

Molecular characterization of *Pseudo-nitzschia* community structure and species ecology in a hydrographically complex estuarine system (Puget Sound, Washington, USA)

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ABSTRACT: Species within the toxic marine diatom genus *Pseudo-nitzschia* coexist in coastal and estuarine waters globally and are difficult to distinguish by microscopy. Here, we describe a sensitive, high throughput PCR-based automated ribosomal intergenic spacer analysis (ARISA) approach to determine the relative abundance of *Pseudo-nitzschia* species within natural communities over space and time. The method was quantitatively validated using simplified mixtures of DNA or ITS1 standards from isolates of *P. pungens*, *P. multiseriata*, and *P. delicatissima*. Relative abundance calculations based on ARISA profiles from these mixtures reflected input ratios, with minor deviations resulting from intraspecific variability. ARISA was used to identify and quantify at least 8 species within Puget Sound and the eastern Strait of Juan de Fuca, Washington, USA: *P. americana*, *P. australis*/*P. seriata*, *P. cuspidata*, *P. delicatissima*, *P. fraudulenta*, *P. fryxelliana*, *P. multiseriata*, and *P. pungens*; genotypes corresponding to *P. pungens* var. *pungens* and *P. pungens* var. *cingulata* were identified by environmental sequencing. The different species were significantly correlated with physical (temperature, salinity), biological (chlorophyll a fluorescence, oxygen), and/or chemical (ammonium, nutrient ratios) factors. The ability to determine shifts in the relative abundance of *Pseudo-nitzschia* species over spatial and temporal scales relevant to dispersion and selection facilitates dissection of the varied mechanisms driving vertical and horizontal species distribution patterns in hydrographically complex systems.

KEY WORDS: Diatom · Puget Sound · Species diversity · Internal transcribed spacer

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INTRODUCTION

The pennate diatom genus *Pseudo-nitzschia* is distributed worldwide across oceanic, coastal, and estuarine environments (Hasle 2002, Lundholm et al. 2004, Trainer et al. 2012). Interest in *Pseudo-nitzschia* taxonomy and ecology has increased over the past few decades, largely because at least 14 species are capable of producing the neurotoxin domoic acid (DA). Toxin production among these species

varies over ~9 orders of magnitude (Trainer et al. 2012, Fernandes et al. 2014). DA accumulates in filter-feeding shellfish and planktivorous fish and can cause DA poisoning in marine vertebrates that feed on contaminated prey (Lefebvre & Robertson 2010). Human consumption of DA-contaminated seafood produces the syndrome amnesic shellfish poisoning (ASP), which can result in permanent short-term memory loss or death (Perl et al. 1990). Production of DA is enhanced in *Pseudo-nitzschia* cultures by ma-

nipulating abiotic and biotic factors (summarized by Bates 1998, Lelong et al. 2012). Although *Pseudo-nitzschia* species commonly occur in phytoplankton assemblages, DA production is not constitutive in the environment and can vary in response to environmental stimuli such as nitrogen speciation and concentration, iron availability, chlorophyll *a* (chl *a*) biomass, pH, and salinity (Armstrong-Howard et al. 2007, Lelong et al. 2012). Recent studies have identified synergistic effects when cultures are simultaneously exposed to multiple stressors (Fuentes & Wikfors 2013, Tatters et al. 2012), highlighting the taxonomic and ecological complexity underlying DA production in the environment (Trainer et al. 2012, Terseleer et al. 2013).

Among the >40 *Pseudo-nitzschia* species currently recognized, complexes composed of species with cryptic (i.e. identical) and pseudo-cryptic (i.e. finely differentiated) morphologies have been identified (Lim et al. 2012, 2013, Trainer et al. 2012, Orive et al. 2013). The use of phylogenetics, electron microscopy, and mating studies has greatly facilitated the recognition of new species within these complexes (Amato et al. 2007, Quijano-Scheggia et al. 2009, Lundholm et al. 2012). The characterization of region-specific as well as cosmopolitan *Pseudo-nitzschia* diversity (Hasle 2002, Orsini et al. 2004, Amato et al. 2007, Hubbard et al. 2008, Casteleyn et al. 2009) suggests that environmental and ecological processes act on multiple spatial (local, regional, and global) and temporal (event, seasonal, annual, decadal) scales to generate and maintain diversity in this genus (Hasle 2002, Lundholm et al. 2010, Ribalet et al. 2010). The emerging message from culture and field studies is that examination of *Pseudo-nitzschia* at appropriate taxonomic scales is necessary to determine the ecological significance of spatiotemporal factors that influence the assembly, growth, maintenance, and toxicity of communities in this harmful algal bloom (HAB) genus.

We previously developed an automated ribosomal intergenic spacer analysis (ARISA) approach that discriminates a majority of known *Pseudo-nitzschia* species in both laboratory and field studies (Hubbard et al. 2008). The approach employs genus-specific PCR primers to amplify a variable region of the internal transcribed spacer 1 (ITS1). Previous work (Hubbard et al. 2008, Marchetti et al. 2008) has demonstrated that the nucleotide sequence and length of the PCR amplicon is diagnostic of different species. Here, we developed a more quantitative ARISA approach for determining relative abundance of the different species, following methodology employed

for bacterial community fingerprinting (Lueders & Friedrich 2003, Brown et al. 2005) and taking into account heterogeneity in *Pseudo-nitzschia* species ITS1 copy numbers per genome (e.g. Penna et al. 2013). We used this approach to examine *Pseudo-nitzschia* species diversity in simplified laboratory communities consisting of isolate DNA, and in environmental communities sampled during June 2006 in the Puget Sound (Washington, USA) estuarine system, encompassing 5 interconnected, but hydrographically distinct, basins and the eastern Strait of Juan de Fuca. The sensitive ARISA approach revealed dynamic shifts in *Pseudo-nitzschia* species composition across horizontal and vertical environmental gradients in Puget Sound, permitting greater insight into the complexity of ecological and physical drivers that contribute to the spatial and temporal distribution of phytoplankton species in estuarine systems.

MATERIALS AND METHODS

Culture conditions and estimation of DNA and ITS1 copy number per cell

Non-axenic diatom isolates of *Pseudo-nitzschia pungens*, *P. multiseriata*, *P. delicatissima*, and *Ditylum brightwellii* from Puget Sound and the outer coast of Washington were provided by the Center for Environmental Genomics, University of Washington, Seattle, USA (Table 1). Cultures were maintained in autoclaved Puget Sound water amended with *f/2* nutrients (Guillard 1975) and grown at 13°C as semi-continuous batch cultures on a 16:8 light:dark cycle of 30 to 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ cool-white light. Increases in chl *a* fluorescence were monitored with a Turner Designs 10-AU fluorometer. Aliquots (5 ml) of mid-to-late exponential cultures were fixed with Lugol's solution for cell counts and cell volume estimates (see below). A 10 ml aliquot of the culture was also diluted into 1000 ml of sterile Puget Sound seawater. Replicate 505 ml subsamples were taken from the diluted culture to simulate environmental subsampling from a Niskin bottle, with 5 ml aliquots from each subsample fixed with Lugol's and the remaining 500 ml filtered onto a 25 mm diameter mixed cellulose filter with 0.45 μm pore size (Millipore), and stored at -80°C until DNA extraction.

For each Lugol's-fixed sample ($n = 3$ per isolate), cells were enumerated using a Nikon Eclipse TS100 inverted light microscope to count at least 30 fields of view or 1000 cells on a Sedgewick-Rafter slide. Api-

Table 1. Summary of species affiliation and origin of *Pseudo-nitzschia* isolates utilized to generate genomic DNA and ITS1 standards for ARISA. ARISA fragment size was predicted from sequence and ARISA data. Cell length and width were determined microscopically for isolates of *P. pungens* and *P. multiseriata* from Puget Sound and were estimated for *P. delicatissima* using genetically similar temperate strains. For cellular DNA content, standard deviations reflect the variability of cell count data, and for ITS1 copies, the variability of 27–36 quantitative PCRs (qPCRs) generated for each isolate. The DNA content for *P. delicatissima* isolate PNWH2O_604 was used in calculations of ITS1 copy number cell⁻¹ for PNWH2O_609. na: not available

Species	Isolate	Date of isolation (mo/yr)	Location of isolation	ARISA fragment size (DNA bp)	Mean ± SD cell length (µm)	Mean ± SD cell width (µm)	Mean ± SD cell volume (µm ³)	DNA content (pg cell ⁻¹)	ITS1 copy number cell ⁻¹ ± SD
<i>P. pungens</i> var. <i>pungens</i>	PNWH2O_GGC3	8/2010	Golden Gardens, WA	142	133.1 ± 1.5	6.0 ± 1.0	3877 ± 1191	0.92 ± 0.62	149 ± 44
	PNWH2O_PnC2	7/2009	Friday Harbor, WA	142	62.1 ± 2.6	5.0 ± 0.9	1286 ± 403	0.78 ± 0.24	227 ± 128
<i>P. pungens</i> var. <i>cingulata</i>	PNWH2O_GGA1	8/2010	Golden Gardens, WA	142	84.7 ± 3.9	3.9 ± 0.8	1063 ± 446	0.86 ± 0.30	404 ± 134
	PNWH2O_GGA3	8/2010	Golden Gardens, WA	142	92.6 ± 4.3	4.1 ± 0.6	1298 ± 399	0.61 ± 0.21	150 ± 44
<i>P. multiseriata</i>	PNWH2O_608	10/2006	Whidbey Basin, WA	142	na	na	na	0.93 ± 0.47	na
	PNWH2O_PC9	7/2010	Penn Cove, WA	144	90.8 ± 2.3	3.8 ± 0.7	1107 ± 415	1.48 ± 0.45	748 ± 203
<i>P. delicatissima</i>	PNWH2O_GGB1	8/2010	Golden Gardens, WA	144	100.3 ± 4.4	4.1 ± 0.7	1348 ± 442	1.63 ± 0.60	406 ± 105
	PNWH2O_604	6/2006	N. Hood Canal	168	na	na	na	0.47 ± 0.30	26 ± 7
	PNWH2O_609	6/2006	N. Hood Canal	168	na	na	na	na	16 ± 11
			WA coast ^a , Bay of Fundy ^b	168 ²	17–66	1–2.3	39–243	na	na

^aStehr et al. (2002)

^bKaczmarek et al. (2008)

cal and transapical axes (in valve view) were measured for 20 randomly selected cells of each isolate with ImageJ software (United States National Institute of Health, Bethesda, Maryland, USA; <http://imagej.nih.gov/ij/>). Calculations of average cell volume considered the linear (60%) and tapered (40%) portions of *Pseudo-nitzschia* cells, and used width as a proxy for depth (Lundholm et al. 2004):

$$\text{Volume} = (0.6 \times L \times W^2) + (0.4 \times 0.5 \times L \times W^2) \quad (1)$$

where L is length and W is width. A subset of the examined cells ($n = 7$) was measured in girdle view. These cells were actively dividing and were excluded from further analysis.

Isolate and environmental (see below) DNA samples were extracted from filtered cells as described by Hubbard et al. (2008) using the DNeasy Plant Mini Kit (Qiagen). Genomic DNA from isolates was quantified using a Nanodrop 1000 Spectrophotometer (Nanodrop Technologies) and PicoGreen[®] assays (Invitrogen) with a SpectraMax M2 microplate reader (Danaher Medical Technologies). The DNA content cell⁻¹ was calculated by dividing average cell abundance ($n = 3$) by the total DNA (using PicoGreen measurements) extracted from each culture.

To generate ITS1 sequence standards, 10 to 30 ng of *P. pungens*, *P. multiseriata*, or *P. delicatissima* isolate DNA were added to 20 µl PCRs containing 0.4 mM deoxynucleoside triphosphates (dNTPs), 0.8 µM 18S-euk F primer, 0.8 µM 5.8S-euk R primer, 2.5 mM MgCl₂, 1× standard reaction buffer, and 0.75 U *Taq* polymerase (Genechoice, Apex[™] Bioresearch Products). Primers and cycling parameters are described in Hubbard et al. (2008). PCR products were excised from a 1% agarose gel, purified using the QIAquick PCR purification kit (Qiagen), ligated into the pCR[®] 4-TOPO[®] vector, and used to transform the Top 10 strain of *Escherichia coli* (Invitrogen). For each species, DNA was isolated from 4 to 6 clones using the QIAprep Spin Miniprep Kit (Qiagen), and the ITS1 inserts from at least 4 clones were sequenced in both directions on an ABI 3730XL high-throughput capillary DNA analyzer. Standards for each species were generated by linearizing the ITS1 plasmid with the restriction enzyme *SpeI* (Invitrogen). Real-time quantitative PCR (qPCR) assays determined ITS1 copy numbers for all *Pseudo-nitzschia* isolates except PNWH2O 608 (due to insufficient DNA). Triplicate 20 µl qPCR amplifications contained 5 µl of isolate DNA or water, 0.8 µM PnallF (5'-TCT TCA TTG TGA ATC TGA-3') and 0.8 µM Pnall R (5'-CTT TAG GTC ATT TGG TT-3') (Hubbard et al. 2008), and iQ SYBR Green Supermix (Bio-Rad Labo-

ratories). Assays were performed with the iCycler iQ Real-Time PCR detection system (Bio-Rad Laboratories) using the following cycling parameters: 95°C for 3 min followed by 50 cycles of 95°C for 10 s, 50.6°C for 50 s, and 72°C for 50 s. Separate runs were conducted for each species and included a dilution series of the corresponding linearized ITS1 standard. The threshold cycle (C_T) was determined for each dilution and compared to the standard curve using the iCycler iQ Optical System Software (Bio-Rad Laboratories) to quantify copy number for ITS1 target DNA. Copy number was normalized to DNA concentration and corresponding cell numbers for each reaction.

Cellular estimates of size (volume), DNA content, and ITS1 copy number for *Pseudo-nitzschia* isolates were examined for normality using Shapiro-Wilk tests (Shapiro et al. 1968) and homogeneity of variances using Levene's test (Levene 1960) in SPSS (IBM software). Differences in the mean values for each metric were analyzed using a 1-way analysis of variance (ANOVA) in conjunction with post hoc analyses.

Puget Sound sample collection

Five Puget Sound basins and the eastern Strait of Juan de Fuca were sampled on 26 to 29 June 2006 from the R/V 'Thomas G. Thompson' during the Puget Sound Regional Synthesis Model (PRISM) biannual hydrographic survey (Fig. 1). Salinity, temperature, and potential density measurements were obtained at 40 stations using an SBE 911*plus*® CTD profiler. Potential density (CTD) was plotted with Ocean Data View (Schlitzer 2012) to identify pycnoclines at vertically profiled stations. Discrete water samples were collected with Niskin bottles mounted on a rosette, generally at 2.5, 5, 10, 20, 30, 40, 50, 100, 150, and 200 m, and near-bottom. For determination of nutrient concentrations, 35 ml of seawater were syringe-filtered through 0.22 µm Whatman filter units (GE Healthcare Biosciences), frozen onboard, and subsequently analyzed with a Technicon Auto-Analyzer II (UNESCO 1994) at the University of Washington Marine Chemistry Lab. For each chloro-

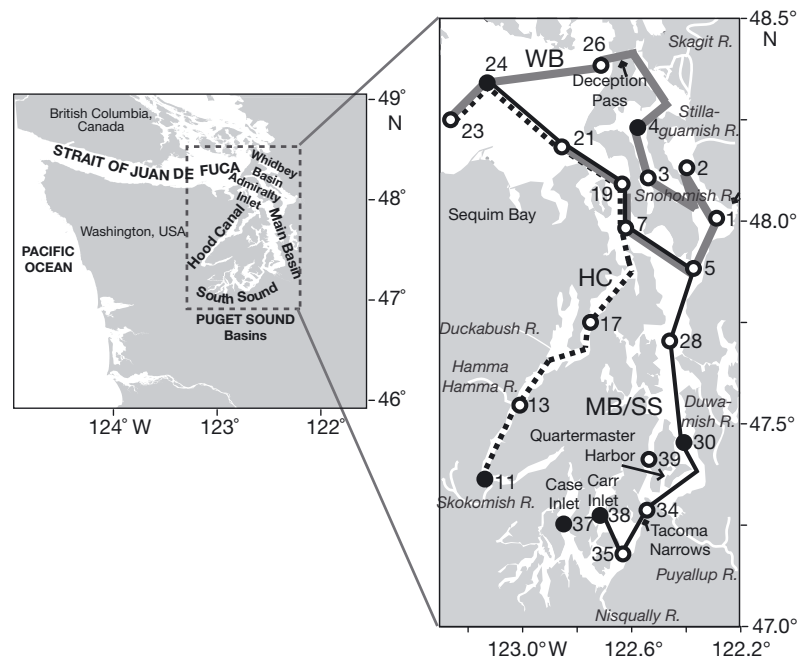


Fig. 1. Washington (USA) coast and estuaries. The Puget Sound study region (dotted rectangle) is expanded (right panel) to indicate ARISA sites sampled from 26 to 28 June 2006. Surface samples (○) and a vertical profile of samples (●) were collected at a subset of PRISM stations. Three transects are indicated: the Strait of Juan de Fuca transect through Whidbey Basin towards Admiralty Inlet (WB, gray line); the Strait transect to Hood Canal (HC, dotted line), and the Strait transect through Main Basin to South Sound (MB/SS, black line)

phyll sample, 65 ml of whole seawater were filtered through 25 mm Whatman glass fiber filters, extracted with acetone, and analyzed with a Turner Designs 10-AU fluorometer. Dissolved oxygen samples were collected in glass bottles and analyzed using modified Winkler titration (Carpenter 1965). Environmental data are available on the Nanoos server: <http://nvs.nanoos.org/CruisePrism>.

A surface net tow (33 µm mesh size) was conducted at 20 stations, and phytoplankton communities were examined onboard the ship with a Nikon Eclipse TS100 inverted light microscope. Water samples for DNA were collected from the surface (i.e. 2.5 m) at the same 20 stations and from 3 to 4 additional depths at the following stations: P24, Strait of Juan de Fuca (5, 50, and 115 m); P30, Main Basin (10, 50, and 80 m); P4, Whidbey Basin (5, 50, and 87 m); P11, Hood Canal (5, 13, 50, and 85.6 m); P37, South Sound (6.5, 10, 30, 52 m); and P38, South Sound (7, 10, and 50 m) (Fig. 1). Subsurface community sample depths were adaptively selected to target distinct water masses, based on pycnoclines identified in the downward CTD cast. For each DNA sample, 500 ml of seawater were filtered onto 2 replicate 0.45 µm HA filters (Mil-

lipore) and stored at -80°C until extraction. Following extraction, environmental DNA was quantified as described above and diluted with sterile water to a concentration of $2\text{ ng }\mu\text{l}^{-1}$.

Amplification for ARISA

Isolate and environmental DNA was amplified for ARISA using the *Pseudo-nitzschia*-specific ITS1 Pnall primer set (Hubbard et al. 2008). Template for ARISA PCRs contained 10 ng of DNA consisting of mixtures of different proportions (2–98%) of *P. pungens*, *P. delicatissima*, or *P. multiseriis* genomic DNA with or without the addition of 5 ng of *D. brightwellii* genomic DNA or environmental genomic DNA. A dilution series of ITS1 sequence standards, derived from target species (*P. delicatissima*, *P. multiseriis*, or *P. pungens*), were added to PCRs to evaluate ARISA limits of detection. Standards of ITS1 DNA were used as template in PCRs containing 1, 10, 100, 500, 1000, and 10 000 ITS1 copies of each species (with *D. brightwellii* DNA added to obtain 10 ng template). Similar concentrations of ITS1 standards from each species were individually added to PCRs containing a cocktail of 5 ng environmental DNA and varying amounts of *D. brightwellii* DNA to obtain 10 ng. For ARISAs containing only genomic environmental template, 10 ng of DNA were added to each PCR.

For each ARISA sample, triplicate 20 μl PCRs contained 0.4 mM dNTPs, 0.4 μM of primer PnallF, 0.4 μM of FAM-labeled primer Pnall R, 0.75 U *Taq* polymerase, 2.5 mM of MgCl_2 , 1 \times standard buffer (Genechoice), and 10 ng template DNA. PCRs consisted of the following cycling parameters: a 2 min denaturing step at 94°C , followed by 32 cycles of 30 s at 95°C , 30 s at 50.6°C , and 60 s at 72°C , with a final 10 min extension at 72°C . PCR products were purified using MultiScreen PCR μ 96 filter plates (Millipore). Triplicate PCRs were pooled, quantified with PicoGreen[®], and diluted to $0.1\text{ ng }\mu\text{l}^{-1}$. For each sample, 1 ng DNA was ethanol precipitated and resuspended with 0.078 μl 10% Tween 20, 9.77 μl sterile water, and 0.15 μl fluorescent size standard Et-ROX 400 (GE Healthcare). Samples were analyzed on a Megabace 1000 capillary sequencer (GE Healthcare). Electropherograms were analyzed using DAX software (Van Mierlo Software). Fluorescent peak height data were extracted from DAX using the online fragment binner Dakster (<http://rocaplab.ocean.washington.edu/cgi/dakster/index.html>) with 0.1 base pair (bp) discrimination, and were manually

grouped into 1-bp bins. The proportion of total peak height attributed to each ARISA peak was computed for isolate and environmental samples. Peaks ranging between 100 and 400 bp that represented greater than 3% total ARISA fluorescence and 15 \times the signal:noise ratio were considered in further analyses. Heat maps were used to display community profiles from individual filters (www.bioinformatics.ubc.ca/matrix2png/bin/matrix2png.cgi).

Raw and relative ARISA peak height data generated from mixed templates of species DNA were tested for normality using a Shapiro-Wilk test. Linear regressions were calculated for peak height data (raw and relative) versus relative concentration of *Pseudo-nitzschia* species DNA added to PCRs (in SPSS).

Environmental clone libraries

Two samples were selected for environmental sequencing, based on ARISA composition, to best capture both species diversity as well as intraspecific diversity within *P. pungens*. Genomic DNA samples from surface waters at Stns P4 (Whidbey Basin) and P30 (Main Basin) were PCR-amplified using the Pnall primers as in Hubbard et al. (2008). PCR products were ligated into the pCR[®] 4-TOPO[®] vector and used to transform *E. coli*, Top 10 strain (Invitrogen). At least 78 transformants were randomly chosen from each library, and the Templi-phi DNA Amplification Kit (GE Healthcare) was used to amplify DNA for sequencing with the DYEnamic ET Dye Terminator Kit (GE Healthcare) on a Megabace 1000 capillary sequencer. Both strands of the Pnall ITS1 amplicon were sequenced to completion with M13 vector primers. Sequences were compiled, edited, and aligned in Sequencher[®] 4.9 (Gene Codes), and deposited in GenBank (accession numbers HM007359–HM007523). *Pseudo-nitzschia* species and intraspecific genotypes (defined by nucleotide polymorphisms) were identified based on ITS1 sequence similarity to sequences in the BLAST nucleotide (nr/nt) database as of March 2013. Rarefaction analyses were conducted with EstimateS software (Colwell 2005).

Comparative community analyses

For environmental ARISA profiles, the non-parametric statistic analysis of similarities (ANOSIM) was used to test for *Pseudo-nitzschia* community structure according to basin origin (PRIMER-E, Clarke &

Warwick 2001); relative fluorescence for individual species was averaged across replicate samples. The Bray-Curtis similarity index (Bray & Curtis 1957, Magurran 2004) considered ARISA fragment composition and the relative abundance of each ARISA fragment in a sample and was utilized to characterize community similarity between replicate filters and to identify spatial and ecological patterns in *Pseudo-nitzschia* communities (in PC-ORD, McCune & Grace 2002). Associations among environmental parameters and *Pseudo-nitzschia* species, based on ARISA data, were determined using the non-parametric Spearman's correlation analysis (in SPSS).

RESULTS

Interspecific variability in cell size, DNA content, and ITS1 copies

Cellular dimensions were similar among *Pseudo-nitzschia pungens* var. *cingulata* isolates GGA1 and GGA3 (3–7 μm wide and 56–100 μm long), *P. pungens* var. *pungens* isolate PnC2 (3–7 μm wide and 56–66 μm long), and *P. multiseriis* isolates PC9 and GGB1 (3–6 μm wide and 85–106 μm long). One isolate of *P. pungens* var. *pungens* was considerably larger (4–8 μm wide and 131–136 μm long; Table 1, Fig. 2A). Size estimates could not be made before the local *P. delicatissima* isolates (604, 609) were lost. Instead, strains from temperate regions with ITS1 sequences identical to 604 and 609 (Stehr et al. 2002, Kaczmarek et al. 2008) were used for *P. delicatissima* size estimates (17–66 μm long, 1–2.3 μm wide; Table 1, Fig. 2A). Statistically significant differences in cell volumes across isolates were identified (1-way ANOVA, $F = 64.516$, $df = 6$, $p < 0.001$), although isolates of *P. multiseriis* and *P. pungens* were not statistically different (Fig. 2A) with the exception of *P. pungens* isolate GGC3, which was 3 times greater in volume than other isolates and was significantly distinct from other isolates (post hoc Games-Howell test, $p < 0.01$). Isolates from both species were estimated to be 1 to 2 orders of magnitude larger in volume than *P. delicatissima* (Table 1).

The average DNA content for the 5 *P. pungens* isolates ranged from 0.61 to 0.93 pg DNA cell⁻¹, despite the larger cell volume of GGC3. *P. multiseriis* isolates on average contained twice as much DNA (1.48–1.63 pg DNA cell⁻¹) as *P. pungens* isolates and 3 times that of the *P. delicatissima* isolates (0.47 pg DNA cell⁻¹; Table 1, Fig. 2B). Isolate differences in DNA per cell were significant (1-way ANOVA, $F = 100.1$,

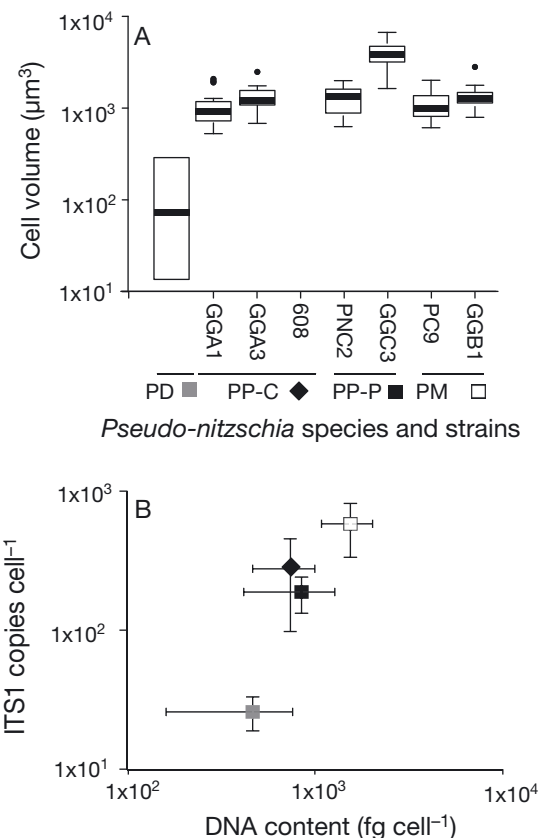


Fig. 2. Comparison of (A) cell volume (μm^3) and (B) DNA content (fg cell⁻¹) and ITS1 copies (cell⁻¹) for isolates of *Pseudo-nitzschia delicatissima* (PD), *P. pungens* var. *cingulata* (PP-C), *P. pungens* var. *pungens* (PP-P), and *P. multiseriis* (PM). Symbols corresponding to each species/variety are the same for both panels. In (A), literature cellular dimensions were used to estimate volume for PD isolates; for other isolates, estimates were derived from 20 measured cells. In (B), PP-C isolate 608 was used for DNA content and ITS1 copy estimates, but not for cell volume estimates. In all boxplots, bars = mean, boxes = 1st and 3rd quartiles, whiskers = 10th and 90th percentiles, and dots = outliers based on variation in estimates of cellular abundance ($n = 3$ per isolate for DNA content) or variation in qPCRs ($n = 27$ –36 PCRs per isolate for ITS1 copies per cell)

$df = 7$, $p < 0.001$), and DNA per cell was significantly different between isolates of *P. multiseriis* and *P. delicatissima*, and *P. multiseriis* and *P. pungens* (post hoc Tukey test, $p < 0.01$), but not among isolates from the same species or between certain isolates of *P. delicatissima* and *P. pungens*. A ratio of 3:2:1 for *P. delicatissima*:*P. pungens*:*P. multiseriis* was used to normalize DNA per cell between species. A log transformation for normality was applied to estimates of ITS1 copies per cell. Species differences in log-transformed ITS1 copies per cell were significant (1-way ANOVA, $F = 353.8$, $df = 2$, $p < 0.001$), and each species was significantly different from the other spe-

cies examined (post hoc Games-Howell test, $p < 0.01$). A positive relationship between DNA per cell and ITS copies per cell was observed (Fig. 2B); however, DNA per cell was not significantly correlated with cell length, width, or volume across isolates or species. On a per cell basis, *P. multiseriata* possessed about 30 times as many ITS1 copies as *P. delicatissima* and about 2.5 times as many as *P. pungens*. On a per DNA basis, this disparity was significantly reduced, and *P. multiseriata* possessed 6.5 times more ITS1 copies pg^{-1} DNA than *P. delicatissima*, and 1.5 times more than *P. pungens* (Table 1, Fig. 2B).

Species quantification with ARISA

Cloned ITS1 DNA standards or genomic DNA from *P. delicatissima*, *P. pungens*, or *P. multiseriata* isolates were added to PCRs to evaluate ARISA performance across a range of cellular equivalents (<1 cell to 10^4 cells). When the template mix was strongly skewed towards *P. delicatissima* genomic DNA (>80%), additional peaks (239 and 280 bp) representing <12% of ARISA fluorescence were detected. These fragment sizes do not correspond to known *Pseudo-nitzschia* species and were not observed in PCRs containing high proportions of cloned *P. delicatissima* ITS1 DNA; these peaks were removed and proportions were recalculated.

Whereas absolute ARISA peak heights were not correlated with the amount of species DNA in mixed samples ($R^2 < 0.2$, not significant), relative ARISA peak heights were positively correlated with relative proportions of *P. delicatissima*, *P. pungens*, and/or *P. multiseriata* DNA in mixed samples (Fig. 3; linear regression, $R^2 = 0.92, 0.88, 0.87$ for each species respectively, $p < 0.01$; $R^2 = 0.87$ for all species, $p < 0.01$). *P. multiseriata* comprised a greater proportion of the ARISA signal in mixed-species templates relative to similar DNA concentrations of *P. pungens* or *P. delicatissima* (Fig. 3). A bias towards *P. multiseriata* was expected, as this species has about 1.5 times as many ITS1 copies fg^{-1} DNA as *P. pungens* and about 6.5 times as many as *P. delicatissima* (Table 1). The observed relative ARISA peak heights were consistent with expected proportions and differed by up to an additional 10%. To evaluate the impact of known differences in the ITS1 fragment length and sequence on the ARISA signal, equivalent numbers ($10^1, 10^2, 10^3, 10^4$, or 10^5) of ITS1 standards from *P. multiseriata*, *P. pungens*, and *P. delicatissima* were combined for PCRs. Slight differences (1.4–5.5%) were observed in ITS1 amplification efficiency be-

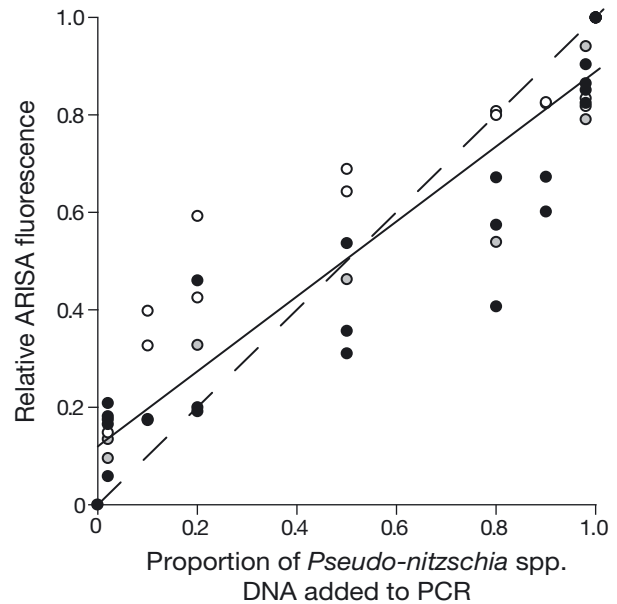


Fig. 3. Proportion of *Pseudo-nitzschia delicatissima* (○), *P. pungens* (●), and/or *P. multiseriata* (○) DNA added to PCRs and corresponding relative ARISA fluorescence. The linear regression (solid line; $y = 0.78x + 0.12$; $R^2 = 0.87$, $p < 0.01$) represents data derived from all 3 species. Dashed line indicates the 1:1 line

tween species, with *P. pungens* amplifying the most efficiently. Thus, the relative ARISA peak height was determined to be a robust indicator of the relative contribution of ITS1 copy numbers or DNA to the total signal for the different species.

ARISA characterization of field assemblages

Surface net tow samples collected throughout Puget Sound and the Strait of Juan de Fuca (Fig. 1) mostly consisted of mixed phytoplankton assemblages. *Pseudo-nitzschia* species were visually observed at all stations, except Stn P38 in Carr Inlet (South Sound), where a bloom of the diatom *Rhizosolenia* sp. dominated the phytoplankton community.

Pseudo-nitzschia species were identified with ARISA in each of the 39 discrete locations sampled, spanning surface and subsurface waters. Ten ARISA fragments were detected. Amplicon lengths 142, 144, 150, 168, 196, 202, 207, and 233, respectively, corresponded to *P. pungens*, *P. multiseriata*, *P. australis*/*P. seriata* (which share an ARISA amplicon size), *P. delicatissima*, *P. americana*, *P. fraudulenta*, *P. fryxelliana*, and *P. cuspidata* (Fig. 4A–C). Environmental ITS1 DNA sequences (from P4 and P30) confirmed the presence of 5 species: *P. pungens*, *P. multiseriata*,

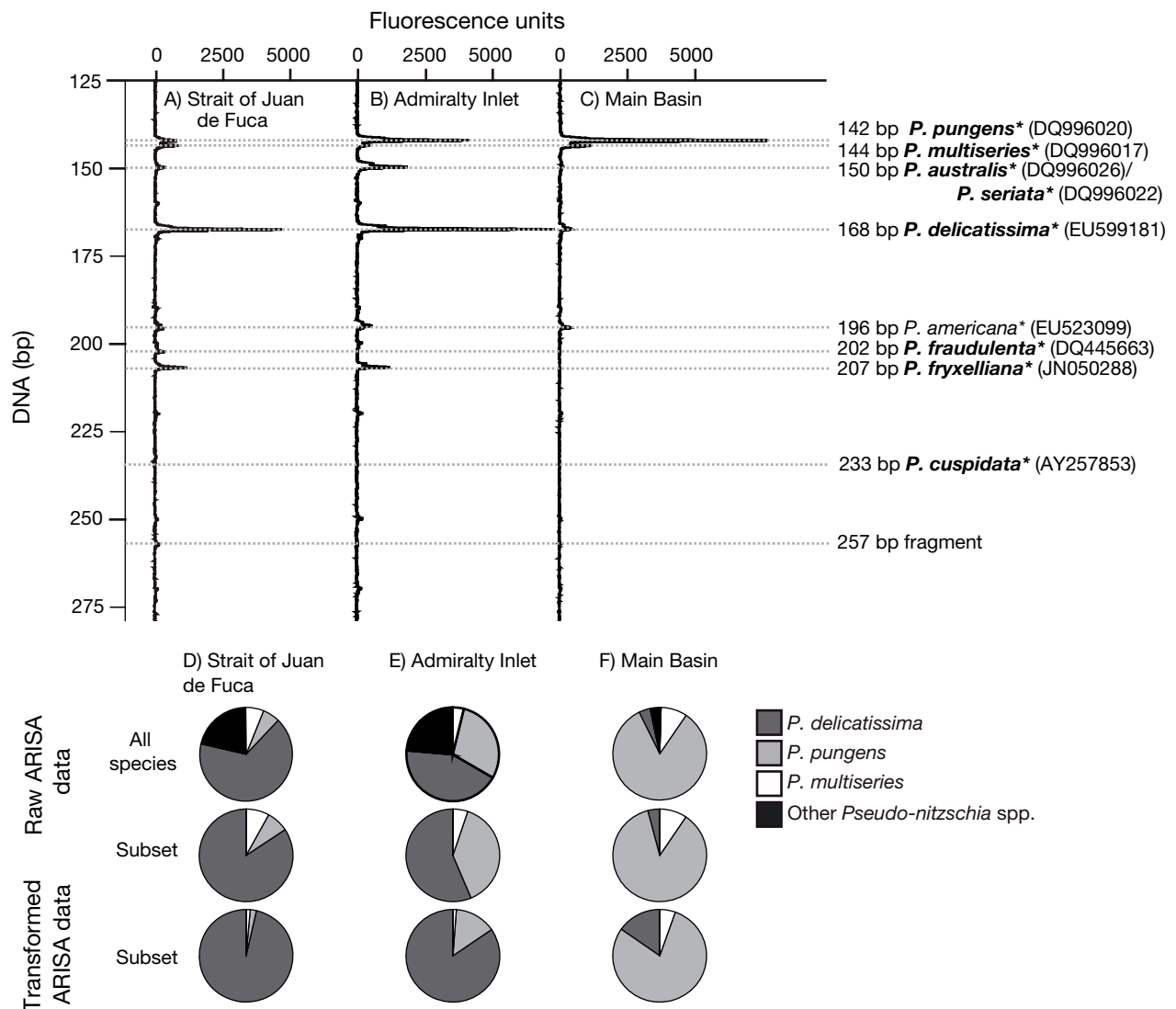


Fig. 4. ARISA profiles for (A) Stn P24 (Strait of Juan de Fuca), (B) Stn P19 (Admiralty Inlet), and (C) Stn P30 (Main Basin). GenBank accession numbers for representative sequences are listed for identified peaks in the ARISA profiles; peak position for *Pseudo-nitzschia cuspidata* is indicated. Species in **bold** and associated sequences were generated from electron microscopy-identified strains, and species with asterisks (*) have been identified in regional environmental sequence databases (Hubbard et al. 2008, present study). (D,E,F) Pie charts below each profile represent the relative proportion of *Pseudo-nitzschia* species in the ARISA profiles, based on relative ARISA peak heights (top: all species; middle: subset of *P. delicatissima*, *P. pungens*, and *P. multiseriis*) or data that were transformed (bottom: same subset), by applying a ratio of 6.5:1.5:1, respectively (predicted from ITS1 copies fg^{-1} DNA) to the ARISA signal to estimate relative species abundance

P. seriata, *P. delicatissima*, and *P. americana*. Rarefaction estimates indicated that species diversity was well, but not fully, sampled by sequencing (not shown). Genotypes for *P. delicatissima*, *P. multiseriis*, *P. americana*, *P. seriata*, *P. pungens* var. *pungens*, and *P. pungens* var. *cingulata* were identical to GenBank sequences from North Atlantic and/or North Pacific isolates. A single 150 bp sequence corresponded to *P. seriata*, and further discrimination between *P. australis*/*P. seriata* was not conducted. Additional

fragments (140 and 257 bp) were identified in a few ARISA profiles (Fig. 4A–C) but did not correspond to known *Pseudo-nitzschia* species and contributed less than 8% to total ARISA fluorescence; these peaks were excluded from subsequent analyses.

Relative ARISA peak heights indicated that 3 species dominated field samples: *P. delicatissima*, *P. pungens*, and to a lesser extent, *P. multiseriis* (Fig. 4D–F, top and middle panels; Fig. 5). Replicate surface samples collected from the same Niskin bot-

tile (Fig. 5) displayed an average Bray-Curtis similarity of 0.92 (ranging from 0.77–0.99), with an average species discrepancy of 2% (up to 16.6%). Greater variability was detected between replicates collected at subsurface depths (average Bray-Curtis similarity 0.79, ranging from 0.56 to 0.99), especially below 20 m (Fig. 5). When relative cell abundances for *P. delicatissima*, *P. pungens*, and *P. multiseriata* were estimated by normalizing to ITS1 copies fg^{-1} DNA (1:1.5:6.5 for *P. multiseriata*:*P. pungens*:*P. delicatissima*), the estimates for *P. delicatissima* accordingly increased by an average of 2.5 \times in each sample (Fig. 4D–F, bottom panel). Communities dominated by either the small-celled species, *P. delicatissima*, or the larger-celled species, *P. pungens*, were similar regardless of whether they were based on the relative ARISA signal or the transformed ARISA signal that approximates relative species abundance (Fig. 4). Because ITS1 copy number fg^{-1} DNA is unknown for most species, the more conservative proportions of ITS1 copies calculated directly from

ARISA data were utilized in subsequent descriptions of communities within Puget Sound, unless otherwise noted.

Environmental and community transitions in Puget Sound

Surface and subsurface samples for ARISA-based species identification were collected from waters that ranged across >9 salinity units, 12°C, >30 μM nitrate, ~3 μM ammonium, >75 μM silicate, >3.5 μM phosphate, and ~20 $\mu\text{g l}^{-1}$ chl *a* (see Table A1 in the Appendix). In general, the chl *a* maxima occurred at either the surface (top 2.5 m) or the nitracline (top 20 m), and decreased below 20 m to less than 1 $\mu\text{g l}^{-1}$. *P. pungens* and *P. delicatissima* were found at most sampling locations, whereas other species (*P. cuspidata*, *P. fraudulenta*, *P. multiseriata*, *P. australis/P. seriata*, *P. fryxelliana*, and *P. americana*) displayed more patchy distributions. Samples from the same

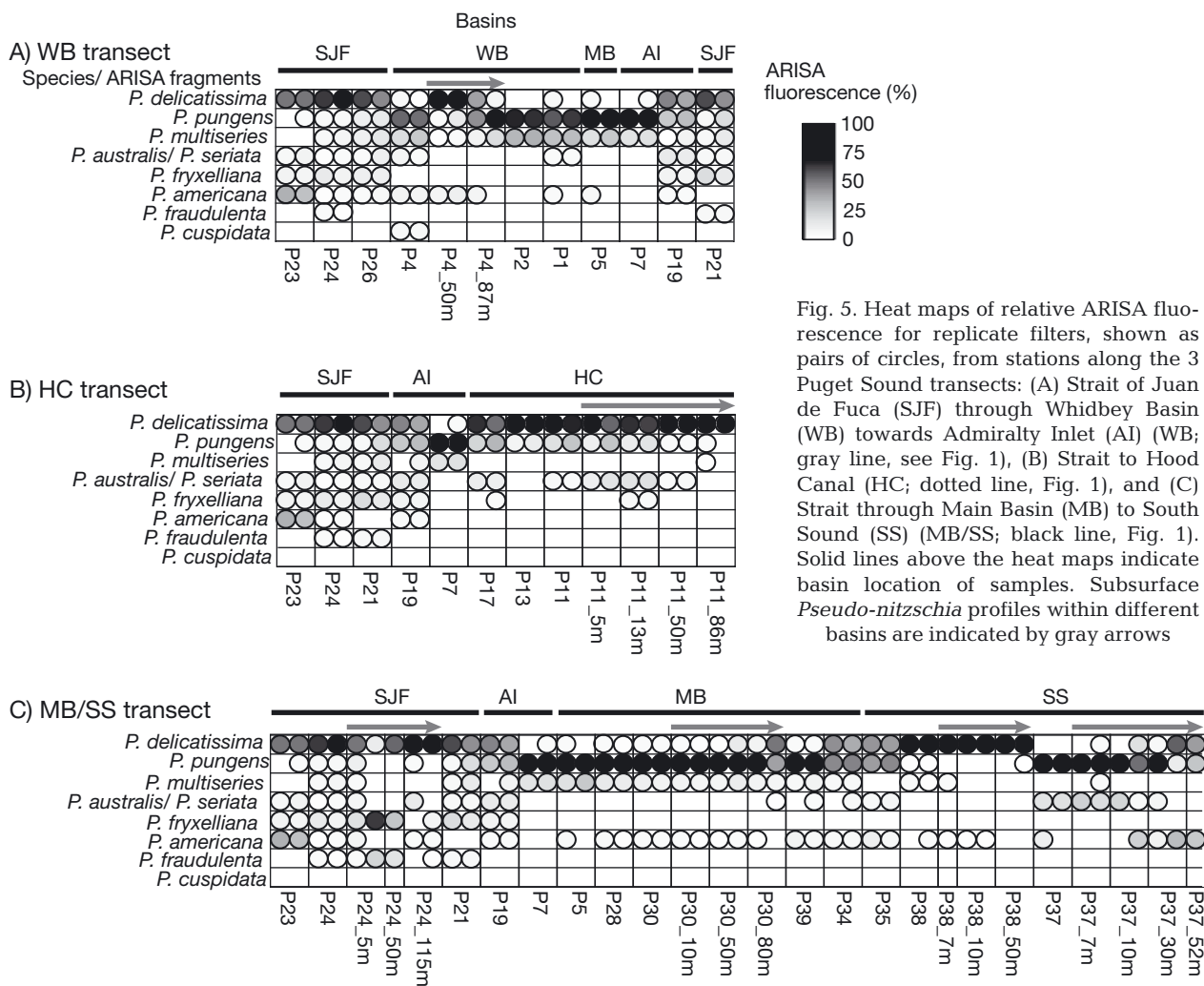


Fig. 5. Heat maps of relative ARISA fluorescence for replicate filters, shown as pairs of circles, from stations along the 3 Puget Sound transects: (A) Strait of Juan de Fuca (SJF) through Whidbey Basin (WB) towards Admiralty Inlet (AI) (WB; gray line, see Fig. 1), (B) Strait to Hood Canal (HC; dotted line, Fig. 1), and (C) Strait through Main Basin (MB) to South Sound (SS) (MB/SS; black line, Fig. 1). Solid lines above the heat maps indicate basin location of samples. Subsurface *Pseudo-nitzschia* profiles within different basins are indicated by gray arrows

basin generally exhibited similar species composition (global ANOSIM value of 0.416, $p < 0.01$).

Stations in the Strait and in northern Admiralty Inlet were generally saltier (29.4–30.5), colder (10.6–11.1°C), less stratified (Fig. 6A–C), and had higher nutrient concentrations and lower biomass than most of Puget Sound (Table A1). Surface and subsurface *Pseudo-nitzschia* communities from the Strait of Juan de Fuca and the northern Admiralty Inlet sill (Stn P19) were dominated by *P. delicatissima* (>60%;

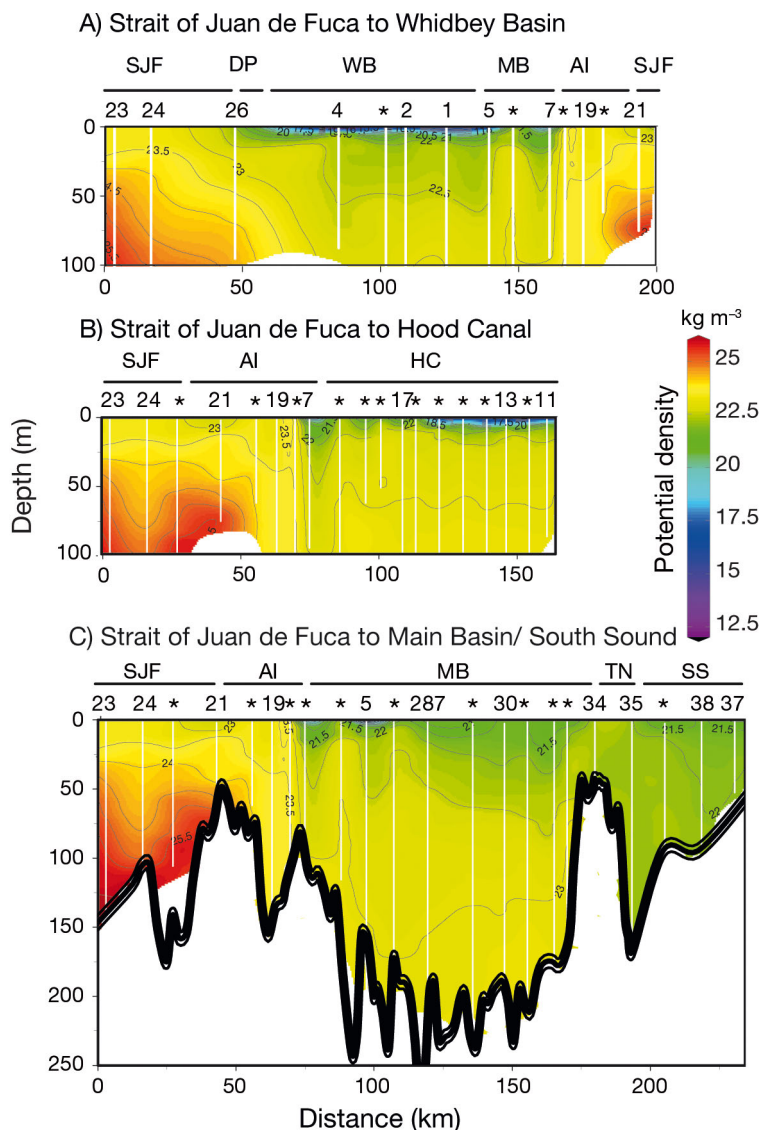


Fig. 6. Potential density along Puget Sound transects. (A) Strait of Juan de Fuca (SJF) to Whidbey Basin (WB), top 100 m; (B) Strait of Juan de Fuca to Hood Canal (HC), top 100 m; and (C) Strait of Juan de Fuca to Main Basin (MB)/South Sound (SS), with bathymetry (thick black lines). Vertical CTD profiles (shown by white lines) were collected at stations where DNA and environmental samples were collected (indicated by numbers) and where only environmental samples were collected (asterisks). AI: Admiralty Inlet; DP: Deception Pass; TN: Tacoma Narrows

Fig. 5A,C). This area was populated by all species except *P. cuspidata*, and represented the only location where *P. fraudulentus* was detected. Proportions of *P. fraudulentus* and *P. fryxelliana* were highest at Stns P24, at 50 m (19 and 46% respectively; Fig. 5C).

Sharp transitions to Puget Sound's more estuarine conditions occurred at the northern shallow sills, Admiralty Inlet and Deception Pass (Fig. 6A–C). A $\sim 2 \text{ kg m}^{-3}$ decrease below 50 m depth and a 2.7 kg m^{-3} decrease in surface density occurred respectively at the northern and southern Admiralty Inlet sills (Fig. 6C). Density on either side of Deception Pass, a narrow, shallow passage connecting the Strait of Juan de Fuca to Whidbey Basin, varied by $>3 \text{ kg m}^{-3}$ at the surface and 1.5 kg m^{-3} at depth (Fig. 6A). These transitions occurred across $<20 \text{ km}$ and were complemented by corresponding shifts in nutrient concentrations, biomass, and the dominant *Pseudo-nitzschia* species (Table A1, Fig. 5A,C). Communities near these well-mixed sill regions were composed of more similar proportions of *P. pungens* and *P. delicatissima*.

Much of Puget Sound was dominated by *P. pungens*, including Whidbey Basin, Main Basin, and parts of South Sound and Hood Canal (Fig. 5A–C). At the surface, Whidbey Basin outflows into the central Main Basin and then into Admiralty Inlet (transect WB; Figs. 1, 5A, & 6A). The highest chl *a* biomass was observed east of Deception Pass, in Whidbey Basin at Stn P4 ($20 \mu\text{g l}^{-1}$), where surface communities were populated by at least 7 *Pseudo-nitzschia* species including *P. cuspidata* (Fig. 5A). Nearly the warmest (15.8°C) and coldest (9.1°C) waters sampled within Puget Sound were observed at this stratified station (Table A1, Fig. 6A). Fewer species were observed below the thermocline, where silicate concentrations were in excess of $80 \mu\text{M}$ (nearly twice the levels detected at less stratified locations), and nitrate concentrations were 8 to $10 \mu\text{M}$ greater than at less stratified stations (Table A1). The other Whidbey and Main Basin stations along the WB transect were similarly dominated by *P. pungens* and populated by *P. delicatissima*, *P. americana*, and *P. multiseriata* (Fig. 5C).

Surface waters in South Sound and the Main Basin generally flow seaward (transect MB/SS). Within South Sound, Carr and Case Inlets were respectively dominated by *P. delicatissima* or *P. pungens* (Fig. 5C). Neither inlet was strongly stratified (Fig. 6C). In Carr Inlet (P38), a *Rhizosolenia* bloom was observed, and *P. delicatissima* represented >90% of surface and subsurface ARISA profiles there (Fig. 5C). In nearby Case Inlet (P37), *P. pungens* dominated, and *P. australis/P. seriata* (5–25%) and *P. americana* (9–24%) were also present. These species comprised a greater fraction of the community at 7 and 10 m (Fig. 5C), where ammonium concentrations were nearly 3 μM , compared to the near-zero values observed in most of Puget Sound (Table A1). Near the Tacoma Narrows sill (P39, P34, P35), roughly equal proportions of *P. pungens* and *P. delicatissima* and low proportions of *P. australis/P. seriata* (Fig. 5C) were observed and were clearly distinct from the *P. pungens*-dominated communities observed farther north in the Main Basin. In the central Main Basin, weak vertical stratification was observed (Fig. 6C), and communities were generally similar across different depths at Stn P30 (Fig. 5C); the only observation of *P. australis/P. seriata* was observed only at 80 m.

The surface waters of the westernmost basin, Hood Canal (transect HC; Fig. 1), were dominated by *P. delicatissima* (>40%) at the northern (P17) and southern (P11) stations (Fig. 5B); *P. pungens* and *P. australis/P. seriata* were also observed at both locations. A *Phaeocystis* sp. bloom occurred mid-canal (P13), with single cells of a small cell-type spe-

cies of *Pseudo-nitzschia* suspended within the gelatinous matrix surrounding *Phaeocystis* colonies. At P13, *P. delicatissima* comprised >90% of the *Pseudo-nitzschia* ARISA fluorescence (Fig. 5B). In central and south Hood Canal, a relatively warm ($\leq 19.8^\circ\text{C}$), fresh (<24.9 psu) surface layer (top 20 m; Fig. 6B) overlaid denser waters that were >10°C colder, >5 saltier, and more nutrient-rich (Table A1). The only occurrence of *P. fryxelliana* within Puget Sound was at the northern Hood Canal station (P17) and at the subsurface chl *a* maximum (13 m) at Stn P11 (Fig. 5B). The only occurrence of *P. multiseriis* in Hood Canal was at 86 m.

Species were significantly correlated with 1 to 8 of the 15 possible environmental factors examined, except for *P. cuspidata*, which was only detected in 1 sample (Table 2). *P. delicatissima* dominated low biomass assemblages at both ends of the observed temperature/salinity/nutrient gradient, and increased relative to other species below the euphotic zone in every basin (Fig. 5). This species was negatively correlated with biomass, whereas *P. pungens* and *P. multiseriis* were positively correlated (Table 2). *P. delicatissima*, *P. fryxelliana*, and *P. fraudulenta* were significantly correlated with high salinity and low oxygen concentrations. With the exception of *P. australis/P. seriata*, which was positively associated with ammonium concentration, correlations between individual species and standing stock nutrient concentrations were not detected (Table 2). Five species demonstrated varied associations with the ratio of nitrate or silicate to other nutrients, especially phosphate (Table 2).

Table 2. Significant Spearman's correlation coefficients for bivariate comparisons of *Pseudo-nitzschia* species and environmental parameters are shown for * $p < 0.05$ and ** $p < 0.01$

	<i>P. pun- gens</i>	<i>P. multi- series</i>	<i>P. australis/ P. seriata</i>	<i>P. deli- catissima</i>	<i>P. fryxel- liana</i>	<i>P. fraud- ulenta</i>	<i>P. amer- icana</i>	<i>P. cus- pidata</i>
Oxygen	0.43**	0.32*	–	–0.38*	–0.46**	–0.42**	–	–
Salinity	–0.52**	–	–	0.39*	0.66**	0.55**	–	–
Temperature	0.36*	–	–	–	–0.50**	–0.45**	–	–
Chlorophyll <i>a</i>	0.63**	0.48**	–	–0.59**	–	–	–	–
Nitrate:silicate	–	–0.33*	–	–	–	–	–	–
Nitrate:phosphate	–	0.37*	–	–	0.42**	0.42**	0.37*	–
Nitrate:ammonium	–0.34*	–	–0.40*	0.39*	–	–	–	–
Phosphate:ammonium	–	–	–0.37*	–	–	–	–	–
Silicon:phosphate	–	–	–0.41**	–	–0.48**	–0.42**	–	–
Silicon:ammonium	–	0.32*	–0.32*	–	–	–	–	–
Silicon:nitrate	–	–	–	–	–	–0.44**	–	–
Ammonium	–	–	0.39*	–	–	–	–	–
Ammonium:silicate	–	–	0.41**	–	–	–	–	–
Ammonium:nitrate	–	–	0.41*	–	–	–	–	–
Ammonium:phosphate	–	–	0.39*	–	–	–	–	–

portions of individual species based on ITS1 copies were highly reproducible when used with the standardized protocols developed for amplification of culture and environmental templates. The sensitivity of the ARISA was demonstrated through successful detection of dilution series of *Pseudo-nitzschia* DNA or ITS1 standards spanning 5 orders of magnitude. Cell size, DNA content, and ITS1 copy number varied across the 3 species most prevalent in Puget Sound during sampling, viz. *P. multiseriis*, *P. delicatissima*, and *P. pungens*. Despite this variation, normalizing ITS1 abundance relative to any of these factors provided relatively little significant improvement in determining trends in proportional abundances across environmental gradients in the present study. Because cellular DNA content and ITS1 copy numbers are not yet known for most *Pseudo-nitzschia* species, the more conservative estimator of relative ITS1 copies can be used as a proxy for relative cell abundance in environmental ARISA. The transition to using more quantitative ARISA data allowed greater insight into the ecology of individual *Pseudo-nitzschia* species in Puget Sound.

P. delicatissima was detected with ARISA in nearly every sample. The Puget Sound *P. delicatissima* was genetically identical to strains isolated from the temperate waters of Denmark (Quijano-Scheggia et al. 2009) and the Bay of Fundy (Kaczmarek et al. 2008). In our study, this species was distributed throughout the water column and comprised >90% of the *Pseudo-nitzschia* community at locations where nearly monospecific phytoplankton blooms were observed. For example, the cells embedded within *Phaeocystis* colonies in Hood Canal were identified as *P. delicatissima*, as has also been described by Sazhin et al. (2007). The colonial matrix of *Phaeocystis* cells may provide protection from grazers, and accumulates certain nutrients and trace metals (see Schoemann et al. 2005). Isolates of *P. delicatissima* appear able to use organic and inorganic substances produced by other phytoplankton to maintain their growth rate (Loureiro et al. 2009). Thus, it is possible that diverse processes (e.g. reduced grazing, flexible nutrient requirements) could contribute to the spatial and/or temporal persistence of *P. delicatissima* in Puget Sound and other temperate estuaries (Kaczmarek et al. 2008, Klein et al. 2010).

P. pungens was also detected throughout Puget Sound. Environmental ITS1 sequences corresponding to the globally distributed *P. pungens* var. *pungens* and the more regionally localized *P. pungens* var. *cingulata* were both detected in 2 adjoining Puget Sound basins, based on ITS1 sequencing of environmental

DNA. An additional ITS1 variant of *P. pungens* var. *cingulata* was detected and appeared to be more prevalent in Whidbey Basin than Main Basin. Sexual hybridization between the *P. pungens* varieties has been documented both in culture (Casteleyn et al. 2009) and in the field (Holtermann et al. 2010), and the 3 dominant *P. pungens* var. *pungens*/*P. pungens* var. *cingulata* genotypes observed in Puget Sound were detected in 1 presumably hybridized strain of *P. pungens* isolated from the Washington coast (NA233, Casteleyn et al. 2009). *P. pungens* var. *pungens* and *P. pungens* var. *cingulata* have previously been detected together in summer samples from the coast of British Columbia (Canada) and the offshore Juan de Fuca eddy (Casteleyn et al. 2009). In contrast, a November 2004 sample from Puget Sound contained only genotypes corresponding to *P. pungens* var. *cingulata* (Hubbard et al. 2008). The coexistence of at least 3 distinct ITS1 types within summer communities in Puget Sound, and the potential maintenance of only *P. pungens* var. *cingulata* within winter communities, demonstrates that intraspecific diversity in *P. pungens* is dynamic in space/time. Furthermore, the genotypes associated with *P. pungens* likely reflect physiologically distinct populations, as has been observed in Puget Sound populations of the diatom *Ditylum brightwellii* (Rynearson et al. 2006). Although *P. multiseriis* and *P. pungens* were similarly correlated with some environmental variables, less intraspecific diversity has been observed, regionally and globally, within *P. multiseriis* (Hubbard et al. 2008, present study).

The community at depth in the Strait of Juan de Fuca was distinct from other locations in Puget Sound. *P. multiseriis*, although present in surface waters, was absent at depth, and the relative contribution from *P. pungens* was minimal (observed in only 1 replicate at 115 m depth). Instead, relatively high proportions of *P. fraudulenta* and *P. fryxelliana* were observed; these species were significantly correlated with salinity, and were likely advected inland from coastal waters via estuarine circulation. The detection of the corresponding ITS1 fragment (207 bp) for *P. fryxelliana* in previously generated ARISA data from the coastal and offshore waters of the eastern Pacific (Hubbard et al. 2008, Ribalet et al. 2010) highlights the sensitivity of ARISA to specifically detect known and novel species diversity. Although *P. fryxelliana* was positively correlated with salinity, its presence in the relatively fresh 13 m Hood Canal sample (Stn P11), and in summer communities from the offshore iron-limited waters of the Pacific (described as *P. sp. 207*; Ribalet et al. 2010) and the west coast of Vancouver Island, British

Columbia (Hubbard et al. 2008), suggests that this recently described species can persist across a broad salinity range. *P. fraudulenta* has previously been observed in estuarine waters north of our survey locations (Horner et al. 1997, Rines et al. 2002), in Puget Sound (Stehr et al. 2002), as well as off the coast of Vancouver Island (Hubbard et al. 2008). This species, observed only in the Strait of Juan de Fuca and northern Admiralty Inlet in the present study, may have a lower tolerance for estuarine conditions than other *Pseudo-nitzschia* species, in which case the distribution of this species may have been restricted by the lower-than-average salinity in Puget Sound during 2006 (Hickey et al. 2009, Sutherland et al. 2011).

P. australis or *P. cuspidata* typically dominate toxic *Pseudo-nitzschia* blooms in Puget Sound and coastal waters of Washington (Trainer et al. 2007), although regional isolates of *P. multiseriata* produce high DA levels in culture (Guannel et al. 2011), and *P. seriata* isolates elsewhere are toxic (Fehling et al. 2004, Fernandes et al. 2014). Our cruise samples from June 2006 were collected 9 mo after separate toxic blooms of *P. cuspidata* and *P. australis* in Puget Sound resulted in shellfish harvest closures (Trainer et al. 2007). During the cruise, *P. cuspidata* was only detected in Whidbey Basin and represented a minor fraction of the community. The *P. australis*/*P. seriata* complex was detected at regions of enhanced mixing, at depth in Main Basin, and in South Sound's Case Inlet, where ammonium concentrations were elevated during the cruise and can exceed $39 \mu\text{mol l}^{-1}$ due to anthropogenic input (www.ecy.wa.gov/). The abundance of *P. australis* in San Francisco Bay (Armstrong-Howard et al. 2007) and *P. seriata* in Scottish waters (Fehling et al. 2006) was positively associated with ammonium concentration, similar to our observations in Puget Sound. During our survey, low DA concentrations (1 ppt) were detected only in shellfish from a harbor in southern Main Basin nearest station P34 (Quartermaster Harbor on Vashon Island; J. Borchert pers. comm.). It is not clear whether the low levels of DA in shellfish could be attributed to low abundance and/or toxicity of *P. australis*/*P. seriata* or to less toxic species such as *P. pungens* or *P. delicatissima*.

It is interesting to consider the spatial and temporal maintenance of *Pseudo-nitzschia* species within Puget Sound, given the influx of coastal waters at depth that provide a source of new diversity into this estuarine system, and the surface outflow of estuarine waters and diversity that can become entrained in alongshore (Hickey et al. 1991) or cross-shelf coastal currents (Mac Fadyen et al. 2005) at the mouth of the Strait of Juan de Fuca. In contrast to the typical

inland flow that occurs at depth, surface coastal waters and their associated phytoplankton communities can be transported into Puget Sound when strong onshore winds cause an overturn of estuarine circulation in the Strait of Juan de Fuca (Hickey et al. 1991). Within a Danish fjord, a genetically stable population of the diatom *Skeletonema marionoi*—differentiated from the oceanic *S. marionoi* population—has been maintained across thousands of generations, despite a lack of strong physical dispersal barriers (Härnström et al. 2011). Cells of the diatom *S. marionoi* form resting stages, which can remain viable within sediment for 100 yr or more (Härnström et al. 2011). Sexual reproduction has been demonstrated for numerous *Pseudo-nitzschia* species, and thin layers of *Pseudo-nitzschia* spp. have been observed in a northern Puget Sound inlet in and below the euphotic zone (Rines et al. 2002); however, limited information is available on the formation and/or viability of sediment resting stages by different species (Zhang et al. 2010, Lelong et al. 2012).

Although stratified, estuarine circulation dominates throughout Puget Sound and the Strait, vertical tidal mixing causes recirculation of waters at shallow sills that separate some basins and sub-basins. Accordingly, residence times for basins and sub-basins in Puget Sound are estimated to vary from less than 1 d to over 180 d (Babson et al. 2006, Sutherland et al. 2011). Some species, including *P. pungens*, *P. delicatissima*, *P. multiseriata*, *P. australis*/*P. seriata*, and *P. fryxelliana*, were detected in the deep waters of basins such as Hood Canal and Whidbey Basin that experience persistent stratification with residence times that span several seasons. Assuming the deep (>50 m) subsurface cells remain viable until re-introduction to the euphotic zone, they have the potential to continuously reseed the species (and genetic) pool in Puget Sound, counteracting the strong winds and tidal and freshwater exchange that over the course of a few days can drive extensive horizontal advection of planktonic communities into or out of Puget Sound (Holbrook et al. 1980, Hickey et al. 1991, Edwards et al. 2007, Moore et al. 2008). Thus, it is likely that species assembly and coexistence throughout Puget Sound is controlled by a combination of stochastic and resource-driven processes. The relative contribution of these different processes can now be considered over broader temporal and spatial scales to better model the physical connectivity of *Pseudo-nitzschia* communities and populations in coastal and inland waters of Washington and their roles in the initiation and maintenance of toxic blooms.

The sensitive molecular detection capabilities implemented here allowed the characterization of diverse *Pseudo-nitzschia* assemblages and a dynamic range of associations between species and environmental conditions from a comprehensive sampling snapshot of Puget Sound. Our study adds to a growing database of studies that identify hypothesized factors related to succession in *Pseudo-nitzschia* species composition across varied spatial or temporal gradients (Almandoz et al. 2008, Kaczmarek et al. 2007, Klein et al. 2010, Olson & Lessard 2010, Ribalet et al. 2010). The integration of species-specific, quantitative approaches such as ARISA into monitoring and research programs may be sufficient to tease apart taxa-specific associations with a matrix of factors involved in the growth and ecology of individual taxa of interest, including greater resolution of the bottom-up and top-down processes that promote HAB formation, toxin production, and bloom decline. Given the taxonomic diversity observed in planktonic field assemblages and even within this single genus, it is interesting to consider the respective ecological and biogeochemical implications of size, DNA content, or cell-based abundance estimates. A compelling question that requires more in-depth consideration is whether some phytoplankton species or genotypes act as biomarkers of distinct physical or chemical environmental conditions which could be informative especially for early HAB detection.

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LITERATURE CITED

- Almandoz GO, Ferreyra GA, Schloss IR, Dogliotti AI and others (2008) Distribution and ecology of *Pseudo-nitzschia* species (Bacillariophyceae) in surface waters of the Weddell Sea (Antarctica). *Polar Biol* 31:429–442
- Amato A, Kooistra WHCF, Ghiron JHL, Mann DG, Pröschold T, Montresor M (2007) Reproductive isolation among sympatric cryptic species in marine diatoms. *Protist* 158: 193–207
- Armstrong-Howard MD, Cochlan WP, Ladizinsky N, Kudela RM (2007) Nitrogenous preference of toxigenic *Pseudo-nitzschia australis* (Bacillariophyceae) from field and laboratory experiments. *Harmful Algae* 6:206–217
- Babson AL, Kawase A, MacCready P (2006) Seasonal and interannual variability in the circulation of Puget Sound, Washington: a box model study. *Atmos-Ocean* 44:29–45
- Bates SS (1998) Ecophysiology and metabolism of ASP toxin production. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) *Physiological ecology of harmful algal blooms*. Springer-Verlag, Heidelberg, p 405–426
- Bray JR, Curtis JT (1957) An ordination of the upland forest communities of Southern Wisconsin. *Ecol Monogr* 27: 325–349
- Brown MV, Schwalbach MS, Hewson I, Fuhrman JA (2005) Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environ Microbiol* 7:1466–1479
- Carpenter JH (1965) The Chesapeake Bay Institute technique for the Winkler dissolved oxygen method. *Limnol Oceanogr* 10:141–143
- Casteleyn G, Evans KM, Backeljau T, D'Hondt S, Chepurnov V, Sabbe K, Vyverman W (2009) Lack of population genetic structuring in the marine planktonic diatom *Pseudo-nitzschia pungens* (Bacillariophyceae) in a heterogeneous area in the Southern Bight of the North Sea. *Mar Biol* 156:1149–1158
- Clarke KR, Warwick RM (2001) Changes in marine communities: an approach to statistical analysis and interpretation. PRIMER-E, Plymouth
- Colwell RK (2005) EstimateS: statistical estimation of species richness and shared species from samples. Available at <http://purl.oclc.org/estimates>
- Edwards K, Kawase M, Sarason C (2007) Circulation in Carr Inlet, Puget Sound, during Spring 2003. *Estuaries Coasts* 30:945–958
- Fehling J, Davidson K, Bolch CJ, Bates SS (2004) Growth and domoic acid production by *Pseudo-nitzschia seriata* (Bacillariophyceae) under phosphate and silicate limitation. *J Phycol* 40:674–683
- Fehling J, Davidson K, Bolch C, Tett P (2006) Seasonality of *Pseudo-nitzschia* spp. (Bacillariophyceae) in western Scottish waters. *Mar Ecol Prog Ser* 323:91–105
- Fernandes LF, Hubbard KA, Richlen M, Smith J and others (2014) Diversity and toxicity of the diatom *Pseudo-nitzschia* Peragallo in the Gulf of Maine, Northwestern Atlantic. *Deep-Sea Res II (Spec edn)* 103:139–162
- Fuentes MS, Wikfors GH (2013) Control of domoic acid toxin expression in *Pseudo-nitzschia multiseriata* by copper and silica: relevance to mussel aquaculture in New England (USA). *Mar Environ Res* 83:23–28
- Guannel ML, Horner-Devine MC, Rocap G (2011) Bacterial community composition differs with species and toxicity of the diatom *Pseudo-nitzschia*. *Aquat Microb Ecol* 64:117–133
- Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) *Culture of marine invertebrate animals*. Plenum Press, New York, NY, p 26–60
- Harnstrom K, Ellegaard M, Andersen TJ, Godhe A (2011) Hundred years of genetic structure in a sediment revived

- diatom population. *Proc Natl Acad Sci* 108:4252–4257
- Hasle GR (2002) Are most of the domoic acid-producing species of the diatom genus *Pseudo-nitzschia* cosmopolites? *Harmful Algae* 1:137–146
- Hickey BM, Thomson RE, Yih H, Leblond PH (1991) Velocity and temperature-fluctuations in a buoyancy-driven current off Vancouver Island. *J Geophys Res Oceans* 96: 10507–10538
- Hickey B, McCabe R, Geier S, Dever E, Kachel N (2009) Three interacting freshwater plumes in the northern California Current System. *J Geophys Res Oceans* 114, doi: 10.1029/2008JC004907
- Holbrook JR, Muench RD, Kachel DG, Wright C (1980) Circulation in the Strait of Juan de Fuca: recent oceanographic observations in the Eastern Basin. NOAA Tech Rep ERL 412-PMEL 33. Pacific Marine Environmental Laboratory, Seattle, WA
- Holtermann KE, Bates SS, Trainer VL, Odell A, Armbrust EV (2010) Mass sexual reproduction in the toxigenic diatoms *Pseudo-nitzschia australis* and *P. pungens* (Bacillariophyceae) on the Washington coast, USA. *J Phycol* 46:41–52
- Horner RA, Garrison DL, Plumley FG (1997) Harmful algal blooms and red tide problems on the US west coast. *Limnol Oceanogr* 42:1076–1088
- Hubbard KA, Rocap G, Armbrust EV (2008) Inter- and intraspecific community structure within the diatom genus *Pseudo-nitzschia* (Bacillariophyceae). *J Phycol* 44: 637–649
- Kaczmarek I, Martin JL, Ehrman JM, LeGresley MM (2007) *Pseudo-nitzschia* species population dynamics in the Quoddy Region, Bay of Fundy. *Harmful Algae* 6:861–874
- Kaczmarek I, Reid C, Martin JL, Moniz MJB (2008) Morphological, biological, and molecular characteristics of the diatom *Pseudo-nitzschia delicatissima* from the Canadian Maritimes. *Botany* 86:763–772
- Klein C, Claquin P, Bouchart V, Le Roy B, Veron B (2010) Dynamics of *Pseudo-nitzschia* spp. and domoic acid production in a macrotidal ecosystem of the eastern English Channel (Normandy, France). *Harmful Algae* 9:218–226
- Lefebvre KA, Robertson A (2010) Domoic acid and human exposure risks: a review. *Toxicol* 56:218–230
- Lelong A, Hégaret H, Soudant P, Bates SS (2012) *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms. *Phycologia* 51:168–216
- Levene H (1960) Robust tests for equality of variances. In: Olkin I, Ghurye SG, Hoeffding W, Madow WG, Mann HB (eds) *Contributions to probability and statistics: essays in honor of Harold Hotelling*. Stanford University Press, Stanford, CA, p 278–292
- Lim HC, Leaw CP, Su SNP, Teng ST and others (2012) Morphology and molecular characterization of *Pseudo-nitzschia* (Bacillariophyceae) from Malaysian Borneo, including the new species *Pseudo-nitzschia circumpora* sp. nov. *J Phycol* 48:1232–1247
- Lim HC, Teng ST, Leaw CP, Lim PT (2013) Three novel species in the *Pseudo-nitzschia pseudodelicatissima* complex: *P. batesiana* sp. nov., *P. lundholmiae* sp. nov., and *P. fukuyoi* sp. nov. (Bacillariophyceae) from the Strait of Malacca, Malaysia. *J Phycol* 49:902–916
- Loureiro S, Jauzein C, Garcés E, Collos Y, Camp J, Vaqué D (2009) The significance of organic nutrients in the nutrition of *Pseudo-nitzschia delicatissima* (Bacillariophyceae). *J Plankton Res* 31:399–410
- Lueders T, Friedrich MW (2003) Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. *Appl Environ Microb* 69: 320–326
- Lundholm N, Hansen PJ, Kotaki Y (2004) Effect of pH on growth and domoic acid production by potentially toxic diatoms of the genera *Pseudo-nitzschia* and *Nitzschia*. *Mar Ecol Prog Ser* 273:1–15
- Lundholm N, Clarke A, Ellegaard M (2010) A 100-year record of changing *Pseudo-nitzschia* species in a sillfjord in Denmark related to nitrogen loading and temperature. *Harmful Algae* 9:449–457
- Lundholm N, Bates SS, Baugh KA, Bill BD, Connell LB, Léger C, Trainer VL (2012) Cryptic and pseudo-cryptic diversity in diatoms with descriptions of *Pseudo-nitzschia hasleana* sp. nov. and *P. fryxelliana* sp. nov. *J Phycol* 48: 436–454
- MacFadyen A, Hickey BM, Foreman MGG (2005) Transport of surface waters from the Juan de Fuca eddy region to the Washington coast. *Cont Shelf Res* 25:2008–2021
- Magurran AE (2004) *Measuring biological diversity*. Blackwell Publishing, Oxford
- Marchetti A, Lundholm N, Kotaki Y, Hubbard K, Harrison PJ, Armbrust EV (2008) Identification and assessment of domoic acid production in oceanic *Pseudo-nitzschia* (Bacillariophyceae) from iron-limited waters in the northeast subarctic Pacific. *J Phycol* 44:650–661
- McCune B, Grace JB (2002) *Analysis of ecological communities*. MjM Software, Gleneden Beach, OR
- Moore SK, Mantua NJ, Newton JA, Kawase M, Warner MJ, Kellogg JR (2008) A descriptive analysis of temporal and spatial patterns of variability in Puget Sound oceanographic properties. *Estuar Coast Shelf Sci* 80:545–554
- Olson MB, Lessard EJ (2010) The influence of the *Pseudo-nitzschia* toxin, domoic acid, on microzooplankton grazing and growth: a field and laboratory assessment. *Harmful Algae* 9:540–547
- Orive E, Perez-Aicua L, David H, Garcia-Etxebarria K, Laza-Martinez A, Seoane S, Miguel I (2013) The genus *Pseudo-nitzschia* (Bacillariophyceae) in a temperate estuary with description of two new species: *Pseudo-nitzschia plurisecta* sp. nov. and *Pseudo-nitzschia abrensis* sp. nov. *J Phycol* 49:1192–1206
- Orsini L, Procaccini G, Sarno D, Montresor M (2004) Multiple rDNA ITS-types within the diatom *Pseudo-nitzschia delicatissima* (Bacillariophyceae) and their relative abundances across a spring bloom in the Gulf of Naples. *Mar Ecol Prog Ser* 271:87–98
- Penna A, Casabianca S, Perini F, Bastianini M, Riccardi E, Pigozzi S, Scardi M (2013) Toxic *Pseudo-nitzschia* spp. in the northwestern Adriatic Sea: characterization of species composition by genetic and molecular quantitative analyses. *J Plankton Res* 35:352–366
- Perl TM, Bedard L, Kosatsky T, Hockin JC, Todd ECD, Remis RS (1990) An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *N Engl J Med* 322:1775–1780
- Quijano-Scheggia SI, Garcés E, Lundholm N, Moestrup O, Andree K, Campi J (2009) Morphology, physiology, molecular phylogeny and sexual compatibility of the cryptic *Pseudo-nitzschia delicatissima* complex (Bacillariophyta), including the description of *P. arenysensis* sp. nov. *Phycologia* 48:492–509
- Ribalet F, Marchetti A, Hubbard KA, Brown K and others

- (2010) Unveiling a phytoplankton hotspot at a narrow boundary between coastal and offshore waters. *Proc Natl Acad Sci USA* 107:16571–16576
- Rines JEB, Donaghay PL, Deksheniaks MM, Sullivan JM, Twardowski MS (2002) Thin layers and camouflage: hidden *Pseudo-nitzschia* spp. (Bacillariophyceae) populations in a fjord in the San Juan Islands, Washington, USA. *Mar Ecol Prog Ser* 225:123–137
- Rynearson TA, Newton JA, Armbrust EV (2006) Spring bloom development, genetic variation, and population succession in the planktonic diatom *Ditylum brightwellii*. *Limnol Oceanogr* 51:1249–1261
- Sazhin AF, Artigas LF, Nejtgaard JC, Frischer ME (2007) The colonization of two *Phaeocystis* species (Prymnesiophyceae) by pennate diatoms and other protists: a significant contribution to colony biomass. *Biogeochemistry* 83:137–145
- Schlitzer R (2012) Ocean Data View. <http://odv.awi.de> (accessed June 2012)
- Schoemann V, Becquevort S, Stefels J, Rousseau V, Lancelot C (2005) *Phaeocystis* blooms in the global ocean and their controlling mechanisms: a review. *J Sea Res* 53:43–66
- Shapiro SS, Wilk MB, Chen HJ (1968) A comparative study of various tests for normality. *J Am Stat Assoc* 63:1343–1372
- Stehr CM, Connell L, Baugh KA, Bill BD, Adams NG, Trainer VL (2002) Morphological, toxicological, and genetic differences among *Pseudo-nitzschia* (Bacillariophyceae) species in inland embayments and outer coastal waters of Washington state, USA. *J Phycol* 38:55–65
- Sutherland DA, MacCready P, Banas NS, Smedstad LF (2011) A model study of the Salish Sea estuarine circulation. *J Phys Oceanogr* 41:1125–1143
- Tatters AO, Fu FX, Hutchins DA (2012) High CO₂ and silicate limitation synergistically increase the toxicity of *Pseudo-nitzschia fraudulenta*. *PLoS ONE* 7:e32116
- Terseleer N, Gypens N, Lancelot C (2013) Factors controlling the production of domoic acid by *Pseudo-nitzschia* (Bacillariophyceae): a model study. *Harmful Algae* 24:45–53
- Trainer VL, Cochlan WP, Erickson A, Bill BD, Cox FH, Borchert JA, Lefebvre KA (2007) Recent domoic acid closures of shellfish harvest areas in Washington State inland waterways. *Harmful Algae* 6:449–459
- Trainer VL, Bates SS, Lundholm N, Thessen AE, Cochlan WP, Adams NG, Trick CG (2012) *Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring and impacts on ecosystem health. *Harmful Algae* 14:271–300
- UNESCO (United Nations Educational, Scientific, and Cultural Organization) (1994) Protocols for the Joint Global Ocean Flux Study (JGOFS) core measurements. Available at http://usjgofs.whoi.edu/protocols_rpt_19.html
- Zhang Y, Lu S, Zhang C, Gao Y (2010) Distribution and germination of viable diatom resting stage cells in sediments of the East China Sea. *Acta Oceanol Sin* 29:121–128

Appendix. Table A1. Characterization of water chemistry in Puget Sound (Washington, USA) basins. Stations surveyed on 26 to 29 June 2006 included Strait of Juan de Fuca (SJF) stations (P21, P23, P24, 26), Admiralty Inlet stations (P7, P19), Main Basin stations (P5, P28, P30, P34, P39), Whidbey Basin stations (P1, P2, P4) and Hood Canal stations (P17, P11, P13). 'Deepest' refers to the depth of the deepest sample as given in the first row. See Fig. 1 in the main text for station locations. na: not available

	Depth (m)	SJF	Admiralty Inlet	Main Basin	Whidbey Basin	South Sound	Hood Canal
Depth of deepest sample (m)		120 (P24)	na	80.2 (P30)	87 (P4)	52 (P37, P38)	85.6 (P11)
Potential density (kg m ⁻³)	2.5	22.2–23.3	22.7–23.2	19.4–22.3	17.4–19.5	21.0–22.0	17.3–21.1
	Deepest	26.0	na	22.8	22.7	22.0–22.2	23.1
Salinity (psu)	2.5	29.4–30.5	27.7–30.5	26.3–29.4	23.8–27.8	28.8–29.3	24.1–29.2
	Deepest	33.3	na	29.7	29.4	29.0–29.1	29.9
Temperature (°C)	2.5	10.6–11.1	10.6–13.3	11.5–14.1	10.9–15.8	11.5–15.3	10.2–19.8
	Deepest	7.8	na	10.4	9.1	11.4–11.7	9.6
Nitrate (μmol l ⁻¹)	2.5	18.9–21.5	3.3–22.3	0.2–20.6	0.3–7.7	4.9–20.6	0–7.8
	Deepest	30.3	na	24.8	31.8	20.4–22.0	34.2
Nitrite (μmol l ⁻¹)	2.5	0.43–0.49	0.17–0.46	0.03–0.05	0.02–0.18	0.23–0.57	0–0.37
	Deepest	0.17	na	0.05	0.25	0.51–0.82	0.16
Ammonium (μmol l ⁻¹)	2.5	0.24–0.60	0.20–0.82	0.09–0.69	0.05–0.17	0.08–1.78	0–0.5
	Deepest	0.07	na	0.02	0.11	0.02–1.69	0.15
Silicate (μmol l ⁻¹)	2.5	39.1–42.0	13.6–42.7	3.6–41.0	10.7–38.1	19.5–41.0	2.2–23.1
	Deepest	49.7	na	45.4	80.4	43.7–44.8	81.0
Phosphate (μmol l ⁻¹)	2.5	1.71–1.89	0.47–1.83	0.21–1.79	0.09–0.60	0.75–1.79	0.11–0.99
	Deepest	2.45	na	2.09	2.82	2.01–2.11	3.65
Chlorophyll <i>a</i> (μg l ⁻¹)	2.5	2.3–6.5	2.9–16.6	3.7–18.2	8.2–20.0	3.7–11.7	1.8–15.7
	Deepest	0.2	na	0.3	0.2	0.5–1.1	na
Dissolved oxygen (mg l ⁻¹)	2.5	6.5–7.3	6.7–11.5	7.9–13.3	12.0–14.4	7.9–11.5	9.7–11.8
	Deepest	3.1	na	6.8	4.3	7.0–7.2	1.4