive detection methods that provide a high level of specificity and sensitivity, particularly for use in monitoring and industry applications (e.g. Rhodes et al., 2013). The sandwich hybridization assay (SHA) satisfies these requirements (Scholin et al., 1997; Miller and Scholin, 1998), and is re-visited in this study to expand the historical probe set available for the potentially noxious HAB genera, *Pseudo-nitzschia* (Greenfield et al., 2008).

The *Pseudo-nitzschia* clade is comprised of approximately forty species described to date. Cells are characteristically pennate (bilaterally symmetrical; with two slightly unequal halves), silicified, needle-like, and fall into one of two overall size categories (Hasle, 1965; Hasle and Syvertsen, 1997): the larger 'seriata' sized cells (with a valve width of >3 μm) and the smaller 'delicatissima' sized cells (with a valve width <3 μm). Cells form a step chain pattern that can range from just two to dozens of cells in length. While some species are distinguishable with light microscopy, the majority can only be delineated into one of the two size groups, with species-level identification requiring

Abbreviations: SHA, sandwich hybridization assay; DA, domoic acid; ESP, environmental sample processor.

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scanning electron microscopy, genetics, or a combination of the two.

To date, twelve species of *Pseudo-nitzschia* have been documented to produce domoic acid (DA; Trainer et al., 2012; Lelong et al., 2012; Teng et al., 2014), a neurotoxin that can accumulate in finfish, shellfish and other invertebrates, and be transferred throughout the food web (e.g. Lefebvre et al., 2002; Kvitek et al., 2008; Trainer et al., 2012). Consumption of aquatic species capable of concentrating toxic *Pseudo-nitzschia* can lead to illness in humans (amnesic shellfish poisoning; Bates et al., 1989; Perl et al., 1990; Todd, 1993) and marine wildlife (domoic acid poisoning; Work et al., 1993; Scholin et al., 2000). Trainer et al. (2012) present a comprehensive review on global distribution of the more damaging *Pseudo-nitzschia* blooms and how they vary in size, duration, toxicity, response to anthropogenic and natural influences, and resulting economic and environmental impacts. It is notable that some regions experience little impact from *Pseudo-nitzschia*, despite the presence of species capable of DA production (see Trainer et al., 2012 and references therein). In stark contrast, some locations (e.g. the California coast) experience recurrent blooms of *Pseudo-nitzschia* that vary drastically in size, duration, toxicity and ecological impacts.

Sandwich hybridization assays have been successfully used for detecting a variety of species, including *Pseudo-nitzschia*, in complex environmental samples via a benchtop platform and a deployable *in situ* instrument (Environmental Sample Processor; ESP; Scholin et al., 1996, 1997; Goffredi et al., 2006; Greenfield et al., 2006, 2008; Roman et al., 2007). The method is homogenate-based and takes advantage of large pools of cellular ribosomal RNA transcripts (Cangelosi et al., 1997), thereby eliminating the need for a potentially biased pre-amplification step. SHA can quantify molecular signatures from multiple species in non-purified lysates across a broad range of sample matrices (e.g. Babin et al., 2005; Goffredi et al., 2006; Greenfield et al., 2006; Metfies et al., 2006; Haywood et al., 2007; Harvey et al., 2013). This assay type has routinely proven comparable to traditional methodologies (e.g. Scholin et al., 1997; Miller and Scholin, 1998; Anderson et al., 2005; Goffredi et al., 2006; Doll et al., 2014).

Several reasons prompted a re-assessment and expansion of current *Pseudo-nitzschia* SHA probes. First, they were developed in the 1990's, and since that time have demonstrated cross-reactivity and varying results (e.g. Cusack et al., 2004; Parsons et al., 1999). With more than one species of *Pseudo-nitzschia* being described per year, some of the variability can be attributed to species that were unknown at the time the probes were developed. Second, while *P. australis* and *P. multiseries* (Hasle) Hasle have historically been implicated in toxic blooms, at least ten other *Pseudo-nitzschia* species can produce DA (one hypothesis is that all species are capable of producing DA under the right conditions; Parsons et al., 1999; Wells et al., 2005). Although laboratory studies typically demonstrate low DA production in these species, there are cases where they can form harmful blooms (e.g. *P. cuspidata* [Hasle] Hasle; Trainer et al., 2009). Indeed, there have been worldwide shifts in *Pseudo-nitzschia* species that depict rising trends in several of these 'low-level' DA producers on seasonal, decadal and centennial time scales (for detailed review see Lelong et al., 2012 and references therein). Third, ongoing successful modeling efforts for *Pseudo-nitzschia* would benefit from high-resolution diversity data for cell abundances (e.g. Anderson et al., 2011). To understand if and how diversity coincides with annual fluctuations of abundances and DA events, an expansion of the current probe set was needed.

This study took advantage of field cruises over a three-year period in two California *Pseudo-nitzschia* 'hotspots' to build a large repository of isolates to use for probe design and/or validation. We re-assessed the original probes for *P. australis* and *P. multiseries* (Scholin et al., 1999), revised probes for two cosmopolitan low domoic acid producers (*P. fraudulenta* and *P. pungens*), and developed probes for two more recently described species (*P. arenysensis* and *P. hasleana*). While isolates were limited in biogeography, these probe sets provide a framework for further in silico and in situ assessments for broader applicability. Furthermore, while this work focuses on SHA probes, the results can be used to inform and guide use of FISH probes that have demonstrated cross-reactivity and labeling variability (Miller and Scholin 1996, 1998; Parsons et al., 1999; Cusack, 2002; Lundholm et al., 2006; Turrell et al., 2008).

The objective of this contribution was to 1) review and update applicability of historical SHA probes (including companion fluorescence in situ hybridization probes) designed for two important *Pseudo-nitzschia* species: *P. australis* and *P. multiseries*; 2) expand the available SHA probe set to target additional species in this genus that are found along the U.S. West Coast; and 3) explore intra-species genetic variation for each probe target.

2. Methods

2.1. Pseudo-nitzschia *strains*

2.1.1. Culture establishment

Cultures were established from single *Pseudo-nitzschia* chains collected from CTD casts and AUV sampling efforts (Zhang et al., 2012) during research cruises in Monterey Bay (central California, USA) and the San Pedro Shelf area (southern California, USA), as well as from net tows collected at Monterey and Santa Cruz wharves (California, USA; Supplemental Table S1). Single chain isolations were washed with media, and then cultured and maintained in 0.2 μm-filtered *f*/2 medium (Guillard, 1975) made with Monterey Bay water (32–34 $^{0/00}$) amended with 106 μM $NaSiO_3$. Growth was supported at 15 °C under a 13:11 h light:dark photoperiod in an environmental chamber illuminated at 142 umol (photons) $m^{-2}\,s^{-1}$ with standard F40 cool white fluorescent tubes.

2.1.2. Culture identification

DNA was extracted from pelleted cells using the DNeasy® Blood and Tissue kit following the manufacturer's protocol (Qiagen, Valencia, CA, USA). The D1-D3 domain of the large ribosomal subunit was targeted for PCR and sequencing using primers D1R-For (Scholin et al., 1994) and D3B-Rev (Nunn et al., 1996) synthesized by Integrated DNA Technologies (Coralville, IA). PCR mixtures contained 1X AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Foster City CA), 0.8 uM of each primer, 1 ul DNA template and molecular biology grade water (MBG; Sigma, St. Louis, MO) to a final volume of 25 ul. The following cycling protocol was used on a GeneAmp® 9700 (Applied Biosystems, Foster City CA): 95 °C for 5 min; 45 cycles of 95 °C 15 s, 58 °C 15 s, 72 °C 1 min; and a final extension of 72 °C for 7 min. PCR products were precipitated using polyethylene glycol (Morgan and Soltis, 1995; 20% w/v polyethylene glycol, mw 8000, 2.5 M NaCl solution), incubated for 20 min, and pelleted by centrifugation at >12,000*g* for 15 min. Pellets were washed with 70% ethanol and allowed to air dry, after which they were re-suspended in 10 ul MBG. PCR products (~800 bp) were prepared for bi-directional sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City CA) in the following 10 ul reactions: 0.5 ul BigDye®, 1X sequencing buffer, 0.08 uM primer, 2 ul PCR product and MBG water to final volume. The following sequence cycling protocol was used on a 3500xl Genetic Analyzer (Applied Biosystems, Foster City CA): 35 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. PCR products were purified using ethanol/EDTA/sodium acetate precipitation, re-suspended in formamide,