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Bacterial community composition differs with species and toxigenicity of the diatom *Pseudo-nitzschia*

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ABSTRACT: Interactions between bacteria and members of the marine diatom genus *Pseudo-nitzschia* may enhance production of the toxin domoic acid (DA) by toxigenic strains of *Pseudo-nitzschia*. To gain a broader understanding of relationships between bacteria and *Pseudo-nitzschia* species, we used automated ribosomal intergenic spacer analysis (ARISA) to assess the composition of the bacterial communities coexisting with 18 *Pseudo-nitzschia* strains representing 6 species. For cultures surveyed across multiple time points and size fractions, the attached and free-living bacterial communities were not significantly distinct from one another, and bacterial composition was stable across diatom growth phases (exponential versus stationary) and approximately 1 yr in culture. Among all cultures, bacterial communities differed significantly with *Pseudo-nitzschia* species and toxigenicity. Toxigenic strains of *Pseudo-nitzschia* hosted fewer bacterial ARISA operational taxonomic units (OTUs), in comparison to nontoxigenic strains. We constructed two 16S rDNA clone libraries to identify bacteria coexisting with 1 *P. multiseries* (toxigenic) and 1 *P. delicatissima* (nontoxigenic) culture. Both cultures hosted members of the *Roseobacter* clade, *Gammaproteobacteria*, and *Flavobacteria*, yet the specific bacteria coexisting with each *Pseudo-nitzschia* strain differed at the genus level or above. Our findings support the hypothesis that bacterial communities respond to DA or other species-specific differences in the environments created by *Pseudo-nitzschia* strains.

KEY WORDS: *Pseudo-nitzschia* · Domoic acid · Phytoplankton–bacteria interactions · ARISA

INTRODUCTION

Toxigenic strains of the marine diatom genus *Pseudo-nitzschia* impact marine ecosystems and human health throughout the world (Bates et al. 1998, Hasle 2002). Domoic acid (DA), the toxin produced by some *Pseudo-nitzschia* species or strains (Bates et al. 1998, Bates 2000) accumulates in shellfish and finfish, causing bird and mammal illness and mortalities, as well as amnesic shellfish poisoning (ASP) in humans (Bates et al. 1989, Wright et al. 1989, Work et al. 1993, Scholin et al. 2000, Lefebvre et al. 2002). Along the coast of Washington (WA), USA, high DA levels have been detected since the early 1990s, resulting in frequent shellfish bed closures (Fryxell et al. 1997, Trainer & Suddleson 2005, Dyson & Huppert 2010). In the inland waters of Puget Sound, WA, 3 DA outbreaks have occurred since 2003 (Bill et al. 2006, Trainer et al. 2007). However, the occurrence of toxigenic blooms of *Pseudo-nitzschia* is unpredictable as we know relatively little about the environmental conditions leading to toxin production (Marchetti et al. 2004, Trainer et al. 2009a,b).

In laboratory studies, DA production is influenced by genetic variability among *Pseudo-nitzschia* species and strains, as well as nutrient limitation and nitrogen source. Variability in toxin production levels can exist among strains of the same toxigenic species of *Pseudo-nitzschia* (Bates 2000, Thessen et al. 2009). However, among the *Pseudo-nitzschia* species capable of DA production, some species tend to produce higher levels of DA in comparison to others. For example, many strains of *P. australis* and *P. multiseries* produce DA at levels of picograms of DA per cell (Bates 1998), and

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these species have caused notably severe DA outbreaks (Bates et al. 1998, Scholin et al. 2000). In comparison, many strains of species including *P. delicatissima*, *P. pungens*, and open-ocean *Pseudo-nitzschia* have not been documented to produce DA, or else produce DA at low levels (as low as fg DA cell^{-1}) (Bates et al. 1998, Bates 2000, Lundholm et al. 2006, Marchetti et al. 2008, Trick et al. 2010). For a single strain in culture, DA levels within the medium typically are highest when cell growth is limited by silicic acid (Bates et al. 1991, Pan et al. 1996b,c), phosphate (Pan et al. 1996a), or iron (Rue & Bruland 2001, Maldonado et al. 2002). Exponentially growing *Pseudo-nitzschia* cells can also produce enhanced levels of DA when using urea as a nitrogen source in comparison to inorganic nitrogen sources (Howard et al. 2007), and field studies have documented DA production by nutrient-replete *Pseudo-nitzschia* cells (Marchetti et al. 2004).

Interactions with bacteria may also enhance DA production by toxigenic strains of *Pseudo-nitzschia*, although the mechanism is unclear. Multiple studies have demonstrated that axenic *P. multiseries* cultures produce lower levels of DA than do nonaxenic cultures (Douglas & Bates 1992, Douglas et al. 1993, Bates et al. 1995, Kobayashi et al. 2009). In particular, DA production was enhanced by up to 2 orders of magnitude upon reintroduction of multiple bacterial strains isolated from *P. multiseries* or the diatom *Chaetoceros* spp. to axenic *P. multiseries* cultures (Bates et al. 1995). Bacteria themselves do not appear to produce DA, even in the presence of *Pseudo-nitzschia* exudates (Bates et al. 2004). Kaczmarska et al. (2005) observed greater bacterial numbers and morphological diversity in higher toxin-producing *P. multiseries* cultures and during the diatom's stationary phase relative to the exponential growth phase and suggested that the level of DA production may be correlated with bacterial diversity or abundance. Bacterial exudates have not been found to enhance DA production by *Pseudo-nitzschia* (Bates 1998, Kobayashi et al. 2009), so there is no direct support for the idea that bacteria produce precursors or signaling molecules critical for DA production by the diatom. To date, only a single field study has examined bacterial abundance in regards to DA levels, and it did not find a correlation (Trainer et al. 2009b).

Although the nature of interactions between *Pseudo-nitzschia* and bacteria has not been determined, bacteria in some *Pseudo-nitzschia multiseries* cultures have been identified as taxa commonly detected with other phytoplankton, including members of the *Roseobacter* clade and other rhodobacters, alteromonads and other *Gammaproteobacteria*, and a member of the *Bacteroidetes* (Stewart et al. 1997, Kobayashi et al. 2003, Kaczmarska et al. 2005). Some of these bacteria may occur intracellularly (Kobayashi et al. 2003), and these same bacterial taxa often co-occur among other diatom and dinoflagellate cultures (Alavi et al. 2001, Hold et al. 2001, Schäfer et al. 2002, Green et al. 2004, Pinhassi et al. 2004, Fandino et al. 2005, Grossart et al. 2005, Jasti et al. 2005, Sapp et al. 2007a). For *Pseudo-nitzschia*, coexisting bacteria have been described only for 1 species, the typically toxigenic species *P. multiseries.*

Bacterial community composition in phytoplankton cultures could be influenced by many factors, beginning with *in situ* composition in the waters from which phytoplankton isolates are obtained. Bacterial composition may be impacted further in response to specific culturing conditions and coexisting algal species. Indeed, phytoplankton-associated bacterial composition has been shown to differ across phytoplankton species or broader taxonomic groups (Pinhassi et al. 2004, Grossart et al. 2005, Jasti et al. 2005). Bacteria have also been demonstrated to have differential responses to algal products such as glycolate (Lau & Armbrust 2006, Lau et al. 2007), polyunsaturated aldehydes (Ribalet et al. 2008), and dimethylsulfoproprionate (DMSP; Malmstrom et al. 2004, Vila et al. 2004, Pinhassi et al. 2005, Merzouk et al. 2008). Thus, in culture, specific bacteria could be enriched in response to the chemical composition of exudates, which can vary among phytoplankton (Myklestad 1995, Biersmith & Benner 1998). Along these lines, there is some evidence that some bacterial strains may utilize DA. Specifically, the growth of some bacteria on a complex medium is enhanced by the presence of DA, while the growth of other strains may be inhibited by it, as evidenced by the creation of clear zones on bacterial lawns (Stewart et al. 1998). In the field, bacteria from regions of *Pseudo-nitzschia* blooms have been shown to degrade DA more quickly than bacteria from other regions (Hagström et al. 2007). Stewart (2008) suggested that long-term culture of toxigenic strains of *Pseudo-nitzschia* may selectively enrich for bacteria with the ability to utilize DA.

To explore whether specific bacterial communities are enriched in response to *Pseudo-nitzschia* species and toxigenicity, we examined bacterial community composition within cultures of 18 *Pseudo-nitzschia* strains representing 6 species. We applied a DNA fingerprinting method — automated ribosomal intergenic spacer analysis (ARISA) — to characterize whole bacterial communities within each of the 18 *Pseudo-nitzschia* cultures. We compared bacterial communities across *Pseudo-nitzschia* species to detect evidence of species-specific associations. In particular, we hypothesized that DA affects bacterial community composition, and thus we compared bacterial communities between toxigenic and nontoxigenic cultures of

Pseudo-nitzschia. Finally, we identified bacteria coexisting with 1 *P. multiseries* culture (toxigenic) and 1 *P. delicatissima* culture (nontoxigenic) using 16S rDNA clone libraries. Our findings support the hypothesis that DA, or another species-specific component of *Pseudo-nitzschia* phycospheres, structures the bacterial communities that coexist with these diatoms.

MATERIALS AND METHODS

***Pseudo-nitzschia* isolations.** Net tow samples were collected from 3 stations in Puget Sound during cruises in June, October, and December 2006 aboard the RV 'Thomas G. Thompson', using a 20 µm pore size plankton net. Net tows were also conducted at Long Beach, WA, in July 2006 and Sequim Bay, WA, in September 2005 and 2006. Approximately 50 ml of net tow samples were stored near *in situ* temperature for fewer than 4 d prior to isolations. Single-cell or single-chain diatoms were pipetted into 1 ml of sterile seawater amended with *f*/20 nutrients (Sigma; Guillard 1975) in 12-well culture plates, with the goal of isolating multiple species from each water sample. Isolates were transferred into sterile seawater amended with *f*/10 nutrients, followed by acclimation to K-medium (Keller et al. 1987).

***Pseudo-nitzschia* strain identification using ITS1 sequencing.** The internal transcribed spacer 1 (ITS1) of new *Pseudo-nitzschia* isolates was sequenced, following the procedure described by Hubbard et al. (2008). Briefly, DNA was extracted from ~20 ml of culture collected on 0.45 µm HA filters (Millipore), using a DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions. Duplicate polymerase chain reactions (PCRs) were performed using the primers 18SF-euk (5′-CTTATCATTTAGAGGAAGGTGAAGTCG-3′) and 5.8SR-euk (5′-CTGCGTTCTTCATCGTTGTGG-3′) and cycling conditions as given in Hubbard et al. (2008). Pooled PCR products were purified using a QIAquick PCR purification kit (Qiagen). Sequencing was conducted at the University of Washington's High-Throughput Genomics Unit or at the Center for Environmental Genomics. Sequences were aligned with reference sequences for *Pseudo-nitzschia* isolates identified by scanning electron microscopy (SEM) (downloaded from GenBank; see Hubbard et al. 2008), using Sequencher 4.8 (Gene Codes). ITS1 sequences of new *Pseudo-nitzschia* isolates were deposited in GenBank under the accession numbers HM138904 to HM138910.

Domoic acid production. We determined *Pseudo-nitzschia* strain toxigenicity during the stationary phase in *f*/2- (Guillard 1975) and K- (Keller et al. 1987) media used for both regular culture maintenance and the experiments in the present study (hereafter referred to as 'toxigenicity'). In addition, we confirmed that stationary phase growth in these media had been induced by silicic acid limitation. Stationary phase growth and silicic acid limitation have been correlated with maximum DA levels in the cultures of many *Pseudo-nitzschia* species and thus are utilized commonly for evaluation of toxigenicity (Bates et al. 1991, Pan et al. 1996b, Bates 1998, Fehling et al. 2004, Lundholm et al. 2006, Marchetti et al. 2008).

Pseudo-nitzschia cultures were grown in duplicate in 0.2 µm filtered, autoclaved Puget Sound seawater amended with nutrients for *f*/2- or K-media through the stationary phase, as determined by daily fluorometer readings (10-AU fluorometer, Turner Designs). For 4 *Pseudo-nitzschia* strains (2 strains maintained in *f*/2-medium; 2 strains maintained in K-medium), nutrient add-back experiments were conducted to confirm silicic acid as the limiting nutrient. Replicate cultures were maintained through the stationary phase, and potential limiting nutrients (the initial medium levels of N, P, or Si sources) were added separately to these nutrient-limited cells. Fluorescence was monitored throughout this experiment, whereby increases in fluorescence upon addition of Si, but not N or P, reflected cell growth in response to the addition of the limiting nutrient.

Domoic acid levels in stationary phase *Pseudo-nitzschia* cultures were determined using direct competitive ASP enzyme-linked immunosorbent assay (ELISA; Biosense Laboratories; Kleivdal et al. 2007). After 1 to 5 d in the stationary phase, 10 ml of culture were filtered onto 0.45 µm HA filters (Millipore) for particulate domoic acid (PDA); 1 ml of filtrate was retained for dissolved domoic acid (DDA). Filters and 1 ml of filtrate were stored at –20°C in the dark. PDA was extracted from filters into 10 ml of ultrapure distilled water (Milli-Q; MilliPore). Sample analysis was performed according to the Biosense protocol. DA in PDA extracts and DDA fractions was quantified according to the working range of the ELISA kit, which corresponded to approximately 10 to 300 pg ml^{-1} of *Pseudo-nitzschia* culture. In our cultures, the working limit of detection was <1.4 fg DA $cell^{-1}$ for both PDA and DDA. This value is >25 times lower than the lowest DA per cell value we report from a culture we characterize as toxigenic.

Pseudo-nitzschia cell counts were conducted to normalize DA measurements for *Pseudo-nitzschia* biomass. From cultures sampled for DA, 3 ml were preserved using 1 ml of 2% glutaraldehyde and stored at 4°C in the dark. Cell counts were performed using a Sedgewick Rafter Cell S50 (Pyser-SGI) and a Nikon Labophot-2 compound microscope. Either 30 fields or 300 cells were counted, excluding empty frustules. For each preserved sample, 3 subsamples were counted and averaged.

Table 1. *Pseudo-nitzschia* cultures (n = 18) analyzed for coexisting bacterial communities, identified by species, strain, domoic acid (DA) production (PDA: particulate DA; DDA: dissolved DA) under silicic acid limitation, geographic origin, person who isolated the strain (Isolated by) and reference. Average of triplicate DA readings (by enzyme-linked immunosorbent assay) is presented. Toxigenic *Pseudo-nitzschia* strains are emphasized in **bold**. BD: below detection limit; X: automated ribosomal intergenic spacer analysis (ARISA) profiles were compared across exponential growth and stationary phases (Exp/Sta), attached and free-living bacterial fractions (AB/FLB), and/or multiple time points separated by repeated culture transfers (Time in culture), within the strain indicated

Pseudo-nitzschia species	Strain	PDA (pg cell^{-1})	DDA (pg cell^{-1})	Exp/Sta	AB/FLB	Time in culture	Origin	Isolated by	Reference
P. australis[a]	03199_1B	21.85	1.63		X		Monterey Wharf, CA	G. Jason Smith	Hubbard et al. (2008)
P. australis[a]	04063_3C	3.15	1.26	X	X		Bodega/Tomales Bay, CA	G. Jason Smith	Hubbard et al. (2008)
P. australis[a]	03184_5D	9.00	3.78				Monterey Wharf, CA	G. Jason Smith	Hubbard et al. (2008)
P. australis[a]	03184_6D	0.20	0.04	X	X		Monterey Wharf, CA	G. Jason Smith	Hubbard et al. (2008)
P. multiseries	CLN-47	0.71	0.17	X	X		Progeny of 2 isolates from eastern Canada[b]	Claude Léger	Mafra Jr et al. (2009)
P. multiseries[a]	PNWH2O A4	5.84	1.32	X	X	X	Puget Sound, WA (Main Basin)	Katherine Hubbard	Hubbard et al. (2008)
P. sp. 233[c]	PNWH2O 233a	13.48	0.85	X	X	X	Sequim Bay, WA 48° 03′ N, 123° 01′ W	Karie Holtermann	Present study
P. delicatissima	PNWH2O 604	BD	BD		X	X	Puget Sound, WA 47° 44.1′ N, 122° 45.7′ W	Michele Guannel	Present study
P. delicatissima	PNWH2O 605	BD	BD	X	X	X	Puget Sound, WA 47° 44.1′ N, 122° 45.7′ W	Michele Guannel	Present study
P. delicatissima	PNWH2O 609	BD	BD				Puget Sound, WA 47° 16.6′ N, 122° 42.5′ W	Michele Guannel	Present study
P. delicatissima	PNWH2O K2	BD	BD				Sequim Bay, WA 48° 03′ N, 123° 01′ W	Karie Holtermann	Present study
P. granii	UWOSP22	BD	BD				NE Pacific 50° N, 145° W	Adrian Marchetti	Present study
P. granii	UWOSP36	BD	BD				NE Pacific 50° N, 145° W	Adrian Marchetti	Marchetti et al. (2008)
P. pungens	PNWH2O 607	BD	BD		X		Puget Sound, WA 48° 1′ N, 122° 18.2′ W	Michele Guannel	Present study
P. pungens	PNWH2O 608	BD	BD				Puget Sound, WA 48° 1′ N, 122° 18.2′ W	Michele Guannel	Present study
P. pungens	PNWH2O LB2	BD	BD				Long Beach, WA 46° 42′ N, 123° 58′ W	Karie Holtermann	Present study
P. pungens	PNWH2O C1	BD	BD	X	X		Puget Sound, WA (Main Basin)	Katherine Hubbard	Hubbard et al. (2008)
P. pungens[a]	PNWH2O 101WB	BD	BD	X	X		Willapa Bay, WA	Katherine Hubbard	Hubbard et al. (2008)

[a]Species identification supported by scanning electron microscopy (SEM)
[b]*P. multiseries* Strain CLN-47 was the progeny of a laboratory cross between Strains CL-147 (isolated from Caribou Harbour, Nova Scotia) and CL-191 (isolated from Deadmans Harbour, Bay of Fundy, New Brunswick)
[c]Not identifiable to species level by SEM or sequencing, although SEM data indicate that this strain does not belong to one of the other species included in the present study (K. Hubbard unpubl. data). This cryptic species is therefore described by *Pseudo-nitzschia* ARISA fragment length (Hubbard et al. 2008)

Laboratory culturing and DNA extraction of bacterial communities coexisting with *Pseudo-nitzschia*. Eighteen *Pseudo-nitzschia* isolates were utilized in the present study, including strains obtained from collaborators and previously identified as *P. australis*, *P. pungens*, *P. granii*, and *P. multiseries* (Table 1). In addition, 1 cryptic strain, PNWH2O 233a, was included. SEM performed on this strain confirmed that it did not belong to any other species examined in the present study (K. Hubbard unpubl. data). We therefore described this strain as a different species, *Pseudo-nitzschia* sp. 233. *Pseudo-nitzschia* strains were cultured in volumes of 20 to 27 ml in replicate glass culture tubes transferred from the same parent culture. Cultures were incubated at 13°C under a 16 h light:8 h dark cycle, except for *P. multiseries* CLN-47, which was incubated at 20°C under 24 h of light. Light levels ranged between 30 and 115 µmol quanta^{-1} m^2. The same culture conditions were used for regular culture maintenance over the course of our study and for the specific sampling time points described here.

Bacterial communities were sampled from attached and free-living size fractions, from exponential growth and stationary phases of *Pseudo-nitzschia*, and over months separated by culture transfers. Between 12 and 15 ml of each *Pseudo-nitzschia* culture were gravity-filtered through 47 mm diameter, 3 µm (Whatman Nuclepore polycarbonate) filters, followed by vacuum filtration through 25 mm diameter, 0.2 µm Supor-200 filters (Pall Life Sciences) to collect attached bacteria (AB; >3 µm) and free-living bacteria (FLB; 0.2 to 3 µm). The pore size of 3 µm, used in other studies to operationally define phytoplankton-attached bacteria (e.g. Sapp et al. 2007a), was the largest pore size (among 1, 2, 3, 5, and 10 µm) that maximized biomass retention of both small (*P. fraudulenta*) and large (*P.*

multiseries) cell types sampled during both exponential growth and stationary phases (data not shown). AB and FLB fractions were collected for all cultures and analyzed for 11 strains (Table 1). Eight cultures, including strains of both typically toxigenic and nontoxigenic species, were sampled at least twice during the diatom growth phase: once during the exponential growth phase and once during the stationary phase (Table 1). One strain of toxigenic *P. multiseries*, CLN-47, was sampled more extensively, on Days 3 and 6 (exponential growth phase) and Days 8, 10, 14, and 17 (stationary phase) in culture. Ten other strains, not sampled during exponential growth phase, were sampled between 2 and 15 d after the beginning of the stationary phase and were predicted to correlate with high DA levels (Bates et al. 1991, Pan et al. 1996b, Bates 1998, Fehling et al. 2004, Lundholm et al. 2006, Marchetti et al. 2008). Four cultures were sampled at 2 or 3 time points separated by repeated transfers in culture (Table 1). Two strains of *P. delicatissima* (commonly a nontoxigenic species; Lundholm et al. 2006) were each sampled at 3 time points: PNWH2O 604 was sampled after 6, 9, and 14 mo following initial isolation, and PNWH2O 605 was sampled after 4, 6, and 9 mo following isolation. Two other strains, including the commonly toxigenic species *P. multiseries*, were sampled at 2 different time points in culture. *P. multiseries* Strain PNWH2O A4 was sampled at 22 and 26 mo following isolation and Strain PNWH2O 233a was sampled at 12 and 14 mo following isolation. Filters were frozen at −80°C until DNA extraction.

Genomic DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen) following pretreatment for gram-positive bacteria. Briefly, filters were agitated in 180 µl enzymatic lysis buffer (20 mM Tris-Cl [pH 8.0], 2 mM sodium EDTA, 1.2% Triton X-100, 20 mg ml^{-1} lysozyme) using a bead-beater (BioSpec Products) without beads for 60 s at maximum speed, and then incubated at 37°C for 30 min. Cells were lysed further by incubating at 70°C for 30 min with 25 µl Proteinase K and 200 µl Buffer AL (Qiagen) and purified using DNeasy mini spin columns and a microcentrifuge according to the DNeasy protocol for gram-positive bacteria. Purified DNA was eluted in 50 µl of Qiagen Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0). DNA was quantified according to binding with the fluorochrome PicoGreen dsDNA Quantitation Reagent (Molecular Probes) and measured using a SpectraMax M2 microplate reader (Molecular Devices Corporation), as described by Ahn et al. (1996).

ARISA of bacterial communities. ARISA (Fisher & Triplett 1999) was conducted to amplify the bacterial ITS region located between the 16S and 23S rDNA regions. PCR was performed using a labeled universal primer 16S 1492F (5′-HEX-GYACACACCGCCCGT-3′) and a bacterial primer 23S 125R (5′-GGTTBYCC-CATTCRG-3′; (Fisher & Triplett 1999), with additional degeneracies as described by Hunt et al. (2006). Each 20 µl PCR mixture contained 1× Mg-free buffer (Promega, GeneChoice), 2.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates (dNTPs), 1 U *Taq* DNA polymerase (Promega, GeneChoice), 0.5 µM of each primer (Operon), and 2 ng genomic DNA. Genomic DNA templates were each amplified in quadruplicate (Polz & Cavanaugh 1998). The PCR amplification began with an initial 4 min denaturation at 94°C, followed by 32 cycles of each of 3 steps: 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min. Extension of the final cycle occurred at 72°C for 10 min. PCR products were stored at 4°C in the dark.

Quadruplicate PCR products were pooled and quantified using PicoGreen and the Spectramax M2 microplate reader. Next, 10 ng DNA was precipitated from pooled PCR product (0.5 to 5 µl) using 27.5 µl biotechnology grade ethanol (Amresco) and 1 µl of 7.5 M ammonium acetate (J. T. Baker) (Sambrook & Russell 2001), followed by resuspension with 7.7 µl loading solution (70% formamide, 1 mM EDTA; Amersham Biosciences) and 0.3 µl of fluorescently labeled internal size standard, CST ROX 60-1500 bp (BioVentures). Samples were analyzed on a MegaBACE 1000 automated capillary sequencer (sequencing mode; injection voltage = 3 kV; injection time = 60 s; run voltage = 5 kV; run time = 370 min).

Analysis of ARISA profiles. The program DAx (van Mierlo Software) was used to identify fragment lengths in ARISA electropherograms. A spectral matrix correction, determined using a MegaBACE Genotyping Test Plate Kit (GE Healthcare), was applied to all electropherograms. Peaks were called using a signal-to-noise ratio of 5 (Fuhrman et al. 2006). Our analysis included only ARISA electropherograms that had a minimum single peak height of 350 relative fluorescence units (RFUs). The presence or absence of peaks (150 to 1500 base pairs, bp, in length) in each electropherogram was summarized using a DAx global sheet. Peak data were then binned using dakster, a Perl binning program (http://rocaplab.ocean.washington.edu/cgi/dakster/index.html), which assigned peaks to variable-sized bins according to fragment length (3 bp bin width for fragments <700 bp, 5 bp bin width for fragments from 700 to 1000 bp, and 10 bp bin width for fragments >1000 bp) (Fuhrman et al. 2006).

ARISA data were analyzed using the statistical software PRIMER 6 (Clarke & Warwick 2001). Pairwise sample comparisons were created using the Sørensen similarity coefficient (Legendre & Legendre 1998) and included operational taxonomic units (OTUs) shared among 2 or more cultures. First, we examined ARISA profiles among individual *Pseudo-nitzschia* strains,

including replicates transferred from the same parent culture. For a subset of cultures (Table 1), we analyzed AB and FLB samples (11 strains), samples collected during exponential and stationary phases (8 strains), and samples collected at 2 or 3 time points separated by repeated transfers in culture (4 strains). Hierarchical cluster analysis was performed on these ARISA data using group-average linking, and samples were considered to be different if they clustered in significantly different groups within a dendrogram according to the similarity profile (SIMPROF) test ($p < 0.05$).

Next, we compared single FLB profiles for each of the 18 *Pseudo-nitzschia* strains. One-way analysis of similarity (ANOSIM) tests were conducted, and a non-metric multidimensional scaling (NMDS) plot was constructed, to compare bacterial communities across *Pseudo-nitzschia* strains according to (1) species (for all species represented by 2 or more strains, thus excluding *Pseudo-nitzschia* sp. 233), (2) toxigenicity, and (3) geographic origin (for Washington, California, and NE Pacific waters, thus excluding the single *P. multiseries* Strain CLN-47 originating from eastern Canada). The resulting p-values for these global statistics were interpreted using the Bonferroni correction for multiple comparisons (Legendre & Legendre 1998). Pairwise comparisons were conducted along with global ANOSIM statistics. Among the pairwise comparisons, moderately high R-values and low p-values indicated groups that were most responsible for driving the overall global results. We based our interpretations of the pairwise species differences more heavily on R- than on p-values, as for many of the pairwise comparisons (due to a limited number of permutations) it would not be possible to achieve low p-values, and a Bonferroni correction applied to these p-values would likely be too conservative (Clarke & Warwick 2001). For OTUs shared by 2 or more *Pseudo-nitzschia* cultures, we performed similarity percentage (SIMPER) analysis, which assesses the contribution of each individual OTU to the overall dissimilarity in bacterial community composition across groups. Finally, we calculated probability based on binomial expansion, or binomial probability (Zar 1974), of the distribution of these shared OTUs, as well as the distribution of all singleton OTUs detected in only 1 toxigenic or only 1 nontoxigenic culture.

Bacterial clone library construction and sequencing. We created 16S–23S rDNA clone libraries from FLB coexisting with 2 Puget Sound *Pseudo-nitzschia* cultures: 1 *P. multiseries* strain (PNWH2O A4, sampled after 22 mo in culture) and 1 *P. delicatissima* strain (PNWH2O 604, sampled after 9 mo in culture). Genomic DNA was amplified using the bacterial-specific primers 16S 27F (5′-AGAGTTTGATCMTGGCTCAG-3′; Lane 1991) and 23S 125R (described in the subsection 'ARISA of bacterial communities'). Quadruplicate PCRs (Polz & Cavanaugh 1998) were prepared for each sample. Each 20 µl PCR mixture contained 2 ng genomic DNA, 1× Mg-free buffer (GeneChoice), 2.5 mM $MgCl_2$, 0.8 mM dNTPs, 1 U *Taq* DNA polymerase (GeneChoice), and 0.5 µM of each primer (Operon). The PCR amplification began with an initial 4 min denaturation at 94°C, followed by 22 cycles each of 3 steps: 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min. Extension of the final cycle occurred at 72°C for 10 min. Following this, a reconditioning step (Thompson et al. 2002) was performed using 3 cycles of additional amplification. For each of the 2 samples, the 4 replicate PCR products were pooled and purified (QIAquick PCR purification kit; Qiagen). Cloning was performed with a TOPO TA Cloning Kit (Invitrogen). Plasmid minipreps were prepared using alkaline lysis and ethanol precipitation (Sambrook & Russell 2001) or a DirectPrep 96 Miniprep Kit (Qiagen).

To select clones for sequencing, we first screened the clones by performing ARISA on plasmid minipreps. ARISA was performed as described above, with minor exceptions. Here, for each clone, a single PCR reaction was performed on approximately 100 ng of DNA from plasmid minipreps. Rarefaction curves were constructed using EstimateS 8.0.0 (Colwell & Coddington 1994) to determine whether each clone library had thoroughly sampled the unique ARISA OTUs from each bacterial community (Gotelli & Colwell 2001).

For each ARISA OTU detected in the clone libraries, 1 to 4 clones were sequenced. High-quality plasmid DNA was extracted using a Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. To obtain complete coverage of both DNA strands, each clone was sequenced at the University of Washington's High-Throughput Genomics Unit (Seattle, WA) using 6 primers: 27F (described earlier in this section) and 1492R (the reverse complement of primer 1492F, described in the subsection 'ARISA of bacterial communities'), as well as the internal bacterial primers 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′), 926F (5′-AAA CTC AAA GGA ATT GAC GG-3′), 515F (5′-GTG CCA GCA GCC GCG GTA A-3′), and 519R (5′-GTA TTA CCG CGG CTG CTG-3′) (Lane 1991).

Contigs were assembled from reads using all 6 primers and curated using the program Sequencher 4.8 (Gene Codes). Each sequence was trimmed to include the 1492R primer and exclude the 27F primer and was checked for chimeras using Bellerophon (Huber et al. 2004). Clones were described as belonging to the same bacterial species if the 16S rDNA sequences were 99% or greater in nucleotide identity (Acinas et al. 2004). A total of 1997 whole 16S rDNA sequences were downloaded from the Ribosomal

Database Project (RDP) on December 11, 2008 (Cole et al. 2005) and used as a reference database in ARB (Ludwig et al. 2004). Clone sequences were fast-aligned against this database and manually curated in ARB. The phylogenetic tree was constructed in PAUP* Version 4.0 (Swofford 2002), using 1324 characters and 73 of the reference sequences. Minimum evolution (ME) analyses were conducted using heuristic search options, with starting trees obtained using neighbor-joining and the tree-bisection-reconnection branch-swapping algorithm. Bootstrap analyses were conducted from 100 replicates (Felsenstein 1985). In addition, maximum likelihood (ML) and maximum parsimony (MP) analyses were performed. Sequences were deposited in GenBank under the accession numbers HM140645 to HM140680.

RESULTS

Pseudo-nitzschia isolate characterization

We obtained 7 *Pseudo-nitzschia* isolates during 3 Puget Sound cruises in 2006, as well as from sampling conducted at 2 other Washington sites in 2005 and 2006 (Table 1). New *Pseudo-nitzschia* isolates were putatively identified as 4 isolates of *P. delicatissima* and 3 isolates of *P. pungens* based upon ITS1 sequencing. The genotypes of these new *Pseudo-nitzschia* isolates were consistent with previous reports of *P. delicatissima* and *P. pungens* ITS1 genotypes detected in NW Pacific waters (Hubbard et al. 2008). Specifically, all 4 *P. delicatissima* isolates represented *P. delicatissima* Genotype 11, which has been previously detected in NW Pacific waters and other regions (Hubbard et al. 2008). These isolates exhibited 100% ITS1 identity to Genotype 11 reference strains, verified by SEM, originating from Denmark (GenBank accession numbers AY257849 and DQ329206) and Portugal (DQ329207) (Lundholm et al. 2006). In contrast, the new *P. pungens* isolates comprised 2 ITS1 genotypes. First, the ITS1 sequence of PNWH2O LB2 was 100% identical to a previously identified Washington *P. pungens* isolate included in the present study, PNWH2O 101WB (DQ996020) (Hubbard et al. 2008), as well as Isolate NA177 Clone 7 (FM207594) obtained from the Juan de Fuca eddy region off the Washington coast (Casteleyn et al. 2009), both confirmed using SEM. Second, the other 2 new isolates, PNWH2O 607 and PNWH2O 608, both differed from PNWH2O LB2 by 3 nucleotides. These isolates, obtained from Puget Sound, were 100% identical to a second genotype of SEM-verified *P. pungens*, exemplified by Isolates NA179 Clone 1 (FM207595) and NA177 Clone 16 (FM207593) from the Juan de Fuca Eddy region (Casteleyn et al. 2009). Each of the *P. pungens* genotypes has been described as different hybrids of 2 clades that co-occur in the NE Pacific (*P. pungens* var. *pungens* and *P. pungens* var. *cingulata*) (Casteleyn et al. 2009).

The resulting culture collection included these 7 new *Pseudo-nitzschia* isolates, as well as 11 isolates provided by collaborators, together representing a total of 5 species from 4 geographic origins (Table 1). The majority of these cultures were from coastal waters, with the exception of 2 strains of *P. granii* from the NE Pacific. Cultures from Washington waters included 2 isolates representing 2 different species, obtained from the same Puget Sound water sample (*P. multiseries* Strain PNWH2O A4 and *P. pungens* Strain PNWH2O C1).

The toxigenicity of these cultures was determined by measuring DA production during the stationary phase, a standard condition for toxigenicity assessment (Bates et al. 1991, Pan et al. 1996b, Bates 1998, Fehling et al. 2004, Lundholm et al. 2006, Marchetti et al. 2008). Nutrient add-back experiments confirmed that silicic acid, rather than N or P sources, was the limiting nutrient in these cultures (data not shown). All strains of species *Pseudo-nitzschia australis* and *P. multiseries*, as well as the cryptic species *Pseudo-nitzschia* sp. 233, produced detectable levels of DA (Table 1). PDA levels ranged between 0.20 and 21.85 pg cell^{-1}, and DDA levels varied between 0.04 and 3.78 pg cell^{-1}. Strains of *P. delicatissima*, *P. granii*, and *P. pungens* did not produce detectable levels of PDA or DDA. Toxigenic and nontoxigenic cultures were obtained from Washington waters. Only toxigenic cultures were obtained from California and eastern Canada waters, and only nontoxigenic cultures were obtained from the NE Pacific. Because the culture conditions used to conduct these DA measurements are the same as those used to maintain the cultures routinely in the laboratory, it is reasonable to assume that the bacterial communities in the cultures we identified here as toxigenic have been exposed to DA repeatedly since they were brought into culture. In contrast, those communities in the cultures where we did not detect DA were likely not exposed to DA repeatedly.

Bacterial community composition in *Pseudo-nitzschia* cultures

We hypothesized that bacterial communities coexisting with each *Pseudo-nitzschia* culture would shift over time in response to culturing conditions and diatom growth phase, and we further predicted that attached and free-living bacterial composition would differ in response to proximity to the diatom. For a subset of cultures (Table 1), we therefore conducted

the following 3 comparisons of bacterial community composition among replicate samples from individual *Pseudo-nitzschia* strains: (1) across multiple time points separated by repeated transfers in culture, (2) between exponential growth and stationary phases following the same transfer, and (3) between AB and FLB fractions. First, bacterial community composition did not differ significantly over time in culture for either nontoxigenic (*P. delicatissima*) or toxigenic (*P. multiseries*, *Pseudo-nitzschia* sp. 233) species. Specifically, the bacterial community composition from *P. delicatissima* Strain PNWH2O 604 was similar across 6, 9, and 14 mo in culture, and bacterial community composition from *P. delicatissima* Strain PNWH2O 605 was similar across 4, 6, and 9 mo in culture. Toxigenic cultures of *P. multiseries* PNWH2O A4 and *Pseudo-nitzschia* sp. PNWH2O 233a did not differ in bacterial community composition across 2 time points separated by approximately 2 mo and repeated transfers in culture. Second, for all 8 *Pseudo-nitzschia* strains tested, bacterial community composition did not differ between exponential growth and stationary phases. In particular, 5 toxigenic strains of *Pseudo-nitzschia* did not exhibit significantly different bacterial community composition during conditions correlated with both low DA levels (exponential growth on inorganic N sources) and high DA levels (silicic acid limitation) (Bates 1998). One toxigenic strain of *P. multiseries*, CLN-47, did not differ significantly in bacterial community composition across samples collected roughly every 3 d during exponential growth and stationary phases of a single transfer. Third, composition of the AB and FLB communities did not differ significantly for 9 of the 11 *Pseudo-nitzschia* strains assessed. However, 1 toxigenic strain of *Pseudo-nitzschia* (PNWH2O 233a) and 1 nontoxigenic strain of *Pseudo-nitzschia* (PNWH2O 101WB) exhibited significantly different AB and FLB community compositions (SIMPROF; $p < 0.05$). Because bacterial community compositions coexisting with a single diatom strain were comparable over time and between the AB and FLB size fractions, the remainder of our analyses included 1 representative FLB sample for each strain, collected during the diatom's stationary phase.

We assessed patterns of bacterial community composition among 18 *Pseudo-nitzschia* cultures representing 5 identified species and 1 unknown species (Table 1). For these samples, the cluster analysis was similar to the ordination conducted for the comparisons described above, but we employed ANOSIM and NMDS because of higher levels of replication among each group. Because we did not obtain either toxigenic or nontoxigenic types of each of the species, from each of the 3 geographic origins, we were unable to statistically separate 3 factors from one another. Therefore, we separately tested each of 3 hypotheses that bacterial community composition varied among (1) cultures of different *Pseudo-nitzschia* species, (2) cultures differing in toxigenicity as assessed by DA production under silicic acid limitation, and (3) cultures derived from different geographic origins (Washington, California, and NE Pacific waters), using 1-way ANOSIM. We interpreted the resulting p-values using the Bonferroni correction for testing 3 hypotheses simultaneously, whereby a p-value less than 0.0167 was significant.

Bacterial community composition differed significantly among *Pseudo-nitzschia* species (Table 2), as observed in the clustering in the NMDS plot (Fig. 1). Pairwise species comparisons conducted along with global ANOSIM statistics indicated that 4 of the 5 total possible pairwise comparisons across toxigenic versus nontoxigenic cultures contributed most strongly to the species differences (as indicated by higher R-values; Table 2). Furthermore, bacterial community composition in nontoxigenic cultures of *Pseudo-nitzschia* was significantly different relative to the bacterial communities in toxigenic cultures (Table 2, Fig. 1). When all 18 cultures were compared (Table 2, Dataset A), bacterial community composition was significantly different in cultures of different geographic origin. However, pairwise comparisons revealed these differences were significant only between open-ocean strains (from the NE Pacific) versus coastal strains (from Washington or California). When only the 16 coastal cultures were compared (Table 2, Dataset B), bacterial communities from coastal *Pseudo-nitzschia* strains did not differ according to origin from Washington versus California waters (Table 2, Dataset B). In the analyses of both datasets, *Pseudo-nitzschia* species and toxigenicity remained as factors that could explain bacterial community structure.

ARISA OTUs correlated with *Pseudo-nitzschia* toxigenicity

In order to evaluate the potential effects of DA on bacterial community composition, we compared the distribution of ARISA OTUs across toxigenic and nontoxigenic cultures of *Pseudo-nitzschia*. Of the 92 total OTUs detected among the 18 *Pseudo-nitzschia* cultures, 59 were singletons (defined as an OTU that was detected once and with only 1 of the *Pseudo-nitzschia* isolates). Forty-nine singletons (83% of the total number of singletons) were detected in a nontoxigenic culture, whereas the remaining 10 (17%) were detected in a toxigenic culture. According to binomial probability, this distribution of singletons across toxigenic ver-

Table 2. Results of analysis of similarity (ANOSIM) tests performed on bacterial communities (automated ribosomal intergenic spacer analysis, or ARISA, data) coexisting with *Pseudo-nitzschia* cultures, analyzed for 2 datasets: all cultures (Dataset A) and only cultures isolated from coastal waters (Dataset B). Bacterial communities were analyzed for patterns according to *Pseudo-nitzschia* species, toxigenicity, and geographic origin. Statistics (R-statistic, p-value, and number of permutations) for both global and pairwise comparisons are presented, where a dash indicates the test was not applicable. Pairwise comparisons that contribute strongly to global test results (moderate to high R- and low p-values) are highlighted in gray, and toxigenic species are emphasized in **bold**, as are global statistics. An asterisk denotes statistical significance (p < 0.0167) after Bonferroni correction

Tests	Dataset A			Dataset B		
	All cultures (n = 18), except as noted			All coastal cultures (n = 16), except as noted		
	R	p	Permutations	R	p	Permutations
SPECIES[a]						
Global	**0.509**	**0.001***	**999**	**0.441**	**0.002***	**999**
Pairwise						
P. australis vs. *P. pungens*	0.644	0.016*	126	0.644	0.016*	126
P. delicatissima vs. *P. pungens*	0.206	0.143	126	0.206	0.143	126
P. australis vs. *P. delicatissima*	0.479	0.057	35	0.479	0.057	35
P. multiseries vs. *P. pungens*	0.609	0.048	21	0.609	0.048	21
P. multiseries vs. ***P. australis***	0.714	0.067	15	0.714	0.067	15
P. multiseries vs. *P. delicatissima*	0.054	0.333	15	0.054	0.333	15
P. pungens vs. *P. granii*	0.664	0.048	21	–	–	–
P. australis vs. *P. granii*	1	0.067	15	–	–	–
P. delicatissima vs. *P. granii*	0.143	0.400	15	–	–	–
P. multiseries vs. *P. granii*	1	0.333	3	–	–	–
TOXIGENICITY						
Global	**0.256**	**0.011***	**999**	**0.287**	**0.010***	**999**
Pairwise	–	–	–	–	–	–
GEOGRAPHIC ORIGIN[b]						
Global	**0.295**	**0.012***	**999**	**0.177**	**0.078**	**999**
Pairwise						
WA vs. CA	0.177	0.078	999	–	–	–
WA vs. NE Pacific	0.331	0.013*	78	–	–	–
CA vs NE Pacific	1	0.067	15	–	–	–

[a]Species test excluded *Pseudo-nitzschia* sp. 233 Strain PNWH2O 233a (single culture of cryptic species)
[b]Origin test excluded *P. multiseries* Strain CLN-47 (single culture from eastern Canada)

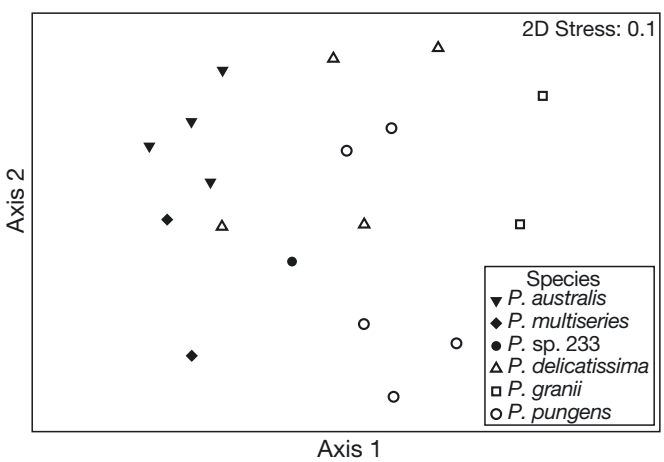

Fig. 1. Nonmetric multidimensional scaling plot describing similarity among whole bacterial profiles coexisting with 18 *Pseudo-nitzschia* cultures representing 6 species, as assessed by automated ribosomal intergenic spacer analysis. Bacterial communities coexisting with toxigenic (closed symbols) and nontoxigenic (open symbols) *Pseudo-nitzschia* cultures are shown

sus nontoxigenic strains was significantly different (p = 0.00017) than the expected distribution if each of the 59 singleton OTUs had an equal chance of occurring in 1 of the 7 toxigenic or 11 nontoxigenic cultures.

Next, we examined the distribution of the 33 OTUs that were detected in 2 or more cultures. Two OTUs were detected only among the toxigenic cultures, 16 OTUs were detected only among the nontoxigenic cultures, and 15 OTUs were detected in both types of culture (Fig. 2). Notably, 2 cultures obtained from the same Puget Sound water sample (toxigenic strain *Pseudo-nitzschia multiseries* PNWH2O A4 and nontoxigenic strain *P. pungens* PNWH2O C1) only shared 1 ARISA OTU, of the total of 6 and 8 OTUs detected per culture, respectively.

To explore which ARISA OTUs were most likely responsible for driving the differentiation in bacterial communities coexisting with toxigenic versus nontoxigenic *Pseudo-nitzschia* cultures, we performed SIMPER analysis. Of the 33 OTUs detected in 2 or more cultures, 22 OTUs contributed <3% to the dissimilar-

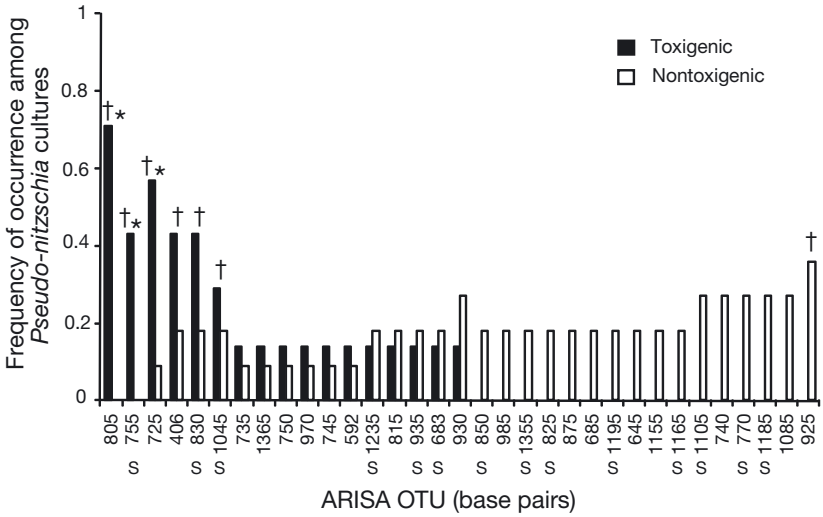

Fig. 2. Distribution of automated ribosomal intergenic spacer analysis (ARISA) peaks, or operational taxonomic units (OTUs), among toxigenic and nontoxigenic *Pseudo-nitzschia* cultures, for OTUs shared among 2 or more cultures. Notations indicate 7 OTUs that together accounted for 39% of the dissimilarity between the bacterial communities coexisting with toxigenic versus nontoxigenic cultures according to similarity percentage (SIMPER) analysis (†) and OTUs identified by probability analysis as being distributed in a significantly different manner between toxigenic and nontoxigenic cultures (*). An S below the ARISA fragment length indicates an OTU identified by 16S rDNA sequencing

ity among the bacterial communities coexisting with toxigenic versus nontoxigenic cultures, or less than the average contribution expected if each OTU had contributed equally. Every one of these 22 OTUs was detected among nontoxigenic cultures. In contrast, the dissimilarity between communities was driven most strongly by 7 OTUs, which together contributed a total of 39% to the overall dissimilarity. These 7 OTUs included 6 OTUs that were found exclusively or predominantly among toxigenic cultures and 1 OTU that was found only among nontoxigenic cultures (Fig. 2).

Because SIMPER is an exploratory, rather than statistically rigorous, analysis, we also examined the distribution of the 33 shared OTUs using binomial probability. Three OTUs exhibited a distribution that was significantly different than the distribution that would be expected if each of the nonsingleton OTUs had an equal probability of occurring in the 7 toxigenic or 11 nontoxigenic cultures. These 3 OTUs (Fig. 2) were also identified by SIMPER analysis as contributing strongly to the dissimilarity between toxigenic and nontoxigenic cultures, and each OTU was detected primarily among the toxigenic cultures: OTUs 805 ($p < 0.05$) and 755 and 725 ($p < 0.1$). Each of these 3 OTUs was detected with >1 *Pseudo-nitzschia* species and with isolates obtained from >1 geographic origin.

Identification of bacteria coexisting with *Pseudo-nitzschia*

We created 16S–23S rDNA clone libraries to identify the ARISA OTUs of bacteria coexisting with each of 2 *Pseudo-nitzschia* cultures, both obtained from Puget Sound: the nontoxigenic *P. delicatissima* Strain PNWH2O 604 and the toxigenic *P. multiseries* Strain PNWH2O A4. We determined ARISA fragment lengths for 95 bacterial clones from *P. delicatissima* (representing 14 distinct OTUs) and 82 bacterial clones from *P. multiseries* (representing 8 distinct OTUs). Of the 177 clones screened by ARISA, only 5 clones possessed ARISA fragment lengths that were not detected in the whole community profiles, including OTU 1305 (from the *P. delicatissima* clone library) and OTU 935 (from the *P. multiseries* clone library). Three clones were neither found in the whole profile nor selected for sequencing: OTUs 550 (*P. multiseries*) and 558 and 1450 (*P. delicatissima*). Rarefaction curves (Fig. 3) demonstrated that the 2 clone libraries were sampled close to saturation in terms of unique ARISA OTUs.

Both ARISA profiles and 16S rDNA clone libraries differed between the 2 *Pseudo-nitzschia* cultures. First, the bacterial communities coexisting with *P. delicatissima* and *P. multiseries* did not share any ARISA OTUs (Fig. 4), despite both cultures originating from Puget Sound. To further assess differences in bacterial community composition between the 2 cultures, the 16S rDNA was sequenced for unique ARISA OTUs detected in the clone libraries. In total, 36 clones were

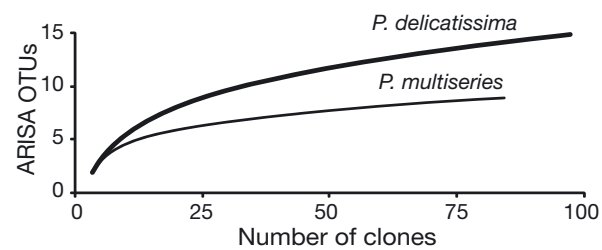

Fig. 3. Rarefaction curves for 16S–23S rDNA clone libraries constructed for bacterial communities coexisting with *Pseudo-nitzschia delicatissima* (Strain PNWH2O 604) and *P. multiseries* (Strain PNWH2O A4). Curves demonstrate the number of distinct automated ribosomal intergenic spacer analysis (ARISA) peaks, or operational taxonomic units (OTUs), represented by bacterial clones

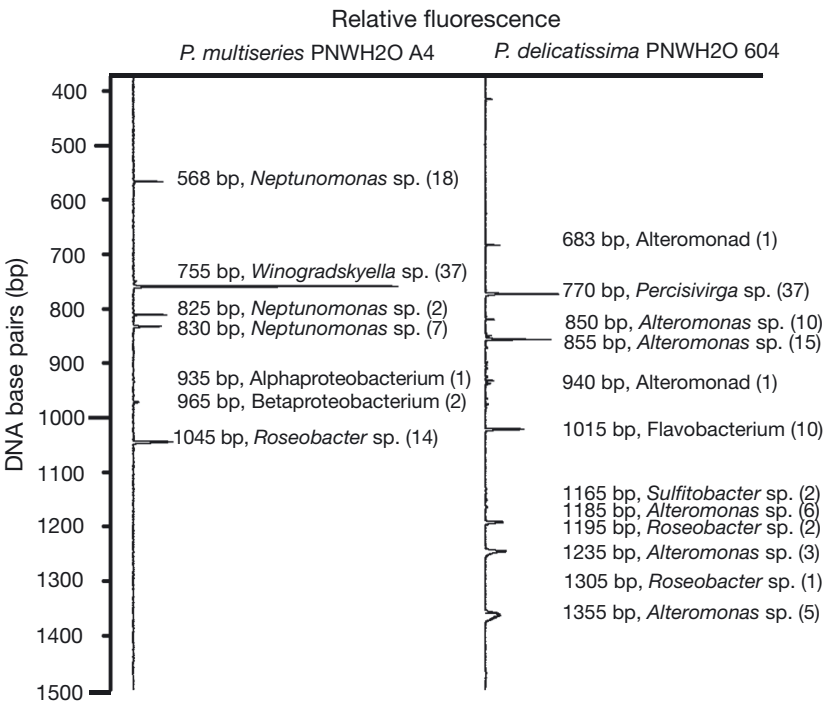

Fig. 4. Automated ribosomal intergenic spacer analysis (ARISA) of bacterial community profiles from *Pseudo-nitzschia multiseries* Strain PNWH2O A4 and *P. delicatissima* Strain PNWH2O 604. Individual ARISA peaks were identified by 16S rDNA sequencing. Numbers in parentheses indicate number of clones representing each ARISA peak in the clone libraries

sequenced, including 21 clones (representing 12 unique ARISA OTUs) derived from *P. delicatissima* and 15 clones (7 ARISA OTUs) derived from *P. multiseries*. Thus, 19 unique ARISA OTUs were identified by 16S rDNA sequencing (Fig. 4), including 14 of the 33 OTUs shared among 2 or more *Pseudo-nitzschia* cultures (Fig. 2). The number of clones representing each ARISA OTU generally corresponded to the intensity (in RFUs) of each peak in the ARISA profile (Fig. 4). In particular, OTU 755, a type that contributed strongly to the differences across all toxigenic and nontoxigenic cultures (Fig. 2), was identified as *Winogradskyella*, a *Flavobacterium*. This bacterium produced the highest-intensity ARISA peak in the *P. multiseries* PNWH2O A4 culture (Fig. 4).

The 16S rDNA sequences revealed that, although generally the same phyla (*Flavobacteria* and *Alpha-* and *Gammaproteobacteria*) were represented in the 2 *Pseudo-nitzschia* cultures, the specific bacteria in the 2 communities differed on a finer taxonomic scale (Fig. 5). Only the *P. multiseries* strain hosted a member of *Betaproteobacteria*. The 2 highest-intensity ARISA peaks in each profile, OTU 755 (*P. multiseries*) and OTU 770 (*P. delicatissima*), were both *Flavobacteria* (Fig. 4). However, the bacteria coexisting with the 2 *Pseudo-nitzschia* strains belonged to different clades (Fig. 5). For example, among the *Gammaproteobacteria* identified in the 2 cultures, *P. multiseries* hosted *Neptunomonas* sp. (order *Oceanospirillales*), whereas *P. delicatissima* hosted alteromonads (order *Alteromonadales*). These 2 strains hosted similar species richness, with 5 species (as defined by >99% 16S rDNA similarity) coexisting with the *P. multiseries* culture and 6 different species coexisting with the *P. delicatissima* culture.

We evaluated whether a single ARISA peak in our cultures represented a single bacterial species or strain by comparing the 16S rDNA sequence identity across clones representing the same ARISA peak. Two or more clones were sequenced for 10 of the 19 ARISA peaks. The clones associated with a single peak were at least 99.0% similar in the 16S rDNA sequence, but exhibited a range of finer scale variability. For 2 clones associated with a *Sulfitobacter* (OTU 1165), the 16S rDNA sequences were 100% identical. Clones associated with each of the other 9 peaks differed by up to 2 bp (*Alteromonas* OTU 850), 3 bp (*Roseobacter* OTU 1045, *Neptunomonas* OTU 568, and *Neptunomonas* OTU 830), 4 bp (*Alteromonas* OTU 1350), 5 bp (*Winogradskyella* OTU 755), 6 bp (Flavobacterium OTU 1015), 7 bp (*Percisivirga* OTU 770), and 14 bp (*Alteromonas* OTU 1185). For each of 2 ARISA peaks from which we sequenced 4 clones (*Winogradskyella* OTU 755 and *Percisivirga* OTU 770), each of the 4 clones possessed a unique 16S rDNA sequence, differing by up to 7 bp. The reported error rate for the *Taq* polymerase used here (8×10^{-6} errors bp^{-1}) would result in <0.3 error in each rDNA sequence reported in the present study (based on 1465 bp amplified for 25 cycles), an order of magnitude lower than the majority of sequence differences we observed.

We also examined whether the same bacterial species or strain produced >1 ARISA peak. Some clones associated with different ARISA OTUs clustered together in the 16S rDNA tree (Fig. 5), exhibiting sequence identities within the same range observed for clones associated with the same ARISA peak. For each of 4 different clades, clones with different ARISA OTUs varied by up to 1 bp (Alteromonad OTUs 683 and 940), 3 bp (*Roseobacter* OTUs 1195 and 1305), 5 bp (*Neptunomonas* OTUs 568, 825, and 830), and 13 bp (*Alteromonas* OTUs 850, 855, 1185, 1235, and 1355).

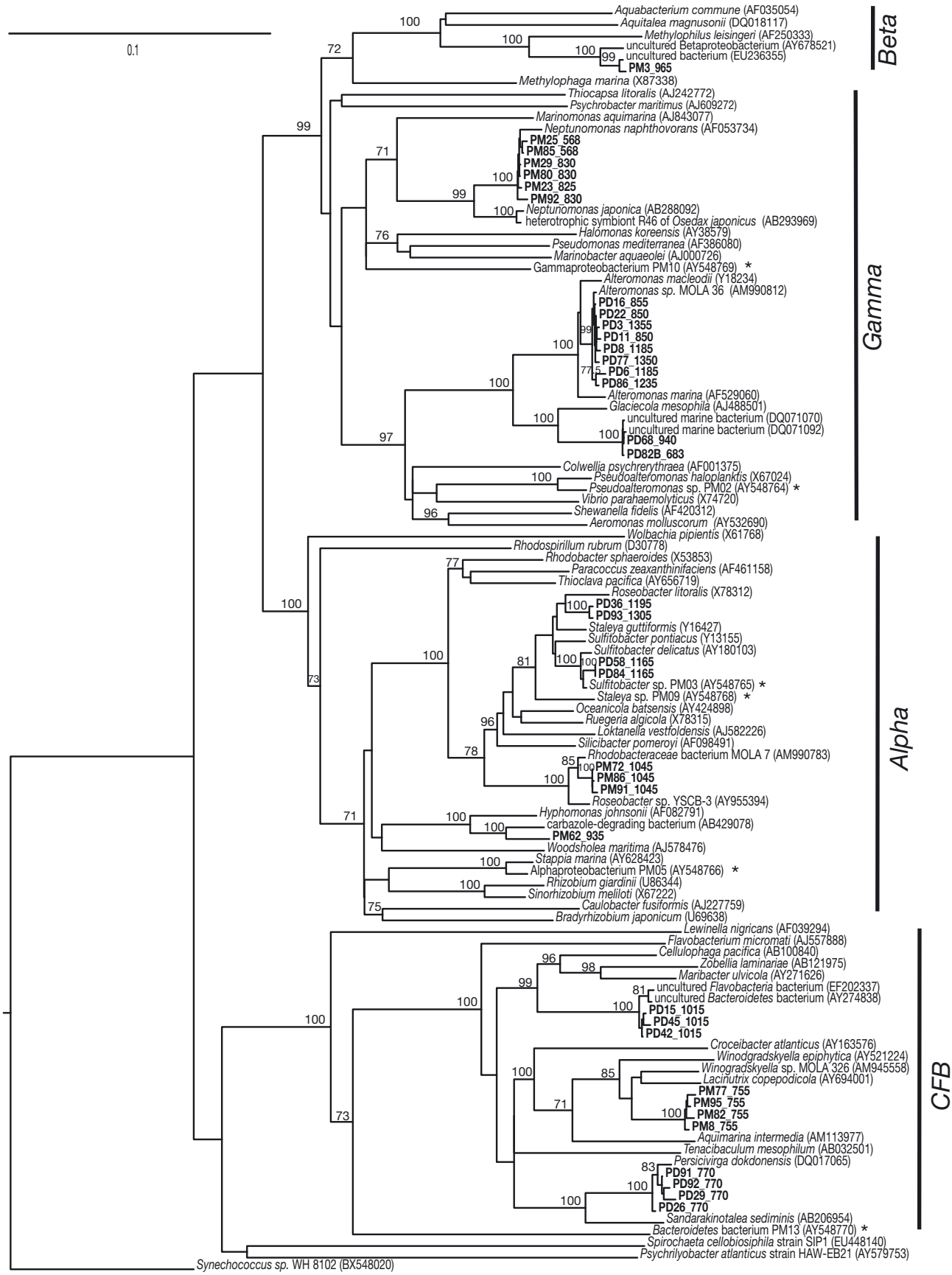
0.1
Aquabacterium commune (AF035054)
Aquitalea magnusonii (DQ018117)
Methylophilus leisingeri (AF250333)
uncultured Betaproteobacterium (AY678521)
uncultured bacterium (EU236355)
PM3_965
Methylophaga marina (X87338)
Beta
Thiocapsa litoralis (AJ242772)
Psychrobacter maritimus (AJ609272)
Marinomonas aquimarina (AJ843077)
Neptunomonas naphthovorans (AF053734)
PM25_568
PM85_568
PM29_830
PM80_830
PM23_825
PM92_830
Neptunomonas japonica (AB288092)
heterotrophic symbiont R46 of Osedax japonicus (AB293969)
Halomonas koreensis (AY38579)
Pseudomonas mediterranea (AF386080)
Marinobacter aquaeolei (AJ000726)
Gammaproteobacterium PM10 (AY548769) *
Alteromonas macleodii (Y18234)
Alteromonas sp. MOLA 36 (AM990812)
PD16_855
PD22_850
PD3_1355
PD11_850
PD8_1185
PD77_1350
PD6_1185
PD86_1235
Alteromonas marina (AF529060)
Glaciecola mesophila (AJ488501)
uncultured marine bacterium (DQ071070)
uncultured marine bacterium (DQ071092)
PD68_940
PD82B_683
Colwellia psychrerythraea (AF001375)
Pseudoalteromonas haloplanktis (X67024)
Pseudoalteromonas sp. PM02 (AY548764) *
Vibrio parahaemolyticus (X74720)
Shewanella fidelis (AF420312)
Aeromonas molluscorum (AY532690)
Gamma
Wolbachia pipientis (X61768)
Rhodospirillum rubrum (D30778)
Rhodobacter sphaeroides (X53853)
Paracoccus zeaxanthinifaciens (AF461158)
Thioclava pacifica (AY656719)
Roseobacter litoralis (X78312)
PD36_1195
PD93_1305
Staleya guttiformis (Y16427)
Sulfitobacter pontiacus (Y13155)
Sulfitobacter delicatus (AY180103)
PD58_1165
PD84_1165
Sulfitobacter sp. PM03 (AY548765) *
Staleya sp. PM09 (AY548768) *
Oceanicola batsensis (AY424898)
Ruegeria algicola (X78315)
Loktanella vestfoldensis (AJ582226)
Silicibacter pomeroyi (AF098491)
Rhodobacteraceae bacterium MOLA 7 (AM990783)
PM72_1045
PM86_1045
PM91_1045
Roseobacter sp. YSCB-3 (AY955394)
Hyphomonas johnsonii (AF082791)
carbazole-degrading bacterium (AB429078)
PM62_935
Woodsholea maritima (AJ578476)
Stappia marina (AY628423)
Alphaproteobacterium PM05 (AY548766) *
Rhizobium giardinii (U86344)
Sinorhizobium meliloti (X67222)
Caulobacter fusiformis (AJ227759)
Bradyrhizobium japonicum (U69638)
Alpha
Lewinella nigricans (AF039294)
Flavobacterium micromati (AJ557888)
Cellulophaga pacifica (AB100840)
Zobellia laminariae (AB121975)
Maribacter ulvicola (AY271626)
uncultured Flavobacteria bacterium (EF202337)
uncultured Bacteroidetes bacterium (AY274838)
PD15_1015
PD45_1015
PD42_1015
Croceibacter atlanticus (AY163576)
Winodgradskyella epiphytica (AY521224)
Winogradskyella sp. MOLA 326 (AM945558)
Lacinutrix copepodicola (AY694001)
PM77_755
PM95_755
PM82_755
PM8_755
Aquimarina intermedia (AM113977)
Tenacibaculum mesophilum (AB032501)
Persicivirga dokdonensis (DQ017065)
PD91_770
PD92_770
PD29_770
PD26_770
Sandarakinotalea sediminis (AB206954)
Bacteroidetes bacterium PM13 (AY548770) *
CFB
Spirochaeta cellobiosiphila strain SIP1 (EU448140)
Psychrilyobacter atlanticus strain HAW-EB21 (AY579753)
Synechococcus sp. WH 8102 (BX548020)

Fig. 5. 16S rDNA phylogenetic tree constructed using minimum evolution (ME) and bootstrap values calculated from 100 replicates in PAUP* Version 4.0. Scale bar indicates 0.1 change per nucleotide. Tree includes 36 bacterial clones coexisting with *Pseudo-nitzschia*, identified by culture (*P. multiseries* Strain PNWH2O A4 [PM] and *P. delicatissima* Strain PNWH2O 604 [PD]), clone number, and automated ribosomal intergenic spacer analysis peak length. *Synechococcus* sp. WH 8102, *Spirochaeta cellobiosiphila* Strain SIP1, and *Psychrilobacter atlanticus* Strain HAW-EB21 were used as outgroups. *Beta*: *Betaproteobacteria*; *Gamma*: *Gammaproteobacteria*; *Alpha*: *Alphaproteobacteria*; *CFB*: *Cytophaga-Flavobacterium-Bacteroides* group; *: bacteria previously identified from *P. multiseries* cultures (Kaczmarska et al. 2005). Compared to the ME tree shown here, additional trees constructed using maximum likelihood and maximum parsimony methods, demonstrated similar topology

DISCUSSION

The present study characterized bacteria coexisting with cultures from several geographic origins, including 5 *Pseudo-nitzschia* species previously unexamined with regards to bacterial community composition. Our findings therefore build upon prior work that characterized bacteria in *P. multiseries* cultures (Bates et al. 1995, Stewart et al. 1997, Kobayashi et al. 2003, Kaczmarska et al. 2005). The high-throughput fingerprinting technique ARISA allowed for rapid comparison of bacterial community composition among multiple cultures. We surveyed toxigenic and nontoxigenic strains of *Pseudo-nitzschia*, as assessed by DA production during the stationary phase induced by silicic acid limitation (Bates et al. 1991, Pan et al. 1996b, Bates 1998, Fehling et al. 2004, Lundholm et al. 2006, Marchetti et al. 2008). These strain-specific toxigenicity assessments were consistent with previous reports of the general toxigenicity of the species examined (Bates 1998, 2000, Lundholm et al. 2006, Marchetti et al. 2008). Although bacterial community composition and DA levels were not measured simultaneously, DA was measured under the same conditions the cultures experienced over the entire course of the study. Therefore, over months of repeated culture transfers in *f*/2- and K-media, we expect that the *Pseudo-nitzschia* cultures characterized as 'toxigenic' consistently produced high levels of DA during the stationary phase under silicic acid limitation, and returned to low or undetectable levels of DA following each transfer during exponential growth on mostly inorganic nitrogen sources (Bates 1998, Howard et al. 2007). Similarly, we assume that the bacterial communities in cultures we characterized as 'nontoxigenic' had very low or no exposure to DA during their time in culture.

Bacterial community composition in phytoplankton cultures likely results from a combination of *in situ* composition in the waters from which phytoplankton isolates are obtained and subsequent selection during the cultivation process based on specific culturing conditions and coexisting algal species. In a brand-new algal culture, even in the absence of exogenous carbon sources as here, bacterial community composition undoubtedly undergoes an initial shift from the *in situ* community due to the cultivation process (Grossart et al. 2005, Sapp et al. 2007b). This shift was not the focus here, and we did not sample our cultures during this initial cultivation period. However, we found that in cultures that were well established (at least 4 mo in culture), the presence of bacterial ARISA types did not differ significantly over a time frame of several months to 1 yr for either nontoxigenic (*Pseudo-nitzschia delicatissima*) or toxigenic (*P. multiseries* and *Pseudo-nitzschia* sp. 233) cultures. Therefore, any initial cultivability-induced changes in bacterial composition had apparently stabilized by the time of our experiments.

We sampled bacterial communities during both exponential growth and stationary phases with the reasoning that bacterial responses to DA (or other physiological differences) could manifest themselves in response to changing DA levels within a single culture or in response to an absolute difference in toxigenicity between cultures. Bacterial communities were similar across exponential growth and stationary phases for 5 toxigenic cultures, as well as for 3 nontoxigenic cultures. Within a single toxigenic culture sampled 6 times over the course of a single transfer (during which DA levels presumably fluctuated), the presence of specific bacterial types did not differ significantly. These findings are consistent with some prior reports (Schäfer et al. 2002, Jasti et al. 2005), but not with others (Grossart et al. 2005, Sapp et al. 2007a). In these latter cases, the stationary phase was greatly advanced (e.g. weeks to months); thus, extensive algal death and lysis could have heavily influenced the culture environment in ways not applicable here. Our data suggest that, within the environment of an established serially transferred batch culture, bacterial communities do not reassemble in response to DA on daily to weekly timescales, but rather that the majority of bacterial types were present throughout different algal growth phases and over time in culture. It is possible that, by the time of sampling for the present study, bacterial communities had already been enriched for DA-tolerant types, giving rise to differences in bacterial composition across toxigenic and nontoxigenic cultures. We further suggest that bacteria may cycle through both attached and free-living stages in the

culture tube, resulting in the observed similarity in bacterial composition across size fractions. Finally, it is worth emphasizing that our measure of community composition was based on the presence or absence of ARISA OTUs across samples and thus presents a conservative view of bacterial community composition. Changes in relative abundance of different members of the community over the growth phase (or across time in culture, geographic origin, or attached compared to free-living) would not be detected here.

We assessed whether geographic origin, *Pseudo-nitzschia* species, and *Pseudo-nitzschia* toxigenicity could explain the patterns observed in the bacterial community composition. The influences of species versus toxigenicity could not be distinguished from one another, because all strains of a particular species were either toxigenic or nontoxigenic. However, both factors were significantly correlated with bacterial composition, indicating that the specific culture environments became enriched for different bacterial types. In contrast, we found that bacterial communities only differed significantly with origin between open-ocean and coastal isolates, likely reflecting bacterial biogeography due to *in situ* environmental conditions. *Pseudo-nitzschia* strains from Washington, in comparison with *Pseudo-nitzschia* strains from California, did not vary significantly in bacterial community composition. Further, we also found that, for cultures of different species obtained from the same origin (and in 1 case the same water sample), bacterial communities differed greatly in terms of ARISA OTUs and 16S rDNA phylogeny, suggesting that other factors besides origin influenced bacterial community composition in the *Pseudo-nitzschia* cultures. These results are in line with the findings of Jasti et al. (2005), who determined that bacterial communities were similar according to coexisting algal type (e.g. the dinoflagellate *Alexandrium* versus nontoxigenic phytoplankton), rather than according to geographic origin. We similarly interpret our data to argue for a greater influence of species and toxigenicity, relative to geographic origin, on bacterial community composition.

The taxonomic identifications of the bacteria in *Pseudo-nitzschia* cultures revealed members of the *Roseobacter* clade, *Flavobacteria*, and *Gammaproteobacteria*, all frequently found with other phytoplankton cultures (Stewart et al. 1997, Alavi et al. 2001, Hold et al. 2001, Schäfer et al. 2002, Kobayashi et al. 2003, Green et al. 2004, Pinhassi et al. 2004, Fandino et al. 2005, Grossart et al. 2005, Jasti et al. 2005, Kaczmarska et al. 2005, Sapp et al. 2007a). However, the level of intraspecific diversity was unexpected; 9 of 10 ARISA peaks sequenced in replicate contained multiple nonidentical 16S rDNA sequences, suggesting that multiple strains of the same bacterial species (>99% 16S rDNA identity; Acinas et al. 2004) coexisted in *Pseudo-nitzschia* cultures. Notably, the bacterial clone libraries were constructed from 2 *Pseudo-nitzschia* strains that had been in culture for a substantial period of time — 9 mo for the *P. delicatissima* strain and 2 yr for the *P. multiseries* strain. Therefore, rather than being a simple community dominated by a few species of clonal bacteria, even culture environments may host substantial microdiversity.

Therefore, across *Pseudo-nitzschia* cultures, differences in both ARISA OTU composition and richness suggested that toxigenic and nontoxigenic cultures support different bacterial communities, due to DA or some other distinction between these cultures. Specifically, ARISA OTU richness was higher among nontoxigenic cultures than among toxigenic cultures. Furthermore, 3 ARISA OTUs were significantly associated with the majority of toxigenic cultures of *Pseudo-nitzschia*, compared to a wider range of OTUs distributed in a generally lower frequency among all nontoxigenic cultures. Kaczmarska et al. (2005) hypothesized that *Pseudo-nitzschia* produces higher levels of DA as a chemical defense against greater numbers or diversity of bacteria. Alternatively, we suggest that, if DA inhibits the growth of some bacteria (Stewart et al. 1998), bacterial richness in toxigenic cultures may instead be lessened (as seen here) due to the inhibitory effects of DA. We hypothesize that, over a time frame of months in culture, toxigenic strains of *Pseudo-nitzschia* cycled between high-DA conditions during the stationary phase and low-DA conditions following each transfer, eventually favored a bacterial community able to persist under both conditions (DA-utilizing and DA-tolerant bacteria). In contrast, nontoxigenic strains of *Pseudo-nitzschia* could potentially harbor both of these types, as well as DA-intolerant bacteria. Differences in bacterial composition would therefore signify a trade-off between types of bacteria that can dominate these environments.

Interactions related to DA should be viewed as a subset of other simultaneously occurring interactions among these diatoms and bacteria, including bacterial vitamin production (Croft et al. 2005), antimicrobial activity through production of compounds such as aldehydes (Ribalet et al. 2008), bacterial dissolution of silica frustules (Bidle & Azam 1999), and bacterial utilization of other dissolved organic carbon compounds produced by the diatom (Azam et al. 1983, Baines & Pace 1991). Ultimately, the enrichment of particular bacteria coexisting with different diatoms will be influenced by the comparative selective pressure on traits underlying any one of these interactions. If DA is the basis for significant interactions among bacteria and *Pseudo-nitzschia*, members of these groups can mutually influence each other; bacteria trigger DA production (Bates et al. 1995), and, in turn, specific bacterial

types become alternatively enriched or diminished in response to this compound.

The current study represents one line of evidence for the hypothesis that DA plays a role in structuring bacterial community composition. Future experiments could identify specific trade-offs in growth experienced by individual cultures of DA-utilizing, DA-tolerant, and DA-intolerant bacteria in response to exposure to DA or its analog glutamate. Whole bacterial community responses to DA could be further explored by measuring changes in both composition and the abundance of specific types in response to supplementing toxigenic and nontoxigenic cultures of *Pseudo-nitzschia* with DA. Here, we demonstrated that bacterial community composition varied significantly with *Pseudo-nitzschia* species and toxigenicity, indicating that the specific culture environments became enriched for different bacterial types. Our findings, in addition to the work of other researchers (Pinhassi et al. 2004, Grossart et al. 2005, Jasti et al. 2005), indicate the existence of algal-specific bacterial communities.

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