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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Abbreviations: SHA (sandwich hybridization assay), DA (domoic acid), ESP (Environmental Sample Processor)

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 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 et al., 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 et al., 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 1). 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^{o}C under a 13:11 hour 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 sec, 58°C 15 sec, 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 sec, 50°C for 5 sec, and 60°C for 4 min. PCR products were purified using ethanol/EDTA/sodium acetate precipitation, re-suspended in formamide, and analyzed on an ABI 3100 capillary sequencer (Perkin Elmer/Applied Biosystems).

2.1.3 Cell enumeration and preservation

Cell counts were performed on an Axioplan2 (Zeiss, Thornwood, NY) using a Sedgewick-rafter counting chamber. For long-term preservation, 2-5 x 10^5 cells (averaged from triplicate counts) from cultures in exponential phase were low-vacuum (5 mmHg) filtered onto multiple (n=30-80 as permitted by cell densities) 25-mm diameter, 0.65 micron pore size Durapore® membrane filters (Millipore, Cork, Ireland). Filters were transferred to 2 mL polypropylene cryovials (Nalgene Nunc International,

Rochester, NY, USA) with sample side facing inward, snap frozen and stored in liquid nitrogen for downstream sandwich hybridization assays. Supporting information for strains used in this study is outlined in Supplementary Table 1.

2.2 Sandwich hybridization assays

2.2.1 Development of capture probes

Table 1 outlines the sandwich hybridization probes assessed in this study. To design new capture probes, all available sequences for the D1-D3 variable region of the LSU rRNA for *Pseudo-nitzschia* species were downloaded from GenBank and aligned using the program Geneious® 9.0.2 (Kearse et al., 2012). Probes were chosen based on 1) specificity (T/G mismatches were ignored, as those are not viable in the presence of the guanidine thiocyanate lysis buffer; Scholin et al., 1996; Van Ness and Chen, 1991), 2) a location within 200 bp of the signal probe (PSDS), and 3) having less than 70% similarity to the signal probe. Once a candidate sequence was determined, OligoTech primer design software (Oligos Etc., Eugene OR) was used to ensure that 1) G+C content was between 40% and 60%, 2) stability of any secondary structures was less than 34°C, and 3) stability of any homodimers was less than 17°C. Capture probes meeting these criteria were synthesized with a biotin and three C9 carbon spacer motif on either the 3' or 5' end of the sequence (Oligos Etc., Eugene, OR or Integrated DNA Technologies, Coralville, IA). Lyophilized stocks were re-suspended in RNase-free MBG water, quantified on a Nanodrop® ND-1000 (Thermo Scientific, Wilmington, DE, USA), aliquoted in 50 µg

increments into 0.5 mL eppendorf tubes, vacuum desiccated (Thermo Savant DNA110 Speedvac; Thermo Electron Corp., Somerset, NJ, USA) and stored in -80^{o}C. Working stocks were reconstituted in 1X TE and probe solutions (400 ng/ul) were made in the presence of streptavidin in a phosphate buffer according to a proprietary formula (Saigene Corporation, Seattle WA). Capture solutions were stored at 4^{o}C.

2.2.2. Preparation of lysates

Two milliliters of lysis buffer (3 M GuSCN, 50 mM Tris, 15 mM EDTA, 2% Sarkosyl and 0.2% SDS (v/v), at pH 8.9) were used to lyse cells archived on 0.65 micron filters as described above. One cryovial was used for specificity testing, while three tubes were pooled for each point of a standard curve. After the addition of lysis buffer, cryovials were incubated at 85^{o}C for 5 min, with one inversion of the tubes at approximately 2.5 min. Lysates were clarified through a sterile, disposable polypropylene syringe fitted with a 0.22 micron Durapore syringe filter. Lysates used for specificity testing of the capture probes were not diluted, as the goal was to challenge the probes with non-target species to determine potential for cross-reactivity. Subsequent 'dose response' curves were established for species demonstrating cross-reactivity during these initial high cell number challenges. Lysates for 'dose response' and standard curves were diluted with lysis buffer to determine range of cross-reactivity and assay linear range, respectively. Lysates were split between plate-based benchtop SHA and array-based SHA on the ESP.

2.2.3 Benchtop sandwich hybridization assays

Benchtop SHA's were carried out on a semi-automated 96-well plate platform fitted with a heated plate stage set at 29ºC (Saigene, Inc., Denver CO, USA). Comprehensive details for preparing and running SHA plates are outlined in Harvey (2014) and elsewhere (Scholin et al., 1999; Goffredi et al., 2006; Haywood et al., 2007; Marin and Scholin, 2010). Measurements in the 0.1 – 3 OD (450) range were considered within the limits of the assay. Duplicate negative controls (lysate only) were included on each plate, and coefficients of variation were used to quality control data within replicates on a plate as well as for assays between plates.

2.2.4 ESP sandwich hybridization assays

Lysates were also loaded onto the Environmental Sample Processor (ESP), an autonomous robotic device with deployment capabilities for *in situ* detection of marine organisms via SHA. A Protran® nitrocellulose membrane (Amersham, Germany) served as the solid support to which the capture probes were attached (detailed information for 'printing' probes can be found in Greenfield et al., 2006; for current study, a Scienion Piezo non-contact printer was used [Berlin, Germany]). Capture probe arrays were housed in titanium pucks and robotics were used to manipulate pucks by adding reagents in a sequential manner to form the 'sandwich' as outlined above (with the exception that the conjugate was prepared as a 1:1000 dilution). Comprehensive details for sample processing onboard the ESP are outlined elsewhere (Goffredi et al., 2006; Greenfield et al., 2006; Roman et al., 2007; Jones et al., 2008). Target molecules were detected via

chemiluminescence and abundances were determined by using the mean of spot intensities for each probe after background was subtracted from the average of four surrounding equal-sized regions and comparing against a species-specific standard curve (Greenfield et al., 2008). Images were acquired after a 40 s exposure with 2 x 2 CCD camera binning. Measurements in the 3,000 – 50,000 CCD range were considered within the limits of the assay. To minimize inter-array variation, array sets from the same batch print were utilized within each standard curve. Negative (lysate only) and positive controls (as outlined in Greenfield et al., 2006) were included for assessing data quality.

2.3 Fluorescence in situ hybridization

Complementary fluorescent in situ hybridization (FISH) probes for *P. australis* (auD1), *P. multiseries* (muD1), and *P. multiseries/P. pseudodelicatissima* (muD2) were previously developed alongside SHA probes (Miller and Scholin, 1996; 1998). Probes were synthesized with a fluorescein molecule on both ends of the oligo (Table 1; Oligos Etc., Eugene, OR, USA). For long time storage, lyophilized stocks were processed as described above. Working stocks were reconstituted in 250 µl 1X TE and stored at 4°C. Cross-reactivity was tested on representative strains of eleven non-target species (Supplementary Table 1), an expansion of results from original probe development. UniR and UniC were used as negative and positive controls, respectively (Miller and Scholin, 1996). The detailed procedure for FISH is outlined in Miller and Scholin (1996, 1998). Cells were viewed using a Zeis Axioplan2 microscope fitted with a fluorescein bandpass

filter set (excitation 465–495 nm; emission 515–555 nm) and a 50 W light source. Images were acquired with an Olympus DP71 camera.

2.4 Performance of SHA and FISH for *P. australis* and *P. multiseries* at bloom threshold cell concentrations.

Fifty thousand cells L^{-1} is considered the threshold for *Pseudo-nitzschia* blooms in monitoring programs (Andersen, 1996; Ajani et al., 2013a; Rhodes et al., 2013; Taylor et al., 2013). In order to determine the reliability of the SHA and FISH assays for *P. australis* and *P. multiseries* at this cell concentration, replicate samples were prepared. One culture for each species (Supplementary Table 1) was counted during exponential phase and diluted to 50,000 cells L^{-1} in fifteen separate 1 liter bottles containing 0.2 µm filtered Monterey Bay water. After gentle mixing, 1 ml was added to replicate tubes prepared for FISH (n=15 for auD1, n=6 for muD1, n=6 for muD2). The remaining volume for each bottle was filtered onto a 0.65 micron pore size Durapore® membrane filter, snap frozen and stored in liquid nitrogen until SHA (benchtop and ESP).

3. Results

3.1 Capture Probe Design

Numerous isolates of *Pseudo-nitzschia* species collected over a three-year period were sequenced in order to predict intra-species variation (Table 2). Representative sequences

were added to alignments containing all available *Pseudo-nitzschia* sequence data from GenBank. Extensive *in silico* analyses were used to identify candidate sites for capture probe development that maximized specificity and met design criteria described above. Figure 1 depicts the position of historical probes (auD1, muD1, muD2), probes that were designed during this study (ary1, frD2, has2, muD3, pung1) and the PSDS *Pseudo-nitzschia* spp. signal probe. While a polymorphic site was uncovered in the set of *P. australis* and *P. fraudulenta* isolates, neither the auD1 probe nor the frD2 probes were located in that region.

3.2 Specificity testing

Capture probes were first tested against a standard curve for the target species to determine dynamic range of the assay (outlined below). Successful probes were challenged with a panel of non-target species to determine potential cross-reactivity. Table 3 provides reactivity (OD 450) values from benchtop SHA obtained for each probe against the non-target species. Species demonstrating potential cross-reactivity (empirically defined by an OD of 0.250 or greater) were further assessed with dose response curves as outlined below.

3.3 Performance of capture probes

3.3.1 *P. arenysensis* (ary1)

A probe for *P. arenysensis* [Quijano-Scheggia, Garcés, Lundholm] was designed after this species showed significant cross-reactivity with the auD1 probe and attempts to re-design the auD1 probe failed. Figure 2 outlines dynamic range of the ary1 assay on both platforms, along with a benchtop SHA dose response curve for *P. australis* cells. On the benchtop platform, the range of *P. australis* cell concentrations needed for cross-reactivity exceeds the dynamic range for detection with the auD1 probe, so in practice the volume filtered would be adjusted to accommodate the auD1 assay (Figure 3a). On the ESP platform, care should be taken when interpreting signal from ary1 when *P. australis* cells are above approximately 64,000 cells L^{-1}, as cell concentrations tested between 75,000 – 200,000 cells L^{-1} did have an impact (data not shown).

3.3.2 *P. australis* (auD1)

The auD1 probe showed significant cross-reactivity with *P. arenysensis* and *P. multistriata* (this latter result agrees with earlier findings from Rhodes et al., 2000 and Orsini et al., 2002). Multiple attempts were made to design a new *P. australis* capture probe, however those probes (n=4) proved unsuccessful in benchtop testing due to poor signal (potentially spanning a break in the rRNA transcript), no improvement in cross-reactivity, or a shift in cross-reactivity to other non-target species. Figure 3a outlines dynamic range of the auD1 assay, plotted with dose response curves for *P. multistriata* (Takano) Takano (the probe is a perfect match to this species). Figures 3b and 3c depict SHA dose response curves for *P. arenysensis* cells (ary1 probe included for comparison). The auD1 probe has one viable mismatch with this species, however it is located at the 5’

end of the transcript (a second mismatch is T/G, which is not viable in the presence of GuSCN [Scholin et al., 1996; Van Ness and Chen, 1991], a component of the lysis buffer). *P. seriata* (Cleve), the most closely related species to *P. australis*, contains one mismatch in the center of the auD1 probe region, however the SHA assay does not appreciably cross-react (Table 3 and data not shown).

3.3.3 *P. fraudulenta* (frD2)

Figure 4 depicts dynamic range of the assay for *P. fraudulenta* (Cleve) Hasle, along with a dose response curve on the ESP platform for *P. multiseries* cells. There is a one base pair mismatch in the center of the probe (C/A) for *P. multiseries* as well as *P. americana* (Hasle), the latter of which did not demonstrate cross-reactivity in initial testing (Table 3). A dose response curve was also performed on *P. australis*, however at 62,500 cells L^{-1}, CCD counts remained below background levels. When challenged with higher concentrations of up to 200,000 cells L^{-1} of *P. australis* cells, OD 450 remained below 0.2. On the ESP platform, care should be taken when interpreting signal from frD2 when *P. australis* cells are above approximately 64,000 cells L^{-1}, as cell concentrations between 75,000 – 200,000 cells L^{-1} did have an impact (data not shown). Of note, frD2 is located approximately 250 bp upstream of the original *P.fraudulenta* probe (frD1) designed for FISH (Miller and Scholin 1996). In silico analyses suggest that a FISH probe based on the frD2 sequence would potentially overcome cross-reactivity that has been reported for the frD1 FISH probe (Miller and Scholin, 1996; Parsons et al., 1999; Lundholm et al., 2006).

3.3.4 *P. hasleana* (has2)

P. hasleana [Lundholm] was found to cross-react with the muD2 probe, which was designed to detect *P. multiseries* and *P. pseudodelicatissima* (Miller and Scholin, 1998; Scholin et al., 1999). This cross-reactivity was not surprising, as *P. hasleana* was recently recognized as part of the *P. pseudodelicatissima* complex (Lundholm et al., 2012). Figure 5 depicts dynamic range of the has2 assay which did not demonstrate significant cross-reactivity with other species tested (Table 3).

3.3.5 *P. multiseries* (muD1, muD2, muD3)

The muD1 probe for *P. multiseries* has always demonstrated a weaker signal compared to muD2 (Miller and Scholin, 1998; Scholin et al., 1999; Greenfield et al., 2008). During this study, the highest cell concentration tested (62,500 cells L^{-1}) resulted in an average OD of 0.120 on the benchtop platform and average CCD count of 14,000 on the ESP platform (data not shown). A new probe (muD3) was designed to address cross-reactivity of muD2 with *P. hasleana*. Figures 6a and 6b depict performance of both probes, and while the signal from muD2 was stronger than muD3 on the benchtop platform, the two assays were comparable on the ESP. The signal from cross-reactivity with *P. hasleana* is reduced with muD3 on both platforms (Figures 7a and 7b). For analysis purposes, this probe set can be used in conjunction with the has2 assay to assess presence of both species.

3.3.6 *P. pungens* (pung1)

Figure 8 outlines the linear range of the probe designed for *P. pungens* [Grunow ex Cleve] and includes a dose response curve for *P. seriata*. Four additional species were examined in greater detail after initial testing showed potential cross-reactivity (Table 3): 1) Initial screening of *P. australis* cells yielded an OD of 0.745, but a subsequent dose response curve resulted in an average OD of 0.141, and a CCD count below background (up to 64,000 cells L^{-1} tested); 2) *P.* sp. 'B' yielded an OD of 0.776, but a second isolate had an OD of 0.378. [This discrepancy may be attributable to a difference in cell health, as two different strains were used. Cultures were lost before a full dose response curve could be initiated.]; 3) *P. hasleana* yielded an OD of 1.276, however a dose response curve on the benchtop platform (up to 375,000 cells L^{-1} tested) resulted in a maximum OD of 0.327. A dose response curve on the ESP (up to 60,000 cells L^{-1} tested) resulted in an average CCD below background; and 4) *P. multistriata* yielded an OD of 1.106, however a dose response curve (15,625 to 125,000 cells L^{1} tested) yielded an average OD of 0.138, and a CCD value below background.

3.4 Performance of FISH probes

Table 4 depicts results for all species from this study tested with previously designed FISH probes for *P. australis* (auD1), *P. multiseries* (muD1) and *P. multiseries/pseudodelicatissima* (muD2). Figure 9 outlines cross-reactivity of the auD1 probe with *P. arenysensis* (six strains tested; panel B). Cross-reactivity was also observed

for *P. multistriata* (one strain tested; data not shown). Figure 10 outlines cross-reactivity of the muD2 probe with *P. hasleana* (six strains tested, panel B).

3.5 Performance of SHA and FISH for *P. australis* and *P. multiseries* at bloom threshold cell concentrations

Replicate samples were used to compare performance of benchtop SHA, ESP SHA and FISH on samples containing 50,000 cell L^{-1} of *P. australis* or *P. multiseries*, the threshold considered for a bloom. Table 5 outlines results for each probe. The muD1 SHA probe has traditionally had a lower signal compared to muD2, and does not appear to be feasible for detecting *P. multiseries* at threshold levels.

4. Discussion

4.1 Sandwich hybridization assays

The sandwich hybridization assay (SHA) is a rapid, robust method for determining abundance of multiple target organisms in lysates generated from a heterogeneous environmental sample (e.g. Babin et al., 2005; Goffredi et al., 2006; Greenfield et al., 2006; Metfies et al., 2006; Haywood et al., 2007). The benchtop platform is amenable to ship operations, where near real-time results (filtration time + one hour) can be used to guide further sampling efforts. SHA probe arrays have also been utilized on moored ESP platforms as a way to sample on greater temporal scales (e.g. Greenfield et al., 2008;

Doucette et al., 2009). In both situations, results can be used to adjust the volume filtered to accommodate rare or bloom-level targets in subsequent samples. Both platforms can be combined with routinely collected contextual data (e.g. salinity, temperature, chlorophyll) for a powerful assessment of organismal population structure.

Use of the SHA method is beneficial within the framework of evaluating population trends over time. For management applications, this relates to monitoring cell concentrations as they shift through bloom initiation, persistence and termination. One caveat for interpreting results is the effects that cell physiology can have on probe reactivity. Miller et al., (2004) demonstrated that the labeling of *P. multiseries* with both the SHA and FISH probes was decreased after cells were grown under nitrate deplete conditions, however there was an increase in labeling intensity with the SHA probe when cells were grown under silicate limitation. Of note, Main et al. also observed a decrease in SHA probe signal related to decreased rRNA abundance for *Heterosigma akashiwo* grown under nitrogen stress (Main et al., 2014). While nutrient conditions are often not measured at the time of sample analyses, the overall trend in populations can still be monitored using these methods. Miller et al., (2004) also point to other instances when SHA and FISH probe results can differ in relation to the physiological status of *Pseudo-nitzschia* cells. Older fragile cells may not survive the processing steps for FISH, leading to an underestimate of abundance. Likewise, SHA can overestimate cell concentrations compared to FISH and microscopy when cells have recently been ingested, in which case genetic material but not whole cells are available for detection (Miller et al., 2004). Main et al. (2014) documented higher SHA probe signal in the early growth stages of *H. akashiwo*, as did Haywood et al. (2007) for *Karenia brevis*, but lower signal during the

light cycle. Other organisms can have their own unique set of caveats, such as the variability in rRNA amounts that Goffredi et al. (2006) attributed to developmental stage of target barnacle species. SHA and FISH can both be challenging when targets are rare, however both are amenable to concentration of cells during the filtration step.

One of the keys to maintaining assay specificity is to continue to assess cross-reactivity with untested species, newly described species, and local isolates. As with any detection method, ongoing groundtruthing efforts (e.g. SEM [Bowers et al., 2016], FISH, PCR) should be incorporated where feasible to further enhance these efforts. This contribution tested previous SHA probes developed to detect *P. australis*, *P. multiseries* and a combination of *P. multiseries* and *P. pseudodelicatissima*. New probes were validated to overcome cross-reactivity and to detect additional *Pseudo-nitzschia* species of interest. Although probe assessments were based on isolates from the California coast, in silico analyses with deposited GenBank sequences, as described below, are suggestive of much broader geographical applications.

4.2 *Pseudo-nitzschia* species-specific sandwich hybridization assays

4.2.1 *P. arenysensis* (ary1)

To our knowledge, this is the first documented report of *P. arenysensis* from the US west coast. The strains in this study were isolated from southern California, however subsequent to this work it was also isolated from Monterey Bay (A. Woods, unpubl. data). This expands the known geographic range from the Atlantic (France), Gulf of

Naples, Gulf of Mexico, and the Mediterranean (Spain), (Quijano-Scheggia et al., 2009; Orive et al., 2010; Lundholm et al., 2012). Lelong et al. (2012) classified this species as non-cosmopolitan, and no blooms or associated DA production have been described thus far. The California isolates had no intra-species diversity and matched two sequences for *P. arenysensis* from the Gulf of Naples (Amato et al., 2007; Ruggiero et al., 2015). It is a recently described species (Quijano-Scheggia et al., 2009), deriving from the *P. delicatissima* complex (Orsini et al., 2004; Lundholm et al., 2006; Amato et al., 2007; Kaczmarska et al., 2008). Cross-reactivity with the auD1 probes (SHA and FISH) for *P. australis*, a common bloom former in US west coast waters, could not be overcome by a re-design of the auD1 probe. Therefore, a new SHA probe, designated ary1, was developed to use in conjunction with results from the auD1 probe. Although a new FISH probe was not realized, labeled *P. arenysensis* cells can often be distinguished from *P. australis* under epifluorescence based on cell size. The linear range of the ary1 assay is above bloom threshold levels (approximately 50,000 cells L^{-1}), although earlier benchtop SHA extended the lower limit of detection to approximately 16,000 cells L^{-1} using a different culture (data not shown). This lower value may be attributable to different factors (e.g. healthier culture used). The other species tested in this study did not appreciably cross-react with the ary1 probe, however results should take into consideration *P. australis* cell concentrations present in a sample. In practice, filtration volume adjustments to maintain the number of *P. australis* cells within the working range of the auD1 probe will limit impacts on cross-reactivity with ary1.

4.2.2 *P. australis* (auD1)

P. australis is a cosmopolitan species that has been documented numerous times from all continents except Asia and Antarctica (Trainer et al., 2012), with the largest, most persistent and highest toxicity blooms occurring along the US west coast. The most destructive of these events have resulted in large marine mammal stranding and mortality events (Fritz et al., 1992; Work et al., 1993; Scholin et al., 2000), as well as large economic losses related to fishery closures (e.g. Trainer et al., 2007). Several other regions have experienced closure of molluscan shellfish harvesting due to the presence of *P. australis* and DA (reviewed in Trainer et al., 2012). *P. australis* sequences from this study were identical to GenBank isolates from Scottish, French, New Zealand and west coast waters (WA and CA). Of note, one sequence from Monterey Bay (isolated in 1991), shares the same polymorphic site as several isolates from this study. Isolates with and without the polymorphic site did not differ in toxin per cell (data not shown). The *P. australis* auD1 SHA probe demonstrates a linear range within cell concentrations preceding bloom levels. Two species isolated during this study were confirmed to cross-react with the auD1 probes for both SHA and FISH (*P. multistriata* and *P. arenysensis*). The range of *P. multistriata* cell concentrations detected with the SHA probe are well within environmentally relevant numbers, however the scale of change in signal on both benchtop and ESP SHA is small within this dynamic range. Labeling with the FISH probe, as well as light microscopy or scanning electron microscopy (SEM) can determine if *P. multistriata* cells are present, as the ends of the cells have a slight curve. Interestingly, *P. arenysensis* cells are more robustly detected with the auD1 probe versus the ary1 probe on the benchtop platform. Nevertheless, the dynamic range of cell

concentrations is the same for both probes and therefore ary1 probe results can be used in analyzing auD1 results. While the auD1 FISH probe also detects *P. arenysensis*, labeled cells are thinner when compared to *P. australis* cells.

4.2.3 *P. fraudulenta* (frD2)

P. fraudulenta has also been described as a cosmopolitan species, having been identified on all continents except Antarctica, with ongoing expansion of geographic distribution (e.g. Stonik et al., 2008; Moschandreou et al., 2010). The isolates from this study match sequences (taking into consideration the two possible nucleotides for the polymorphic site) available in GenBank from around the globe (North America, Europe and Africa). There is some genetic diversity among additional sequences identified as *P. fraudulenta* in GenBank which may hint at cryptic diversity within this species.

P. fraudulenta can develop into bloom concentrations, as documented in the Gulf of California in 2006 (Gárate-Lizárraga et al., 2007). During that event, *P. fraudulenta* dominated the phytoplankton assemblage, reaching cell densities of 5 to 13 x 10^5 cells L^{-1} with associated DA levels of 24 to 52 ng on concentrated net tow filters. More recently, during a month long deployment in Monterey Bay, qualitative culturing efforts and downstream genetic fingerprinting techniques pointed to a *Pseudo-nitzschia* assemblage dominated by *P. fraudulenta* (Bowers et al., in prep.). While sampling in the southern part of the bay revealed cell concentrations for the 'seriata' size class (which includes *P. fraudulenta*) ranging from 15,000 to over 400,000 cells L^{-1}, FISH probes on samples from the north part of the bay collected on the same days did not detect *P. australis* or *P.*

multiseries. Furthermore, two deployed ESPs showed little to no cells present for these two species (the frD2 probe was not included on the arrays). DA levels in samples collected from various platforms were low to negative, with some episodic spikes that may or may not have been attributable to other *Pseudo-nitzschia* species present. Although *P. fraudulenta* isolates from this bloom were not tested for DA, toxic strains have been reported from Monterey Bay during other efforts (Wells et al., 2005; pers. obs.).

In addition to Monterey Bay, DA production by *P. fraudulenta* has been documented in isolates from New Zealand (Rhodes et al., 1998) and the Chesapeake Bay (Thessen et al., 2009). Low toxin levels were reported for these isolates, however a recent study by Tatters et al. (2012) used *P. fraudulenta* as a model species to relate a dramatic rise in cellular DA production with regards to ocean acidification. While numerous studies have shown toxicity to increase when laboratory strains of *Pseudo-nitzschia* experience silicate limitation, Tatters et al., found a synergistic effect in increased cellular DA levels with end-of-century projected CO_2 levels when *P. fraudulenta* strains were reared in acidified seawater. These findings suggest the potential for near-future shifts in toxin production, and the sensitivity of the frD2 probe will aid efforts to monitor this species in natural assemblages. Results utilizing this probe should be analyzed with respect to the probe set for *P. multiseries* in order to assess any influence on signal from this species. The frD2 SHA probe was designed more than two hundred base pairs away from the original FISH probe for *P. fraudulenta* (frD1; Miller and Scholin, 1996), which may serve as a feasible site for a new FISH probe that would overcome reported variability in labeling intensity (Turrell et al., 2008) and cross-reactivity (Miller and

Scholin, 1996; Miller and Scholin, 1998; Parsons et al., 1999; Cusack, 2002; Lundholm et al., 2006).

4.2.4 *P. hasleana* (has2)

A probe was designed for *P. hasleana* since this species was found to significantly cross-react with both the SHA and FISH muD2 probes. This cross-reactivity was not unexpected, as the muD2 probe was originally designed to detect *P. pseudodelicatissima* in addition to *P. multiseries* (Miller and Scholin, 1998; Scholin et al., 1999). *P. pseudodelicatissima* has since been recognized to be a complex of species that until recently included *P. hasleana* (Lundholm et al., 2012). The known distribution of this species has included Washington (Lundholm et al., 2012), Japan (Lundholm et al., 2012), Spain (Penna et al., 2007; Orive et al., 2010; Lundholm et al., 2012), Australia (Ajani et al., 2013b), and Italy (Ruggiero et al., 2015) however historical reports of other '*delicatissima*' size class species may have been *P. hasleana* (Lundholm et al., 2012). To our knowledge, this is the first documented report of this species in California. No blooms or toxicity have been reported to date. The sequences from this study did not exhibit any intraspecies variation and were a 100% match to the limited data available on GenBank. The sensitivity of the has2 probe will allow for detection of this species at background levels, and those results can be used to assess results from the muD2 and muD3 SHA probe set. Although a new FISH probe was not designed for *P. hasleana*, the thinner cell size is amenable to distinction from the larger *P. multiseries* cells.

4.2.5 *P. multiseries* (muD1/muD2/muD3)

P. multiseries is a cosmopolitan species documented from all continents except Antarctica (Trainer et al., 2012). This species was linked to the first amnesic shellfish poisoning (ASP) event in humans from consumption of blue mussels (*Mytilus edulis*) contaminated with DA (Bates et al., 1989). Subsequent to that event, several regions experienced closure of molluscan shellfish harvesting due to the presence of toxic *P. multiseries* (reviewed in Trainer et al., 2012). Those events sparked interest in examining current and historical records for phytoplankton identification, which in turn revealed a shift away from this species in favor of other *Pseudo-nitzschia* spp. (e.g. Jester et al., 2009; Lundholm et al., 2010; Lelong et al., 2012; G.J. Smith pers. comm.). This species has become a model choice for a variety of laboratory-based studies related to nutrient uptake, bacterial interactions, DA production, allelopathy, detailed molecular characterization, etc. (reviewed in Lelong et al., 2012 and Trainer et al., 2012). Recent elucidation of the *P. multiseries* transcriptome (project number 402006, JGI portal; Nordberg et al., 2014), associated plastid genome (Cao et al., 2016), mitochondrial genome (Yuan et al., 2016) and gene expression studies (e.g. Boissonneault et al., 2013; Di Dato et al., 2015) will help put experimental results into perspective and open new possibilities for understanding the genus as a whole.

P. multiseries sequences from this study did not exhibit any intra-species variation and were identical to all GenBank isolates deposited (Australia, Eastern Canada, Italy, Japan, South Africa, southeast Russia, US east and west coasts). All three SHA probes demonstrate a linear range within cell concentrations preceding bloom levels. One

species assessed in this study, *P. hasleana*, was found to cross-react with the muD2 probe (SHA and FISH) originally designed to detect *P. multiseries* and *P. pseudodelicatissima*. While the newly designed muD3 probe reduces the signal from *P. multiseries*, it also diminishes the impact from *P. hasleana* cells and analysis with this probe set should include results from the has2 probe to further define community structure. Although the muD2 FISH probe also detects *P. hasleana*, labeled cells be distinguishable from *P. multiseries* cell based on size. As more species are recognized within the *P. pseudodelicatissima* complex, cross-reactivity of muD2 and muD3 should be assessed.

4.2.6 *P. pungens* (pung1)

P. pungens is a cosmopolitan species documented from all continents except Antarctica (Trainer et al., 2012). Toxic isolates have been confirmed from New Zealand (Rhodes et al., 1996), Washington (Baugh et al., 2006) and California (Bates et al., 1998; Bowers et al., in prep.). Thus far, measurements of toxin production have been low, and no major blooms have been associated with impacts to marine health or economic loss. However, interesting global shifts have been observed in this species (e.g. Lundholm et al., 2010; Trainer et al., 2012 and references therein), and at least three geographic clades have been recognized based on higher resolution molecular and structural analyses (e.g. Evans et al., 2005; Adams et al., 2009; Castelyn et al., 2009, 2010; Lim et al., 2014). These studies have greatly expanded our understanding of phytoplankton population structure on a worldwide scale.

P. pungens sequences from this study did not exhibit any intra-species variation. Interestingly, they confirm a one nucleotide difference shared with two strains isolated from Monterey Bay in the mid-1990's that no other *P. pungens* sequences in GenBank possess. Beyond the one nucleotide difference, the isolates from this study were identical to strains from a broad geographic range (Australia, Malaysia, Portugal, South Africa, Turkey [Black Sea], US east and west coasts, Vietnam). The two original Monterey Bay *P. pungens* strains stand apart from this group, as they share two insertion/deletion sites. The pung1 probe does not span the single nucleotide difference nor the insertion-deletion sites, making it conducive to detecting global ribotypes reported to date. The SHA probe works across a linear range that is within cell concentrations preceding and well into bloom levels. Extensive testing of species that showed potential cross-reactivity revealed that *P. seriata* cells demonstrate the greatest impact to probe signal, particularly on the ESP platform. Although the other species had less of an influence, care should be taken while interpreting results when these other species are in high abundance. The pung1 SHA probe was designed in the same region as the original FISH probe for *P. pungens* (puD1; Miller and Scholin, 1996), however pung1 incorporates fourteen additional base pairs and could serve as a potential site for a new FISH probe that would overcome reported variability in labeling intensity (Miller and Scholin, 1998) and cross-reactivity (Cusack et al., 2002; Turrell et al., 2008).

4.3 Conclusions

As the number of *Pseudo-nitzschia* species and associated bloom events continues to persist worldwide, it is imperative to employ rapid methods of detection in order to fully understand population dynamics. Those results contribute to a better understanding of *Pseudo-nitzschia* ecology and allow stakeholders to take preventative actions (e.g. increase monitoring efforts, warn affected communities) in order to protect local economies and assess public health risks.

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Table 1. Summary of rRNA-targeted SHA probes (captures and signal) and fluorescence in situ hybridization (FISH) probes used in this study. Sequence symbols for mixed -mers in FISH probes were as follows: K [G or T], W [A or T], and M [A or C]. Capture probes were labeled with a motif of biotin and three C9 spacers at the 3' or 5' end. The signal probe was labeled with a motif of dioxigenin and one C9 spacer at both the 3' and 5' ends. The signal had an additional dig-C9 motif label located internally, denoted by *. FISH probes were labeled with fluorescein at both ends of the sequence. Tm's were determined by OligoTech primer software.

Probe type	**Probe name**	**Target organism**	**Sequence (5' – 3')**	**Tm (°C)**	**Reference**
SHA capture	auD1	*Pseudo-nitzschia australis*	AAATGACTCACTCCACCAGGCGG-(C9x3)-biotin	75.3	Scholin et al., 1999
	muD1	*P. multiseries*	AAATGACTCACTCTGCCAGG-(C9x3)-biotin	68.3	Scholin et al., 1999
	muD2	*P. multiseries/ pseudodelicatissima*	AGCCCACAGCGCCCAAGCCA-(C9x3)-biotin	76.5	Scholin et al., 1999
	ary1	*P. arenysensis*	biotin-(C9x3)-TTGACAACGACTCACTCCACCAG	73.5	this study
	frD2	*P. fraudulenta*	biotin-(C9x3)-TCAACCAACCCAAACCCACGCAAGCT	77.6	this study
	has2	*P. hasleana*	AAAGTAAACCCACGCAAGCCCACAG-(C9x3)-biotin	75.8	this study
	muD3	*P. multiseries*	biotin-(C9x3)-CAAACTCACGCAAGCCCACAG	72.8	this study
	pung1	*P. pungens*	biotin-(C9x3)-ACTCACTTTACCAGGCGGACGGGA	77.3	this study
SHA signal	PSDS	*Pseudo-nitzschia* spp.	dig-(C9) CTCTTTAACTCTCTT*TTCAAAGTTCTTTGCATC-(C9)-dig	74.5	Scholin et al., 1999
FISH	auD1 BF	*Pseudo-nitzschia australis*	fluor-AAATGACTCACTCCACCAGG-fluor	45	Miller and Scholin 1998
	muD1 BF	*P. multiseries*	fluor-ATGACTCACTCTGCCA-fluor	45	Miller and Scholin 1998
	muD2 BF	*P. multiseries/ pseudodelicatissima*	fluor-AAGCCCACAGCGCCCAAGCC-fluor	55	Miller and Scholin 1998
	uniC BF	positive control	fluor-GWATTACCGCGGCKGCTG-fluor	45	Miller and Scholin 1998
	uniR BF	negative control	fluor-CAGCMGCCGCGGTAATWC-fluor	45	Miller and Scholin 1998

Table 2. Total number of isolates sequenced for each *Pseudo-nitzschia* species (D1-D3 LSU rRNA) during this study. SPS = San Pedro Shelf, CA; MB = Monterey Bay, CA. Intra-species variation observations are based on alignments of the total number of isolates for each species.

species	SPS 2013	MB 2013	SPS 2014	MB 2015	Intra-species variation?
P. americana	1	---	11	---	no
P. arenysensis	---	---	10	---	no
P. australis	63	3	20	219	yes[1]
P. cuspidata	---	---	3	---	no
P. sp. 'A'	3	---	1	3	no
P. sp. 'B'	1	---	13	---	no
P. fraudulenta	10	434	38	11	yes[1]
P. hasleana	43	---	74	---	no
P. sp. 'C'	8	---	15	1	yes[1]
P. multiseries	26	---	18	37	no
P. multistriata	---	---	1	---	n/a
P. pungens	77	---	153	30	no
P. seriata	---	---	---	14	no

[1]One polymorphic site.

Table 3. Specificity testing of SHA probes against a panel of non-target species. Values are OD 450 readings from the benchtop SHA platform. Fields with * denote species that demonstrated potential cross-reactivity (empirically defined by an OD of 0.250 or greater) and were further subjected to dose response curves.

		OD 450 values for SHA capture probes							
	No. of cells tested	auD1	ary1	frD2	has2	muD1	muD2	muD3	pung1
P. americana	5 x 10^5	0.065	0.112	0.108	0.052	0.056	0.054	0.058	0.066
P. arenysensis	5 x 10^5	3.126*	--	0.107	0.085	0.061	0.058	0.060	0.135[b]
P. australis	5 x 10^5	--	0.320*	0.243*	0.063	0.073	0.067	0.085	0.745*
P. cuspidata	5 x 10^5	0.069	0.113	0.103	0.060	0.062	0.056	0.064	0.583[a]
P. sp. 'A'	2.5 - 5 x 10^5	0.063	0.123	0.093	0.053	0.062	0.060	0.069	0.183
P. sp. 'B'	5 x 10^5	0.058	0.091	0.111	0.066	0.059	0.076	0.068	0.776*
P. fraudulenta	5 x 10^5	0.068	0.144	--	0.080	0.061	0.063	0.068	0.212
P. hasleana	5 x 10^5	0.061	0.092	0.105	--	n.t.	1.288[d]*	0.481[d]*	1.276*
P. sp. 'C'	5 x 10^5	0.069	0.092	0.145	0.132	0.067	0.068	0.081	0.142
P. multiseries	5 x 10^5	0.072	0.137	0.436*	0.178	--	--	--	0.156
P. multistriata	5 x 10^5	3.747*	0.177	0.081	0.073	0.061	0.056	0.063	1.106*
P. pungens	5 x 10^5	0.066	0.109	0.151	0.070	0.060	0.058	0.067	--
P. seriata	2 x 10^5	0.081	0.060	0.128	0.060	0.062	0.058	0.062	0.785*

[a]cultures expired before cells could be archived for dose response curve.
[b]3 x 10^5 cells used for testing.
[c]not tested; the muD1 FISH probe (which overlaps the muD1 SHA probe 100%) was negative against twenty-three *P. hasleana* cultures
[d]1.67 x 10^5 cells used for testing.

Table 4. Cross-reactivity of FISH probes for *P. australis* (auD1), *P. multiseries* (muD1) and *P. multiseries/pseudodelicatissima* (muD2) with non-target species.

	FISH probe		
	auD1	muD1	muD2
P. americana	--	--	--
P. arenysensis	+/++	--	--
P. australis	++	--	--
P. cuspidata	--	--	--
P. sp. 'A'	--	--	--
P. sp. 'B'	--	--	--
P. fraudulenta	--	--	--
P. hasleana	--	--	++
P. sp. 'C'	--	--	--
P. multiseries	--	++	++
P. multistriata	+	--	--
P. pungens	--	--	--
P. seriata	--	--	--

Table 5. Comparison of FISH, benchtop SHA and ESP SHA performance at bloom threshold levels of 50,000 cell L^{-1} for *P. australis* and *P. multiseries.* nt = not detectable

	FISH (# cells on filter +/- SD)	Benchtop SHA (OD 450 +/- SD)	ESP SHA (CCD +/- SD)
auD1	35 +/- 11	1.104 +/- 0.179	14,280 +/- 2423
muD1	40 +/- 13	nt	6906 +/- 1513
muD2	41 +/- 14	1.26 +/- 0.10	23,280 +/- 4522
muD3	n/a	0.800 +/- 0.016	31,996 +/- 6784

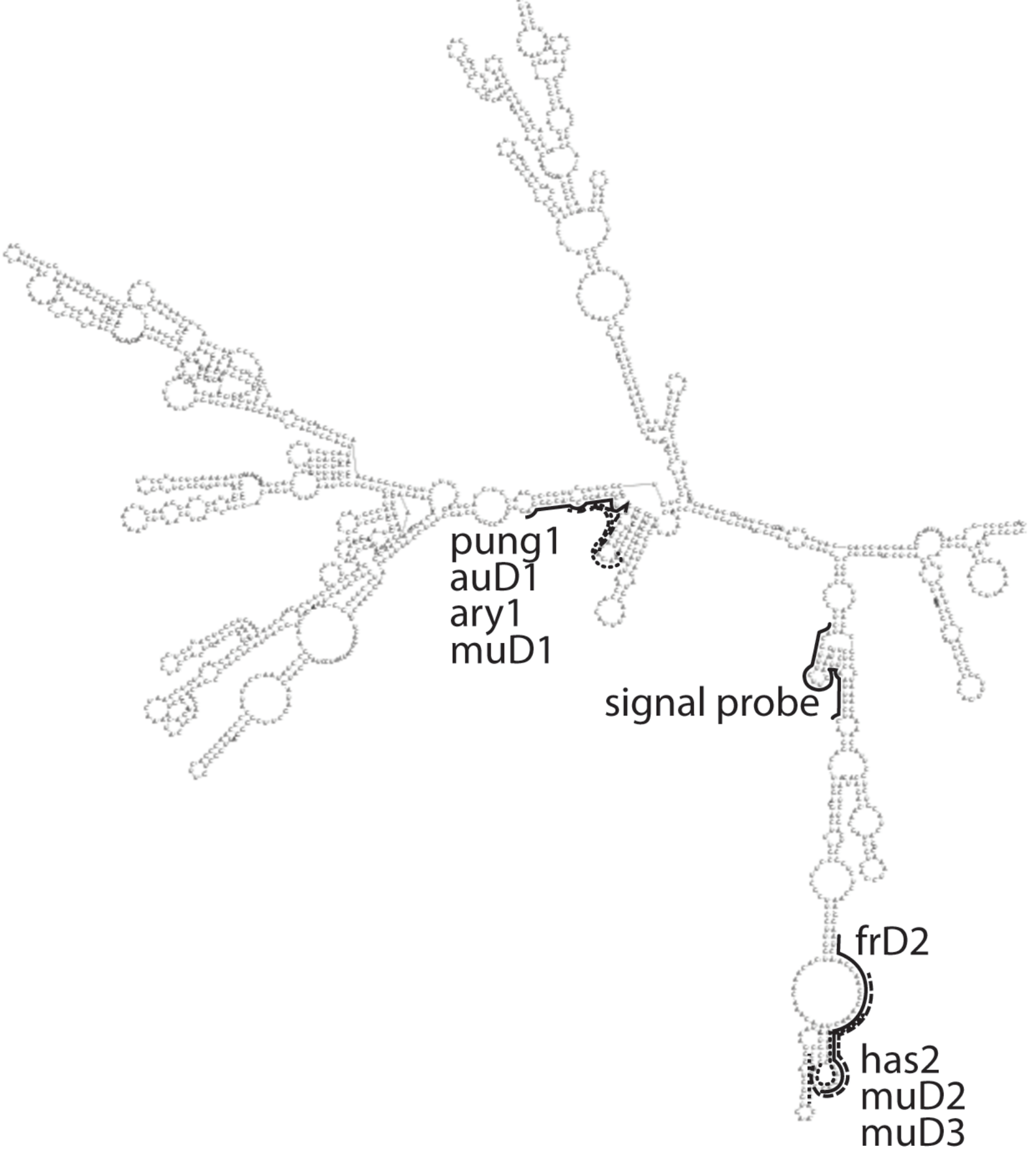
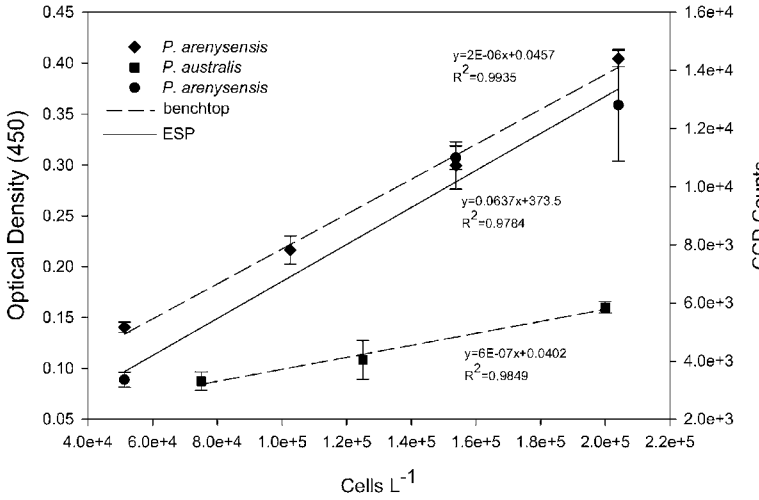

pung1
auD1
ary1
muD1
signal probe
frD2
has2
muD2
muD3

Figure 2


P. arenysensis
P. australis
P. arenysensis
benchtop
ESP
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R2=0.9935
y=0.0637x+373.5
R2=0.9784
y=6E-07x+0.0402
R2=0.9849
Optical Density (450)
CCD Counts
Cells L-1
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
1.6e+4
1.4e+4
1.2e+4
1.0e+4
8.0e+3
6.0e+3
4.0e+3
2.0e+3
4.0e+4
6.0e+4
8.0e+4
1.0e+5
1.2e+5
1.4e+5
1.6e+5
1.8e+5
2.0e+5
2.2e+5

Figure 3a

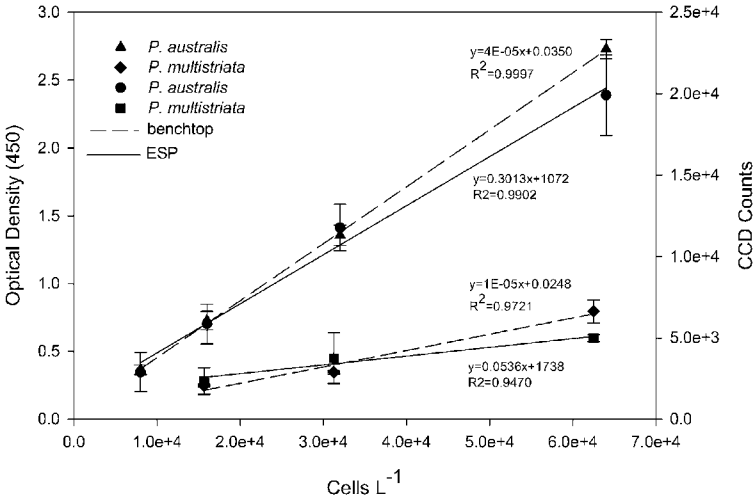

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Optical Density (450)
2.5e+4
2.0e+4
1.5e+4
1.0e+4
5.0e+3
0.0
CCD Counts
P. australis
P. multistriata
P. australis
P. multistriata
benchtop
ESP
y=4E-05x+0.0350
R2=0.9997
y=0.3013x+1072
R2=0.9902
y=1E-05x+0.0248
R2=0.9721
y=0.0536x+1738
R2=0.9470
0.0
1.0e+4
2.0e+4
3.0e+4
4.0e+4
5.0e+4
6.0e+4
7.0e+4
Cells L-1

Figure 3b

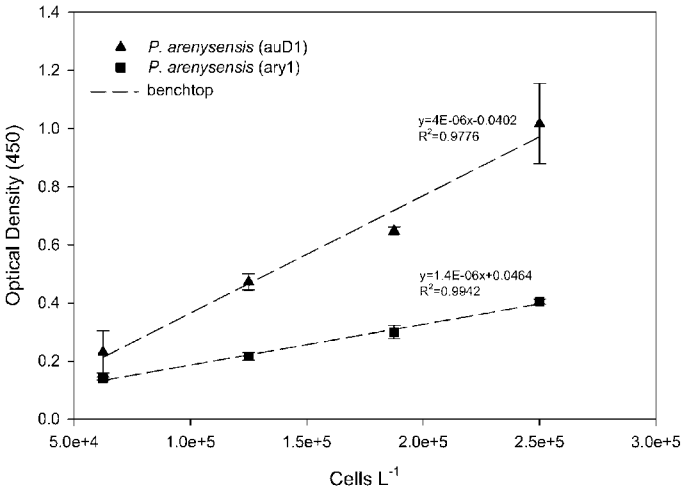

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Optical Density (450)
P. arenysensis (auD1)
P. arenysensis (ary1)
benchtop
y=4E-06x-0.0402
R2=0.9776
y=1.4E-06x+0.0464
R2=0.9942
5.0e+4
1.0e+5
1.5e+5
2.0e+5
2.5e+5
3.0e+5
Cells L-1

Figure 3c

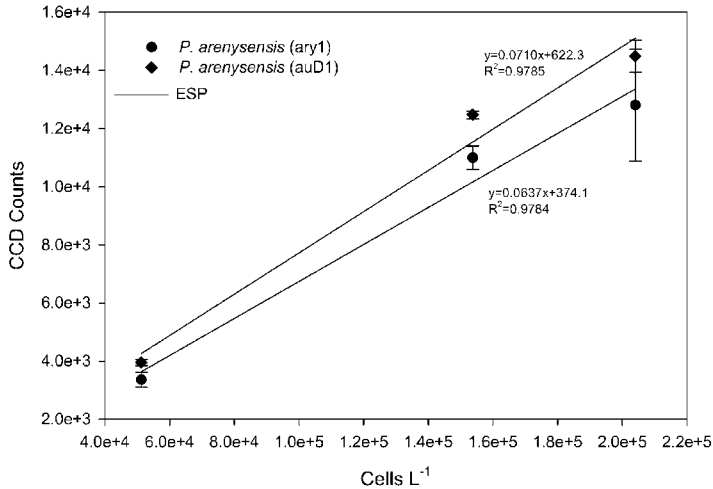
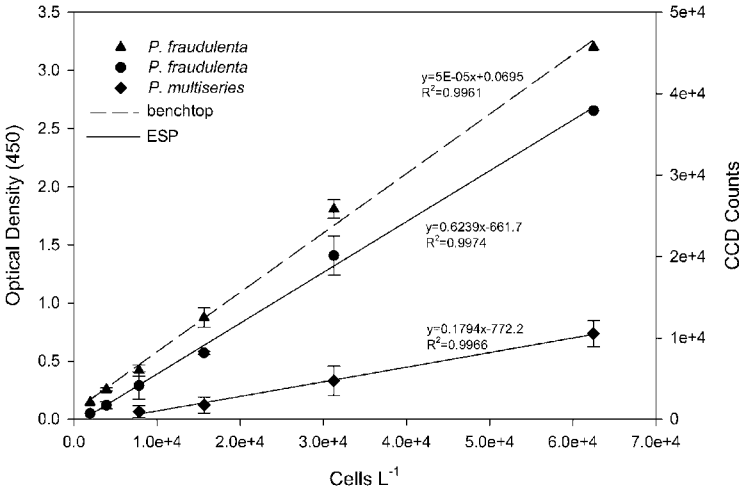

1.6e+4
1.4e+4
1.2e+4
1.0e+4
8.0e+3
6.0e+3
4.0e+3
2.0e+3
CCD Counts
4.0e+4
6.0e+4
8.0e+4
1.0e+5
1.2e+5
1.4e+5
1.6e+5
1.8e+5
2.0e+5
2.2e+5
Cells L-1
P. arenysensis (ary1)
P. arenysensis (auD1)
ESP
y=0.0710x+622.3
R2=0.9785
y=0.0637x+374.1
R2=0.9784

Figure 4


Optical Density (450)
CCD Counts
Cells L-1
P. fraudulenta
P. fraudulenta
P. multiseries
benchtop
ESP
y=5E-05x+0.0695
R2=0.9961
y=0.6239x-661.7
R2=0.9974
y=0.1794x-772.2
R2=0.9966
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
1e+4
2e+4
3e+4
4e+4
5e+4
0.0
1.0e+4
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3.0e+4
4.0e+4
5.0e+4
6.0e+4
7.0e+4

Figure 5

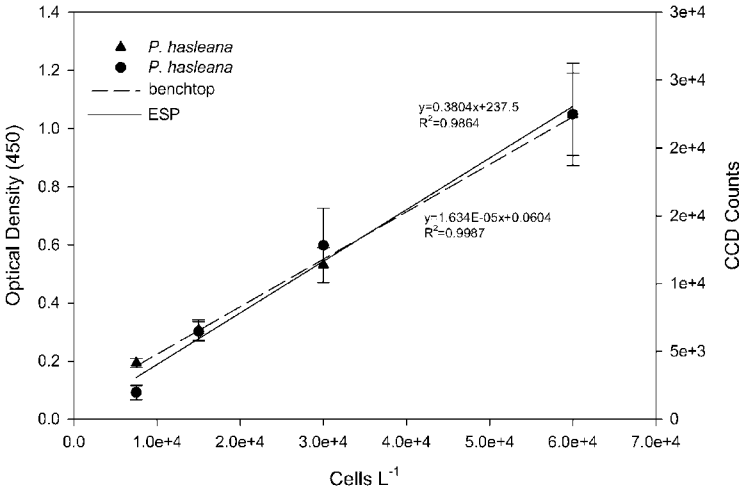

P. hasleana
P. hasleana
benchtop
ESP
y=0.3804x+237.5
R2=0.9864
y=1.634E-05x+0.0604
R2=0.9987
Optical Density (450)
CCD Counts
Cells L-1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
5e+3
1e+4
2e+4
2e+4
3e+4
3e+4
0.0
1.0e+4
2.0e+4
3.0e+4
4.0e+4
5.0e+4
6.0e+4
7.0e+4

Figure 6a

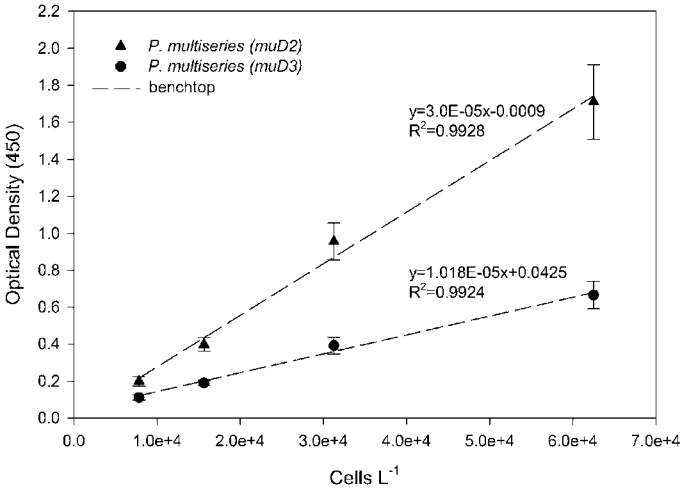

P. multiseries (muD2)
P. multiseries (muD3)
benchtop
y=3.0E-05x-0.0009
R2=0.9928
y=1.018E-05x+0.0425
R2=0.9924
Optical Density (450)
Cells L-1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
1.0e+4
2.0e+4
3.0e+4
4.0e+4
5.0e+4
6.0e+4
7.0e+4

Figure 6b

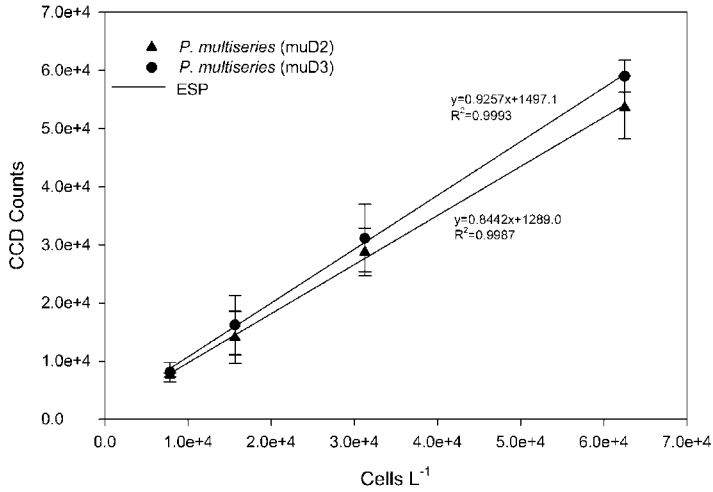

7.0e+4
6.0e+4
5.0e+4
4.0e+4
3.0e+4
2.0e+4
1.0e+4
0.0
CCD Counts
P. multiseries (muD2)
P. multiseries (muD3)
ESP
y=0.9257x+1497.1
R2=0.9993
y=0.8442x+1289.0
R2=0.9987
0.0
1.0e+4
2.0e+4
3.0e+4
4.0e+4
5.0e+4
6.0e+4
7.0e+4
Cells L-1

Figure 7a

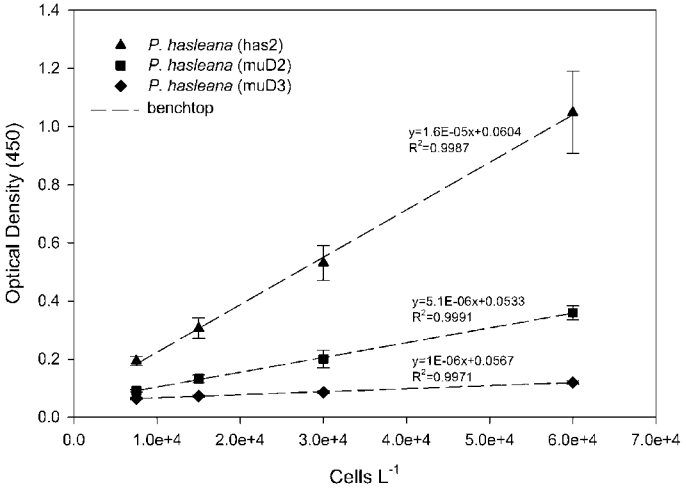

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Optical Density (450)
P. hasleana (has2)
P. hasleana (muD2)
P. hasleana (muD3)
benchtop
y=1.6E-05x+0.0604
R2=0.9987
y=5.1E-06x+0.0533
R2=0.9991
y=1E-06x+0.0567
R2=0.9971
0.0
1.0e+4
2.0e+4
3.0e+4
4.0e+4
5.0e+4
6.0e+4
7.0e+4
Cells L-1

Figure 7b

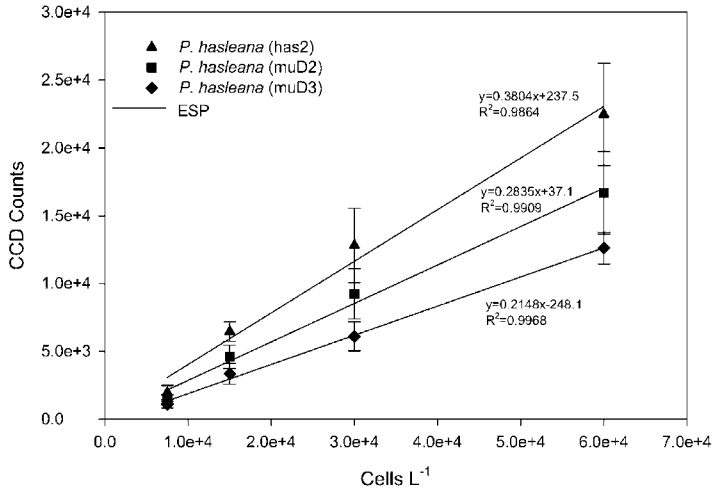
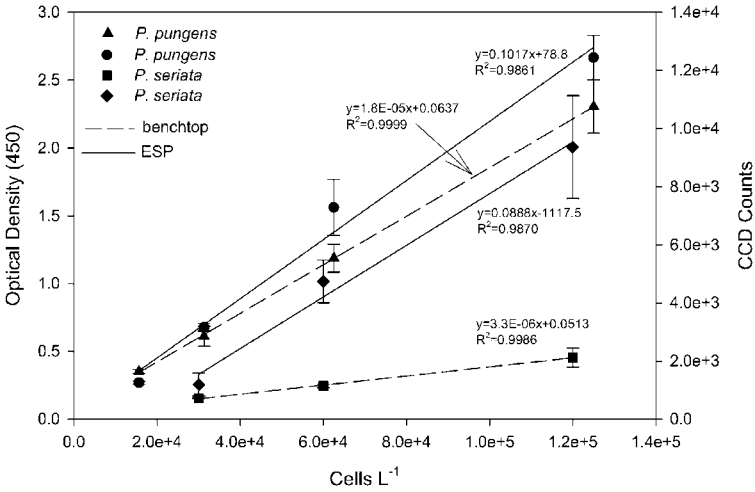
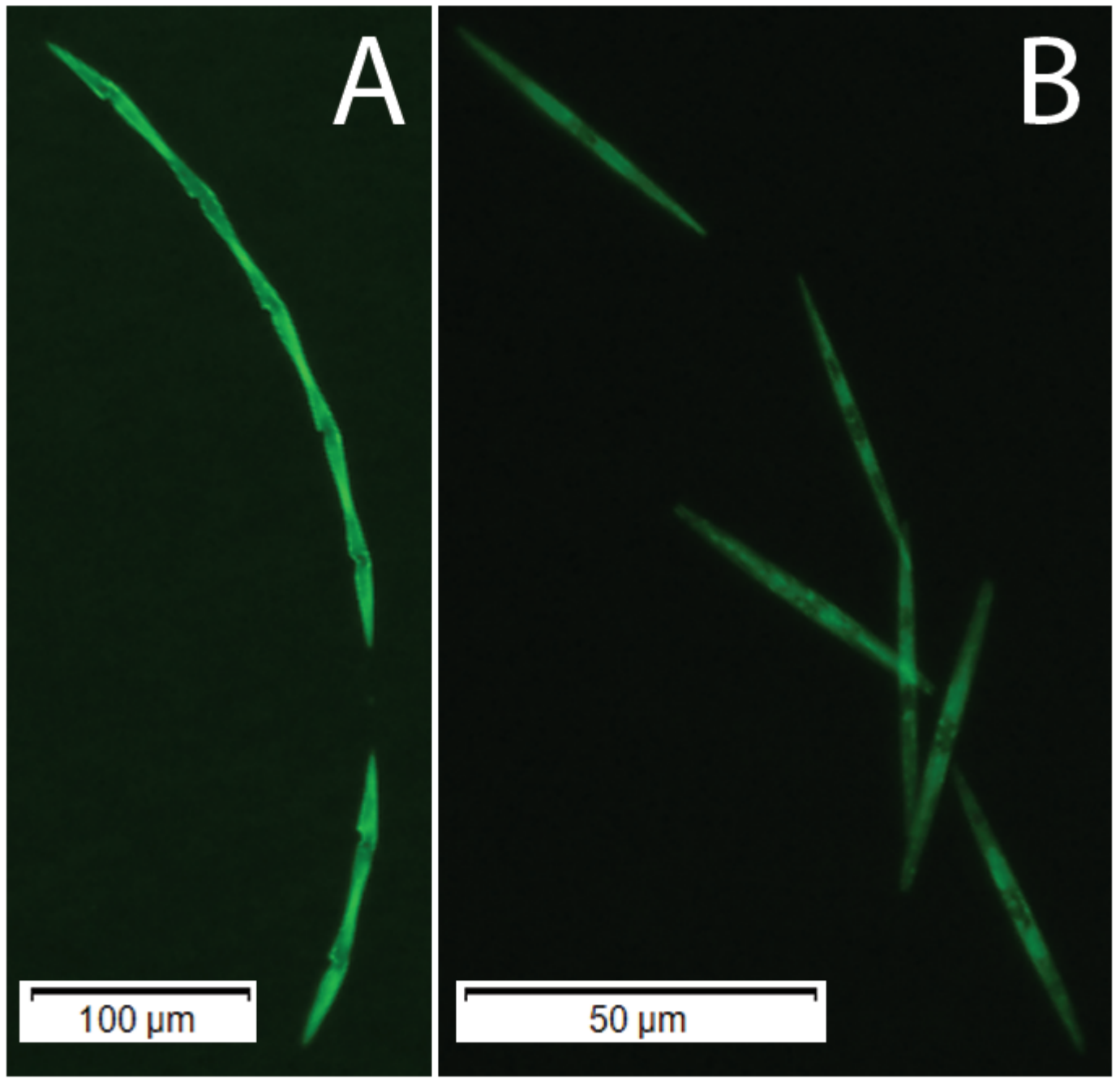
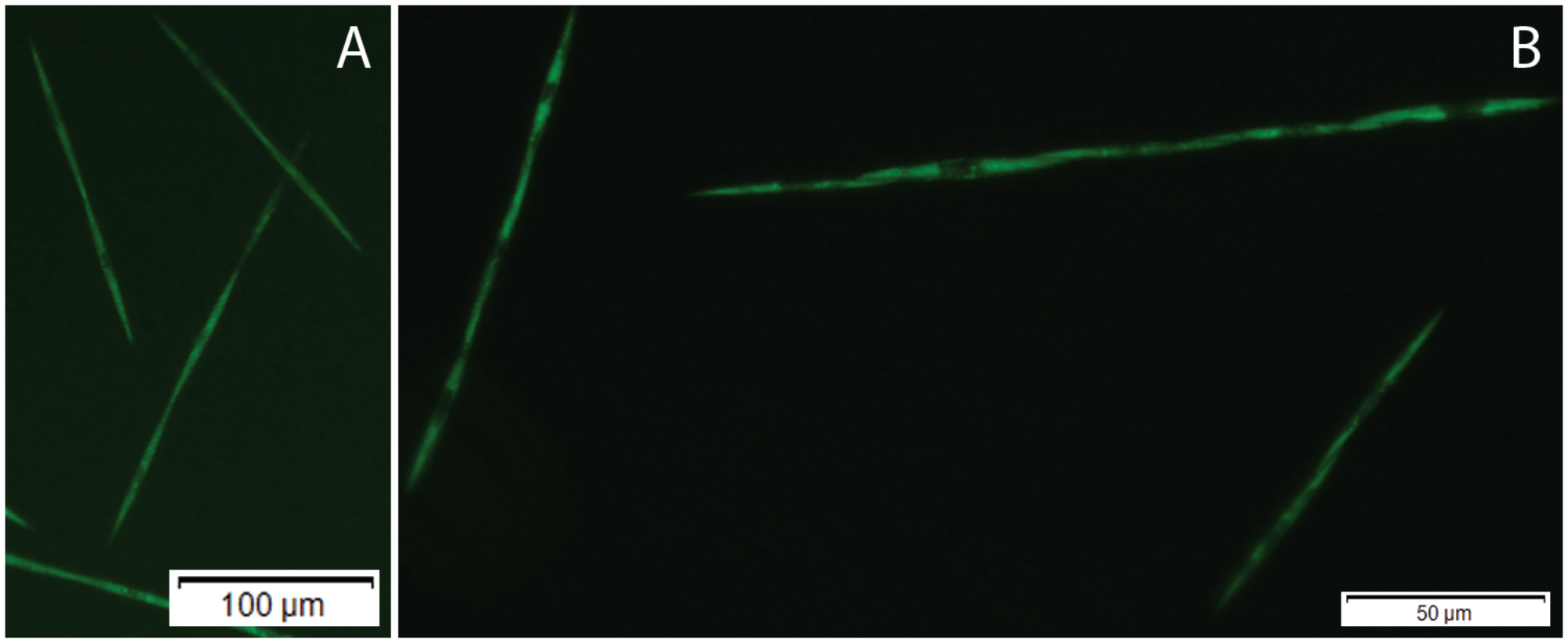

3.0e+4
2.5e+4
2.0e+4
1.5e+4
1.0e+4
5.0e+3
0.0
CCD Counts
P. hasleana (has2)
P. hasleana (muD2)
P. hasleana (muD3)
ESP
y=0.3804x+237.5
R2=0.9864
y=0.2835x+37.1
R2=0.9909
y=0.2148x-248.1
R2=0.9968
0.0
1.0e+4
2.0e+4
3.0e+4
4.0e+4
5.0e+4
6.0e+4
7.0e+4
Cells L-1

Figure 8


P. pungens
P. pungens
P. seriata
P. seriata
benchtop
ESP
y=0.1017x+78.8
R2=0.9861
y=1.8E-05x+0.0637
R2=0.9999
y=0.0888x-1117.5
R2=0.9870
y=3.3E-06x+0.0513
R2=0.9986
Optical Density (450)
CCD Counts
Cells L-1
0.0
0.5
1.0
1.5
2.0
2.5
3.0
2.0e+3
4.0e+3
6.0e+3
8.0e+3
1.0e+4
1.2e+4
1.4e+4
2.0e+4
4.0e+4
6.0e+4
8.0e+4
1.0e+5
1.2e+5
1.4e+5


A
B
100 µm
50 µm


A
100 µm
B
50 µm