

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

Michele L. Guannel¹, M. Claire Horner-Devine², Gabrielle Rocap^{1,*}

¹University of Washington School of Oceanography, Box 357940, Seattle, Washington 98195, USA

²University of Washington School of Aquatic and Fishery Sciences, Box 355020, Seattle, Washington 98195, USA

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. multiseriis* (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

Resale or republication not permitted without written consent of the publisher

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. multiseriis* produce DA at levels of picograms of DA per cell (Bates 1998), and

*Corresponding author. Email: rocap@ocean.washington.edu

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. multiseriis* 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. multiseriis* or the diatom *Chaetoceros* spp. to axenic *P. multiseriis* 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). Kaczmarek et al. (2005) observed greater bacterial numbers and morphological diversity in higher toxin-producing *P. multiseriis* 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 multiseriis* 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, Kaczmarek 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. multiseriis*.

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 dimethylsulfopropionate (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, Bier-smith & 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. multiseriata* 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'-CTGCGTTCATCGTTGTGG-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 re-

ferred 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. Toxicogenic *Pseudo-nitzschia* strains are emphasized in **bold**. BD: below detection limit; X: automated ribosomal inter-genic 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

<i>Pseudo-nitzschia</i> species	Strain	PDA (pg cell ⁻¹)	DDA (pg cell ⁻¹)	Exp/Sta	AB/FLB	Time in culture	Origin	Isolated by	Reference
<i>P. australis</i> ^a	03199_1B	21.85	1.63	X	X		Monterey Wharf, CA	G. Jason Smith	Hubbard et al. (2008)
<i>P. australis</i> ^a	04063_3C	3.15	1.26	X	X		Bodega/Tomales Bay, CA	G. Jason Smith	Hubbard et al. (2008)
<i>P. australis</i> ^a	03184_5D	9.00	3.78				Monterey Wharf, CA	G. Jason Smith	Hubbard et al. (2008)
<i>P. australis</i> ^a	03184_6D	0.20	0.04	X	X		Monterey Wharf, CA	G. Jason Smith	Hubbard et al. (2008)
<i>P. multiseriis</i> ^a	CLN-47	0.71	0.17	X	X		Progeny of 2 isolates from eastern Canada ^b	Claude Léger	Mafrà Jr et al. (2009)
<i>P. multiseriis</i> ^a	PNWH20 A4	5.84	1.32	X	X	X	Puget Sound, WA (Main Basin)	Katherine Hubbard	Hubbard et al. (2008)
<i>P. sp. 233</i> ^c	PNWH20 233a	13.48	0.85	X	X	X	Sequim Bay, WA 48° 03' N, 123° 01' W	Karie Holtermann	Present study
<i>P. delicatissima</i>	PNWH20 604	BD	BD	X	X	X	Puget Sound, WA 47° 44.1' N, 122° 45.7' W	Michele Guannel	Present study
<i>P. delicatissima</i>	PNWH20 605	BD	BD	X	X	X	Puget Sound, WA 47° 44.1' N, 122° 45.7' W	Michele Guannel	Present study
<i>P. delicatissima</i>	PNWH20 609	BD	BD	X	X	X	Puget Sound, WA 47° 16.6' N, 122° 42.5' W	Michele Guannel	Present study
<i>P. delicatissima</i>	PNWH20 K2	BD	BD	BD	BD	BD	Sequim Bay, WA 48° 03' N, 123° 01' W	Karie Holtermann	Present study
<i>P. granii</i>	UWOSP22	BD	BD	BD	BD	BD	NE Pacific 50° N, 145° W	Adrian Marchetti	Present study
<i>P. granii</i>	UWOSP36	BD	BD	BD	BD	BD	NE Pacific 50° N, 145° W	Adrian Marchetti	Marchetti et al. (2008)
<i>P. pungens</i>	PNWH20 607	BD	BD	BD	BD	BD	Puget Sound, WA 48° 1' N, 122° 18.2' W	Michele Guannel	Present study
<i>P. pungens</i>	PNWH20 608	BD	BD	BD	BD	BD	Puget Sound, WA 48° 1' N, 122° 18.2' W	Michele Guannel	Present study
<i>P. pungens</i>	PNWH20 LB2	BD	BD	BD	BD	BD	Long Beach, WA 46° 42' N, 123° 58' W	Karie Holtermann	Present study
<i>P. pungens</i>	PNWH20 C1	BD	BD	X	X	X	Puget Sound, WA (Main Basin)	Katherine Hubbard	Hubbard et al. (2008)
<i>P. pungens</i> ^a	PNWH20 101WB	BD	BD	X	X	X	Willapa Bay, WA	Katherine Hubbard	Hubbard et al. (2008)

^aSpecies identification supported by scanning electron microscopy (SEM)

^b*P. multiseriis* 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)

^cNot 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. multiseriis* (Table 1). In addition, 1 cryptic strain, PNWH20 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. multiseriis* CLN-47, which was incubated at 20°C under 24 h of light. Light levels ranged between 30 and 115 $\mu\text{mol quanta}^{-1} \text{m}^{-2}$. 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 non-toxigenic 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 \times 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. multiseriata* 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. multiseriata* 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'-AGAGTTTGATCMTG-GCTCAG-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 μ l PCR mixture contained 2 ng genomic DNA, 1 \times Mg-free buffer (GeneChoice), 2.5 mM MgCl₂, 0.8 mM dNTPs, 1 U *Taq* DNA polymerase (GeneChoice), and 0.5 μ M 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 Mini-prep 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. multiseriis* 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. multiseriis*, 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 nontoxicogenic (*P. delicatissima*) or toxicogenic (*P. multiseriis*, *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. Toxicogenic cultures of *P. multiseriis* 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 toxicogenic 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 toxicogenic strain of *P. multiseriis*, 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 toxicogenic strain of *Pseudo-nitzschia* (PNWH2O 233a) and 1 nontoxicogenic 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 toxicogenic or nontoxicogenic types of each of the species, from each of the 3 geographic origins, we were unable to statisti-

cally 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 toxicogenicity 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 toxicogenic versus nontoxicogenic cultures contributed most strongly to the species differences (as indicated by higher R-values; Table 2). Furthermore, bacterial community composition in nontoxicogenic cultures of *Pseudo-nitzschia* was significantly different relative to the bacterial communities in toxicogenic 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 toxicogenicity remained as factors that could explain bacterial community structure.

ARISA OTUs correlated with *Pseudo-nitzschia* toxicogenicity

In order to evaluate the potential effects of DA on bacterial community composition, we compared the distribution of ARISA OTUs across toxicogenic and nontoxicogenic 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 nontoxicogenic culture, whereas the remaining 10 (17%) were detected in a toxicogenic culture. According to binomial probability, this distribution of singletons across toxicogenic 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						
<i>P. australis</i> vs. <i>P. pungens</i>	0.644	0.016*	126	0.644	0.016*	126
<i>P. delicatissima</i> vs. <i>P. pungens</i>	0.206	0.143	126	0.206	0.143	126
<i>P. australis</i> vs. <i>P. delicatissima</i>	0.479	0.057	35	0.479	0.057	35
<i>P. multiseriis</i> vs. <i>P. pungens</i>	0.609	0.048	21	0.609	0.048	21
<i>P. multiseriis</i> vs. <i>P. australis</i>	0.714	0.067	15	0.714	0.067	15
<i>P. multiseriis</i> vs. <i>P. delicatissima</i>	0.054	0.333	15	0.054	0.333	15
<i>P. pungens</i> vs. <i>P. granii</i>	0.664	0.048	21	–	–	–
<i>P. australis</i> vs. <i>P. granii</i>	1	0.067	15	–	–	–
<i>P. delicatissima</i> vs. <i>P. granii</i>	0.143	0.400	15	–	–	–
<i>P. multiseriis</i> vs. <i>P. granii</i>	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	–	–	–

^aSpecies test excluded *Pseudo-nitzschia* sp. 233 Strain PNWH2O 233a (single culture of cryptic species)
^bOrigin test excluded *P. multiseriis* Strain CLN-47 (single culture from eastern Canada)

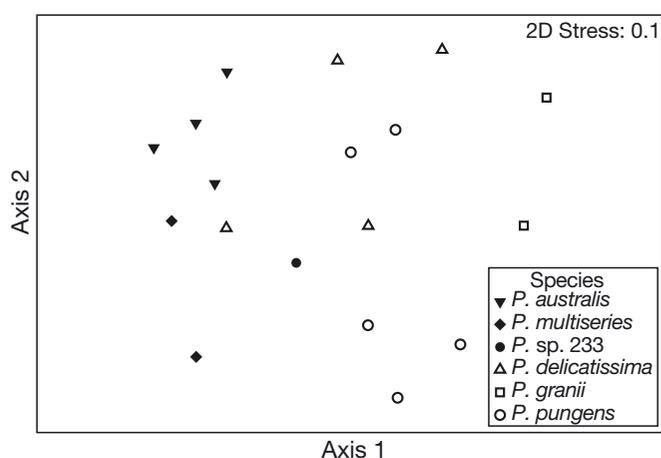


Fig. 1. Nonmetric multidimensional scaling plot describing similarity among whole bacterial profiles coexisting with 18 *Pseudo-nitzschia* cultures representing 6 species, as assessed by automated ribosomal intergenic spacer analysis. Bacterial communities coexisting with toxigenic (closed symbols) and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

sus nontoxicogenic 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 nontoxicogenic 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 nontoxicogenic 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 multiseriis* PNWH2O A4 and nontoxicogenic 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 nontoxicogenic *Pseudo-nitzschia* cultures, we performed SIMPER analysis. Of the 33 OTUs detected in 2 or more cultures, 22 OTUs contributed $<3\%$ to the dissimilar-

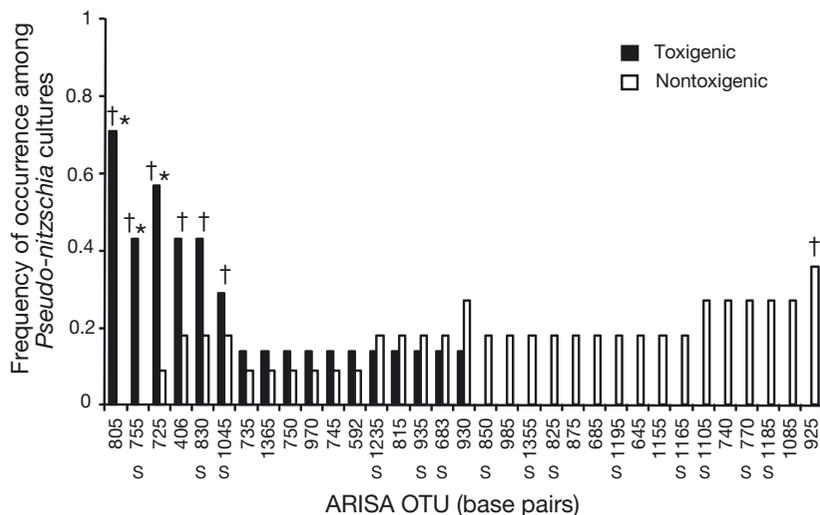


Fig. 2. Distribution of automated ribosomal intergenic spacer analysis (ARISA) peaks, or operational taxonomic units (OTUs), among toxicogenic and nontoxicogenic *Pseudo-nitzschia* cultures, for OTUs shared among 2 or more cultures. Notations indicate 7 OTUs that together accounted for 39% of the dissimilarity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

ity among the bacterial communities coexisting with toxicogenic versus nontoxicogenic cultures, or less than the average contribution expected if each OTU had contributed equally. Every one of these 22 OTUs was detected among nontoxicogenic 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 toxicogenic cultures and 1 OTU that was found only among nontoxicogenic 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 toxicogenic or 11 nontoxicogenic cultures. These 3 OTUs (Fig. 2) were also identified by SIMPER analysis as contributing strongly to the dissimilarity between toxicogenic and nontoxicogenic cultures, and each OTU was detected primarily among the toxicogenic 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 nontoxicogenic *P. delicatissima* Strain PNWH2O 604 and the toxicogenic *P. multiseriis* 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. multiseriis* (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. multiseriis* clone library). Three clones were neither found in the whole profile nor selected for sequencing: OTUs 550 (*P. multiseriis*) 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. multiseriis* 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

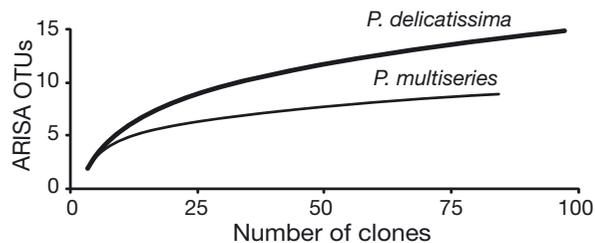


Fig. 3. Rarefaction curves for 16S–23S rDNA clone libraries constructed for bacterial communities coexisting with *Pseudo-nitzschia delicatissima* (Strain PNWH2O 604) and *P. multiseriis* (Strain PNWH2O A4). Curves demonstrate the number of distinct automated ribosomal intergenic spacer analysis (ARISA)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

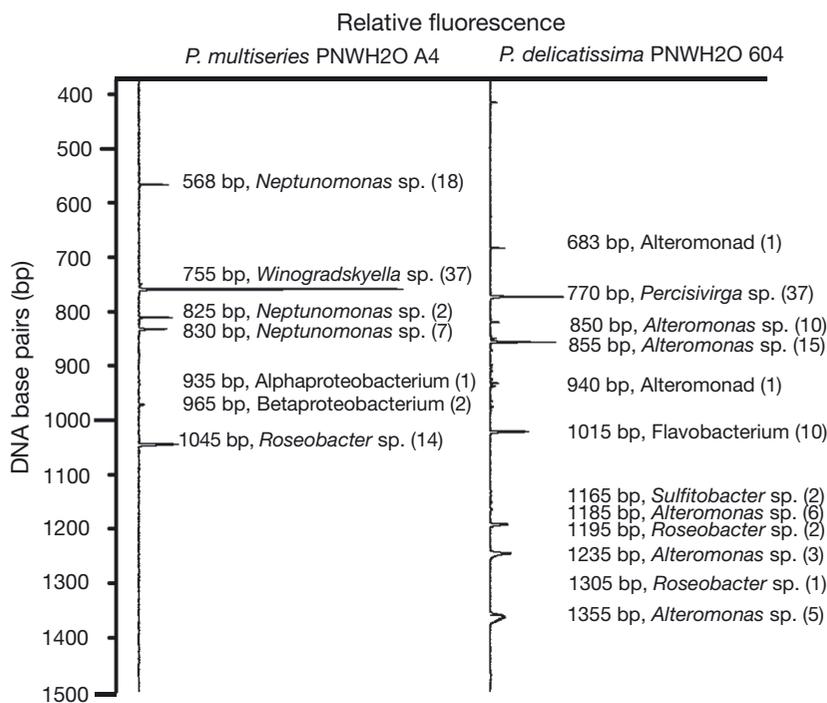


Fig. 4. Automated ribosomal intergenic spacer analysis (ARISA) of bacterial community profiles from *Pseudo-nitzschia multiseriis* Strain PNWH2O A4 and *P. delicatissima* Strain PNWH2O 604. Individual ARISA peaks were identified by 16S rDNA sequencing. Numbers in parentheses indicate number of clones 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. multiseriis*. 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. multiseriis* 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. multiseriis* strain hosted a member of *Betaproteobacteria*. The 2 highest-intensity ARISA peaks in each profile, OTU 755 (*P. multiseriis*) 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. multiseriis* 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. multiseriis* 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).

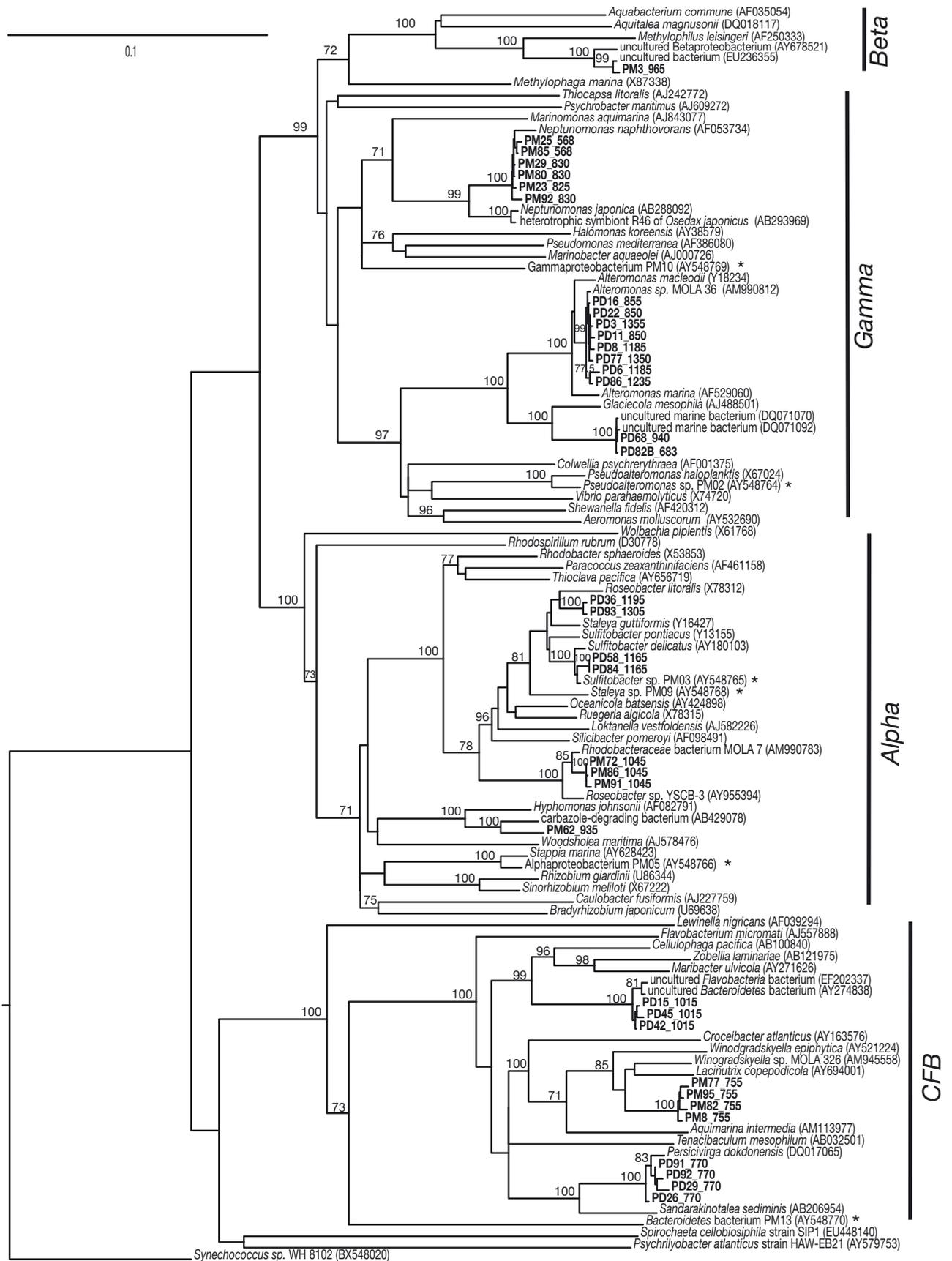


Fig. 5. 16S rDNA phylogenetic tree constructed using minimum evolution (ME) and bootstrap values calculated from 100 replicates in PAUP* Version 4.0. Scale bar indicates 0.1 change per nucleotide. Tree includes 36 bacterial clones coexisting with *Pseudo-nitzschia*, identified by culture (*P. multiseriis* 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. multiseriis* cultures (Kaczmarek 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. multiseriis* cultures (Bates et al. 1995, Stewart et al. 1997, Kobayashi et al. 2003, Kaczmarek 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. multiseriis* 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, Kaczmarek 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. multiseriata* 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. Kaczmarek 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.

Acknowledgements. We thank Stephen S. Bates, Karie Holtermann, Katherine Hubbard, Adrian Marchetti, and G. Jason Smith for providing *Pseudo-nitzschia* cultures. Gwendolyn Hannam assisted with culturing, and Diana Haring helped with DNA clone libraries. Katherine Hubbard and Rhonda Marohl-Morales conducted *Pseudo-nitzschia* ITS1 sequencing and identification for some cultures. We thank Katherine Hubbard for helpful comments and discussions. Constructive comments of 3 anonymous reviewers improved the focus and clarity of this manuscript. This work was supported by the Pacific Northwest Center for Human Health and Ocean Sciences (National Institute of Environmental Health: P50 ES012762 and National Science Foundation: OCE-0434087) and by a Predoctoral Traineeship in Oceans and Human Health from the Pacific Northwest Consortium to M.L.G.

LITERATURE CITED

- Acinas SG, Klepac-Ceraj V, Hunt DE, Pharino C, Ceraj I, Distel DL, Polz MF (2004) Fine-scale phylogenetic architecture of a complex bacterial community. *Nature* 430: 551–554
- Ahn SJ, Costa J, Emanuel JR (1996) PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. *Nucleic Acids Res* 24:2623–2625
- Alavi M, Miller T, Erlandson K, Schneider R, Belas R (2001) Bacterial community associated with *Pfiesteria*-like dinoflagellate cultures. *Environ Microbiol* 3:380–396
- Azam F, Fenchel T, Field JG, Gray JS, Meyer-Reil LA, Thingstad F (1983) The ecological role of water-column microbes in the sea. *Mar Ecol Prog Ser* 10:257–263
- Baines SB, Pace ML (1991) The production of dissolved organic matter by phytoplankton and its importance to bacteria: patterns across marine and freshwater systems. *Limnol Oceanogr* 36:1078–1090
- Bates SS (1998) Ecophysiology and metabolism of ASP toxin production. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) *The physiological ecology of harmful algal blooms*. Springer-Verlag, Heidelberg, p 405–426
- Bates SS (2000) Domoic-acid-producing diatoms: another genus added! *J Phycol* 36:978–983
- Bates SS, Bird CJ, de Freitas ASW, Foxall R and others (1989) Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from Eastern Prince Edward Island, Canada. *Can J Fish Aquat Sci* 46: 1203–1215
- Bates SS, deFreitas ASW, Milley JE, Pocklington R, Quilliam MA, Smith JC, Worms J (1991) Controls on domoic acid production by the diatom *Nitzschia pungens* f. *multiseries* in culture: nutrients and irradiance. *Can J Fish Aquat Sci* 48:1136–1144
- Bates SS, Douglas DJ, Doucette GJ, Léger C (1995) Enhancement of domoic acid production by reintroducing bacteria to axenic cultures of the diatom *Pseudo-nitzschia multiseries*. *Nat Toxins* 3:428–435
- Bates SS, Garrison DL, Horner RA (1998) Bloom dynamics and physiology of domoic-acid-producing *Pseudo-nitzschia* species. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) *Physiological ecology of harmful algal blooms*. Springer-Verlag, Heidelberg, p 267–292
- Bates SS, Gaudet J, Kaczmarek I, Ehrman JM (2004) Interaction between bacteria and the domoic-acid-producing diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle; Can bacteria produce domoic acid autonomously? *Harmful Algae* 3:11–20
- Bidle KD, Azam F (1999) Accelerated dissolution of diatom silica by marine bacterial assemblages. *Nature* 397:508–512
- Biersmith A, Benner R (1998) Carbohydrates in phytoplankton and freshly produced dissolved organic matter. *Mar Chem* 63:131–144
- Bill BD, Cox FH, Horner RA, Borchert JA, Trainer VL (2006) The first closure of shellfish harvesting due to domoic acid in Puget Sound, Washington, USA. *Afr J Mar Sci* 28: 435–440
- Casteleyn G, Adams NG, Vanormelingen P, Debeer AE, Sabbe K, Vyverman W (2009) Natural hybrids in the marine diatom *Pseudo-nitzschia pungens* (Bacillariophyceae): genetic and morphological evidence. *Protist* 160:343–354
- Clarke KR, Warwick RM (2001) Changes in marine communities: an approach to statistical analysis and interpretation, 2nd edn. PRIMER-E, Plymouth
- Cole JR, Chai B, Farris RJ, Wang Q and others (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res* 33:D294–D296
- Colwell RK, Coddington JA (1994) Estimating terrestrial biodiversity through extrapolation. *Philos Trans R Soc Lond B Biol Sci* 345:101–118
- Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature* 438:90–93
- Douglas DJ, Bates SS (1992) Production of domoic acid, a neurotoxic amino acid, by an axenic culture of the marine diatom *Nitzschia pungens* f. *multiseries* Hasle. *Can J Fish Aquat Sci* 49:85–90
- Douglas DJ, Bates SS, Bourque LA, Selvin RC (1993) Domoic acid production by axenic and non-axenic cultures of the pennate diatom *Nitzschia pungens* f. *multiseries*. In: Smayda T, Shimizu Y (eds) *Toxic phytoplankton blooms in the sea*. Elsevier, Amsterdam, p 595–600
- Dyson K, Huppert DD (2010) Regional economic impacts of razor clam beach closures due to harmful algal blooms (HABs) on the Pacific coast of Washington. *Harmful Algae* 9:264–271

- Fandino LB, Riemann L, Steward GF, Azam F (2005) Population dynamics of *Cytophaga-Flavobacteria* during marine phytoplankton blooms analyzed by real-time quantitative PCR. *Aquat Microb Ecol* 40:251–257
- Fehling J, Green DH, Davidson K, Bolch CJ, Bates SS (2004) Domoic acid production by *Pseudo-nitzschia seriata* (Bacillariophyceae) in Scottish waters. *J Phycol* 40:622–630
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Fisher MM, Triplett EW (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl Environ Microbiol* 65:4630–4636
- Fryxell GA, Villac MC, Shapiro LP (1997) The occurrence of the toxic diatom genus *Pseudo-nitzschia* (Bacillariophyceae) on the West Coast of the USA, 1920–1996: a review. *Phycologia* 36:419–437
- Fuhrman JA, Hewson I, Schwalbach MS, Steele JA, Brown MV, Naeem S (2006) Annually reoccurring bacterial communities are predictable from ocean conditions. *Proc Natl Acad Sci USA* 103:13104–13109
- Gotelli NJ, Colwell RK (2001) Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. *Ecol Lett* 4:379–391
- Green DH, Llewellyn LE, Negri AP, Blackburn SI, Bolch CJS (2004) Phylogenetic and functional diversity of the cultivable bacterial community associated with the paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum*. *FEMS Microbiol Ecol* 47:345–357
- Grossart HP, Levold F, Allgaier M, Simon M, Brinkhoff T (2005) Marine diatom species harbour distinct bacterial communities. *Environ Microbiol* 7:860–873
- 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
- Hagström JA, Granéli E, Maneiro I, Barreiro A, Petermann A, Svensen C (2007) Release and degradation of amnesic shellfish poison from decaying *Pseudo-nitzschia multiseries* in presence of bacteria and organic matter. *Harmful Algae* 6:175–188
- Hasle GR (2002) Are most of the domoic acid-producing species of the diatom genus *Pseudo-nitzschia* cosmopolites? *Harmful Algae* 1:137–146
- Hold GL, Smith EA, Rappé MS, Maas EW and others (2001) Characterisation of bacterial communities associated with toxic and non-toxic dinoflagellates: *Alexandrium* spp. and *Scrippsiella trochoidea*. *FEMS Microbiol Ecol* 37:161–173
- Howard MDA, 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
- Hubbard KA, Rocab G, Armbrust EV (2008) Inter- and intraspecific community structure within the diatom genus *Pseudo-nitzschia* (Bacillariophyceae). *J Phycol* 44:637–649
- Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* 20:2317–2319
- Hunt DE, Klepac-Ceraj V, Acinas SG, Gautier C, Bertilsson S, Polz MF (2006) Evaluation of 23S rRNA PCR primers for use in phylogenetic studies of bacterial diversity. *Appl Environ Microbiol* 72:2221–2225
- Jasti S, Sieracki ME, Poulton NJ, Giewat MW, Rooney-Varga JN (2005) Phylogenetic diversity and specificity of bacteria closely associated with *Alexandrium* spp. and other phytoplankton. *Appl Environ Microbiol* 71:3483–3494
- Kaczmarek I, Ehrman JM, Bates SS, Green DH, Léger C, Harris J (2005) Diversity and distribution of epibiotic bacteria on *Pseudo-nitzschia multiseries* (Bacillariophyceae) in culture, and comparison with those on diatoms in native seawater. *Harmful Algae* 4:725–741
- Keller DK, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. *J Phycol* 23:633–638
- Kleivdal H, Kristiansen SI, Nilsen MV, Briggs L (2007) Single-laboratory validation of the Biosense direct competitive enzyme-linked immunosorbent assay (ELISA) for determination of domoic acid toxins in shellfish. *J AOAC Int* 90:1000–1010
- Kobayashi K, Kobiyama A, Kotaki IY, Kodama M (2003) Possible occurrence of intracellular bacteria in *Pseudo-nitzschia multiseries*, a causative diatom of amnesic shellfish poisoning. *Fish Sci* 69:974–978
- Kobayashi K, Takata Y, Kodama M (2009) Direct contact between *Pseudo-nitzschia multiseries* and bacteria is necessary for the diatom to produce a high level of domoic acid. *Fish Sci* 75:771–776
- Lane DJ (1991) 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, New York, NY, p 115–147
- Lau WWY, Armbrust EV (2006) Detection of glycolate oxidase gene *gldD* diversity among cultured and environmental marine bacteria. *Environ Microbiol* 8:1688–1702
- Lau WWY, Keil RG, Armbrust EV (2007) Succession and diel transcriptional response of the glycolate-utilizing component of the bacterial community during a spring phytoplankton bloom. *Appl Environ Microbiol* 73:2440–2450
- Lefebvre KA, Bargu S, Kieckhefer T, Silver MW (2002) From sanddabs to blue whales: the pervasiveness of domoic acid. *Toxicol* 40:971–977
- Legendre P, Legendre L (1998) *Numerical ecology*, 2nd English edn. Elsevier Science BV, Amsterdam
- Ludwig W, Strunk O, Westram R, Richter L and others (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* 32:1363–1371
- Lundholm N, Moestrup Ø, Kotaki Y, Hoef-Emden K, Scholin C, Miller P (2006) Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. *J Phycol* 42:464–481
- Mafra LL Jr, Léger C, Bates SS, Quilliam MA (2009) Analysis of trace levels of domoic acid in seawater and plankton by liquid chromatography without derivatization, using UV or mass spectrometry detection. *J Chromatogr A* 1216:6003–6011
- Maldonado MT, Hughes MP, Rue EL, Wells ML (2002) The effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*. *Limnol Oceanogr* 47:515–526
- Malmstrom RR, Kiene RP, Kirchman DL (2004) Identification and enumeration of bacteria assimilating dimethylsulfoniopropionate (DMSP) in the North Atlantic and Gulf of Mexico. *Limnol Oceanogr* 49:597–606
- Marchetti A, Trainer VL, Harrison PJ (2004) Environmental conditions and phytoplankton dynamics associated with *Pseudo-nitzschia* abundance and domoic acid in the Juan de Fuca eddy. *Mar Ecol Prog Ser* 281:1–12
- 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 north-east subarctic Pacific. *J Phycol* 44:650–661
- Merzouk A, Levasseur M, Scarratt M, Michaud S, Lizotte M, Rivkin RB, Kiene RP (2008) Bacterial DMSP metabolism

- during the senescence of the spring diatom bloom in the Northwest Atlantic. *Mar Ecol Prog Ser* 369:1–11
- Myklestad SM (1995) Release of extracellular products by phytoplankton with special emphasis on polysaccharides. *Sci Total Environ* 165:155–164
- Pan YL, Rao DVS, Mann KH (1996a) Changes in domoic acid production and cellular chemical composition of the toxic diatom *Pseudo-nitzschia multiseriata* under phosphate limitation. *J Phycol* 32:371–381
- Pan Y, Rao DVS, Mann KH, Brown RG, Pocklington R (1996b) Effects of silicate limitation on production of domoic acid, a neurotoxin, by the diatom *Pseudo-nitzschia multiseriata*. 1. Batch culture studies. *Mar Ecol Prog Ser* 131:225–233
- Pan Y, Rao DVS, Mann KH, Li WKW, Harrison WG (1996c) Effects of silicate limitation on production of domoic acid, a neurotoxin, by the diatom *Pseudo-nitzschia multiseriata*. 2. Continuous culture studies. *Mar Ecol Prog Ser* 131:235–243
- Pinhassi J, Sala MM, Havskum H, Peters F, Guadayol O, Malits A, Marrase C (2004) Changes in bacterioplankton composition under different phytoplankton regimens. *Appl Environ Microbiol* 70:6753–6766
- Pinhassi J, Simo R, Gonzalez J, Vila M and others (2005) Dimethylsulfoniopropionate turnover is linked to the composition and dynamics of the bacterioplankton assemblage during a microcosm phytoplankton bloom. *Appl Environ Microbiol* 71:7650–7660
- Polz MF, Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* 64:3724–3730
- Ribalet F, Intertaglia L, Lebaron P, Casotti R (2008) Differential effect of three polyunsaturated aldehydes on marine bacterial isolates. *Aquat Toxicol* 86:249–255
- Rue E, Bruland K (2001) Domoic acid binds iron and copper: a possible role for the toxin produced by the marine diatom *Pseudo-nitzschia*. *Mar Chem* 76:127–134
- Sambrook S, Russell DW (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York, NY
- Sapp M, Schwaderer A, Wiltshire K, Hoppe HG, Gerdt G, Wichels A (2007a) Species-specific bacterial communities in the phycosphere of microalgae? *Microb Ecol* 53:683–699
- Sapp M, Wichels A, Gerdt G (2007b) Impacts of cultivation of marine diatoms on the associated bacterial community. *Appl Environ Microbiol* 73:3117–3120
- Schäfer H, Abbas B, Witte H, Muyzer G (2002) Genetic diversity of 'satellite' bacteria present in cultures of marine diatoms. *FEMS Microbiol Ecol* 42:25–35
- Scholin CA, Gulland F, Doucette GJ, Benson S and others (2000) Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 403:80–84
- Stewart JE (2008) Bacterial involvement in determining domoic acid levels in *Pseudo-nitzschia multiseriata* cultures. *Aquat Microb Ecol* 50:135–144
- Stewart JE, Marks LJ, Wood CR, Risser SM, Gray S (1997) Symbiotic relations between bacteria and the domoic acid producing diatom *Pseudo-nitzschia multiseriata* and the capacity of these bacteria for gluconic acid/gluconolactone formation. *Aquat Microb Ecol* 12:211–221
- Stewart JE, Marks LJ, Gilgan MW, Pfeiffer E, Zwicker BM (1998) Microbial utilization of the neurotoxin domoic acid: blue mussels (*Mytilus edulis*) and soft shell clams (*Mya arenaria*) as sources of the microorganisms. *Can J Microbiol* 44:456–464
- Swofford DL (2002) *PAUP*: phylogenetic analysis using parsimony (and other methods)* 4.0, Beta edn. Sinauer Associates, Sunderland, MA
- Thessen AE, Bowers HA, Stoecker DK (2009) Intra- and interspecies differences in growth and toxicity of *Pseudo-nitzschia* while using different nitrogen sources. *Harmful Algae* 8:792–810
- Thompson JR, Marcelino LA, Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Res* 30:2083–2088
- Trainer VL, Suddleson M (2005) Monitoring approaches for early warning of domoic acid events in Washington State. *Oceanography (Wash DC)* 18:228–237
- 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, Hickey BM, Lessard EJ, Cochlan WP and others (2009a) Variability of *Pseudo-nitzschia* and domoic acid in the Juan de Fuca eddy region and its adjacent shelves. *Limnol Oceanogr* 54:289–308
- Trainer VL, Wells ML, Cochlan WP, Trick CG and others (2009b) An ecological study of a massive bloom of toxic *Pseudo-nitzschia cuspidata* off the Washington State coast. *Limnol Oceanogr* 54:1461–1474
- Trick CG, Bill BD, Cochlan WP, Wells ML, Trainer VL, Pickell LD (2010) Iron enrichment stimulates toxic diatom production in high-nitrate, low-chlorophyll areas. *Proc Natl Acad Sci USA* 107:5887–5892
- Vila M, Simo R, Kiene RP, Pinhassi J, Gonzalez JM, Moran MA, Pedros-Alio C (2004) Use of microautoradiography combined with fluorescence *in situ* hybridization to determine dimethylsulfoniopropionate incorporation by marine bacterioplankton taxa. *Appl Environ Microbiol* 70:4648–4657
- Work TM, Barr B, Beale AM, Fritz L, Quilliam MA, Wright JLC (1993) Epidemiology of domoic acid poisoning in brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) in California. *J Zoo Wildl Med* 24:54–62
- Wright JLC, Boyd RK, de Freitas ASW, Falk M and others (1989) Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. *Can J Chem* 67:481–490
- Zar JH (1974) *Biostatistical analysis*, 1st edn. Prentice-Hall, Englewood Cliffs, NJ

Editorial responsibility: Jed Fuhrman,
Los Angeles, California, USA

Submitted: May 6, 2010; Accepted: April 14, 2011
Proofs received from author(s): July 11, 2011