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Marine microbial biogeography from microscopic to global scales: ecology of the diatom *Pseudo-nitzschia*

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ABSTRACT

Marine microbial biogeography from microscopic to global scales:
distributions of the diatom *Pseudo-nitzschia* and co-existing bacteria

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Members of the marine diatom genus *Pseudo-nitzschia* are globally distributed, biologically productive, remarkably diverse, and responsible for Harmful Algal Blooms (HABs) through the production of the neurotoxin, domoic acid. This dissertation explores three major dimensions of life which are important to the biogeography and toxigenicity of *Pseudo-nitzschia*, employing methods of culturing, genetic fragment analysis techniques, collection of standard oceanographic data, and *in silico* analysis of publicly available DNA sequences. First, we report species-specific bacterial communities associated with *Pseudo-nitzschia* cultures; furthermore, these communities varied according to culture toxigenicity. Second, *Pseudo-nitzschia* communities were responsible for moderate DA levels in the northern Benguela Upwelling Zone, potentially caused by species previously reported as very weakly toxigenic. Throughout the South Atlantic Ocean, novel and known *Pseudo-nitzschia* types were detected, with communities exhibiting biogeographic patterns that varied with abiotic ocean conditions. Third, newly constructed nuclear- and chloroplast-based phylogenies suggest the existence of two

major *Pseudo-nitzschia* clades, which exhibit similar morphological and physiological characters. Together, these findings describe *Pseudo-nitzschia* as an organism that is tightly coupled with its biotic and abiotic surroundings.

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DEDICATION

For Greg and Milo

INTRODUCTION

Until recently, the very existence, not to mention the significance, of marine microbial biogeography was largely underappreciated. To the average human, the ocean may appear to be an interconnected, dynamic environment, which lacks the structure that is easily observable among more familiar terrestrial ecosystems. Furthermore, in keeping with the Greek meaning of 'planktos' (drifting), planktonic marine microorganisms can be quickly dispersed throughout the oceans. These two factors may lead to a misunderstanding of the Baas-Becking hypothesis (Baas-Becking, 1934) as, simply, "Everything is everywhere."

While evidence exists that very few barriers to dispersal actually exist for marine microorganisms (Fenchel & Finlay, 2004), their survival and persistence clearly varies in time and space within the marine environment (e.g. Fuhrman et al. 2006). This is the second part of the Baas-Becking hypothesis, "the environment selects." The distributions of marine microorganisms, like those of terrestrial macroorganisms, are influenced by the same types of present-day conditions, including parameters that are both biotic (interactions with other organisms, such as predation and mutualisms) and abiotic (environmental properties of their surroundings, such as temperature and salinity). Observed patterns in biogeography arise through feedback between organism and its biotic and abiotic environment. These interactions become encoded in an organisms' genome, as the traits that influence the organism's ecological success give rise to evolutionary adaptations.

In particular, diatoms epitomize a classic biogeographic and ecological problem, the 'paradox of the plankton' (Hutchinson, 1961). Diatoms tend to coexist in communities that comprise many similar species, and also similar strains of the same species. Based upon Gause's (1934) competitive exclusion principle, a dominant species is predicted to outcompete a less dominant

one by virtue of being more efficient at nutrient utilization, or some other single aspect of diatom's survival. According to Gause, we should not see the abundance of similar species co-existing in time or space. This is Hutchinson's paradox, and what are the solutions? Two major ideas have been proposed. First, rapid fluctuations in diatoms' turbulent habitats may create a state of disequilibrium, thus never allowing one type to dominate over another. In addition, increasingly greater diversity is being revealed among similar-appearing species, likely allowing closely related organisms to occupy distinct niches. (Note that, by Hutchinson's definition, each species occupies a distinct "niche." Throughout this dissertation, I will use a broader definition of the term, whereby "niche" implies a distinct set of abiotic and biotic conditions that together allow an ecotype, species, or clade of organisms to thrive relative to members of other groups.)

Furthermore, diatoms represent a major group that is important due to their biological productivity and ability to thrive across a wide range of environmental conditions. Although all photosynthetic organisms in the ocean are responsible for roughly half of the global oxygen production (Field, Behrenfeld, Randerson, & Falkowski, 1998), diatoms alone contribute about 20% of global productivity (Nelson, Treguer, Brzezinski, Leynaert, & Queguiner, 1995). Broadly speaking, diatoms tend to dominate turbulent coastal environments rather than the open ocean, exhibiting a trade-off between high growth rates on high nutrients and ability to tolerate fluctuating conditions (diatoms) and superior growth on low nutrients and in stratified waters (picoplankton, dinoflagellates). However, if given the opportunity, diatoms within open ocean waters can rapidly multiply and dominate nutrient-enriched incubations (De Baar et al., 2005).

The diatom genus *Pseudo-nitzschia* includes approximately 37 species, many of which often co-occur and possess broad ranges (Hasle 2002; Lelong et al. 2012). In addition to exemplifying the flexible physiology and cosmopolitan distribution characteristic of diatoms in general, *Pseudo-nitzschia* offers unique opportunities to investigate a range of applied and basic research questions. Because it is an organism that causes harmful algal blooms, or HABs, a

great deal of research has been collected on its distribution. The toxin produced by *Pseudo-nitzschia*, domoic acid (DA), can have severe physiological impacts, such as neurological problems, gastrointestinal illness, and death (as summarized by Trainer et al. (2012)). There is no cure or treatment for DA poisoning, which in humans is called Amnesic Shellfish Poisoning (ASP).

Currently, approximately 14 *Pseudo-nitzschia* species are known to produce DA (Lelong et al., 2012), and strains of a single species can vary in toxigenicity (Bates 2000). In the field, both nutrient-limited and healthy *Pseudo-nitzschia* cells can produce DA, but specific environmental conditions have not been correlated conclusively with most DA outbreaks. In the laboratory, *Pseudo-nitzschia* produce higher levels of DA in response to multiple stimuli, most widely documented under conditions of nutrient limitation (Bates 1998). In addition, exponentially-growing *P. multiseries*, as well as cells in stationary phase, can produce elevated levels of DA in response to bacteria (Bates et al. 1995). DA production is an example of the metabolic complexity of diatoms, as its metabolic pathway, ecological or physiological function, and precise “triggering” conditions are unknown.

In this dissertation, I explore three major dimensions of life which are important to the biogeography of *Pseudo-nitzschia*. These themes include interactions among organisms (Chapter 1), environmental regimes that influence species distribution (Chapter 2), and phylogeny based upon genetic, morphological, and physiological patterns (Chapter 3). All three of these themes hold important implications for the toxigenicity and ecological success of this diatom.

Interactions among organisms (Chapter 1)

Interactions between diatoms and bacteria greatly influence marine ecosystem function, as both types of organisms represent the abundant and diverse foundations of marine food webs. Diatoms release a large proportion of primary production extracellularly, where it is utilized by heterotrophic bacteria as part of the microbial loop (Azam et al., 1983; Baines & Pace, 1991). The bulk of bacteria-diatom interactions, such as the basis of energy flow in the microbial loop, may represent an example of diffuse coevolution, whereby a broad group of bacteria have sustained associations over time with a broad group of phytoplankton. Thus, the biogeographical distributions of diatoms and marine bacteria can be closely linked.

Tight types of coevolution can maintain species-specific associations between bacteria and diatoms. Bacterium-diatom associations include a range of interactions, from antagonism (Bidle & Azam, 1999) to mutualism (Amin et al., 2009). Each pair of cells likely interacts in more than one of these ways. For example, a unique association enables the nitrogen-fixing cyanobacterium *Richelia* to exist widely among *Rhizosolenia* at the expense of its ability to persist as a free-living organism (Villareal, 1990); this trade-off supports the specificity of *Richelia*'s habitat. Such tight interactions between bacteria and diatoms likely influenced the evolutionary history of diatoms. Cyanobacteria and other bacteria are responsible for endowing diatoms with their chloroplasts and mitochonidria, respectively. In addition, a surprisingly high level of horizontal gene transfer has occurred from diverse types of bacteria to diatoms, likely endowing different diatoms with unique metabolic capabilities (Bowler et al., 2008).

In the broad context of evolutionarily and ecologically important interactions between bacteria and diatoms, I investigate the specificity of associations between *Pseudo-nitzschia* and bacteria.

This work was inspired in part due to the reported enhancement of DA production upon addition of bacteria to *Pseudo-nitzschia* cultures. Additionally, each culture of *Pseudo-nitzschia* and associated bacteria represents one environmental regime (from the perspective of the bacteria), whereby organic compounds released by the diatom are utilized by heterotrophic bacteria. Such cultures can represent incubations that can be expected to foster neutral or beneficial types of interactions between bacteria and diatoms. In this Chapter, I investigate the hypothesis that ***Pseudo-nitzschia* species and domoic acid production influence bacterial community composition**.

Abiotic environmental drivers of *Pseudo-nitzschia* community composition (Chapter 2)

The abiotic environmental controls on *Pseudo-nitzschia* species distribution are not fully understood. Some species have been correlated with different ranges of salinity (Thessen, Dortch, Parsons, & Morrison, 2005), and a subset of species are restricted to polar regions due to temperature tolerances (Hasle 2002). Generally, however, strains of the same species of *Pseudo-nitzschia* can exhibit wide ranges in tolerances of salinity, temperature, and nutrient preferences (as summarized by Lelong et al. (2012). In Chapter 2, I hypothesize that ***Pseudo-nitzschia* community composition reflects distinct patterns of abiotic environmental conditions**.

To date, much of *Pseudo-nitzschia* research has been conducted in the Northern Hemisphere (Hasle 2002). In Chapter 2, I further hypothesize that **novel and known genotypes of *Pseudo-nitzschia* gave rise to toxigenic blooms in the Benguela Upwelling Zone**. The geographic location of this study also provided an opportunity to test the specificity of the fragment analysis technique, Automated Ribosomal Intergenic Spacer Analysis, developed to identify *Pseudo-nitzschia* species (Hubbard et al. 2008), on the opposite part of the globe from

the Pacific Northwest waters in which the method was first tested. Furthermore, little information exists about *Pseudo-nitzschia* species composition and toxigenic blooms in the northern Benguela Upwelling Zone; this study sought to measure both of these.

In the field of harmful *Pseudo-nitzschia* bloom research, one major overarching question focuses on characterizing the genetic and environmental components that together trigger DA outbreaks. DA production and *Pseudo-nitzschia* community composition have been most widely documented in the Northern Hemisphere and coastal areas. Do the same cosmopolitan *Pseudo-nitzschia* species produce DA everywhere? If novel genotypes are found to produce DA, what is the common genetic thread that conveys the ability to produce DA? Answers to these questions will inform HAB forecasting efforts.

Phylogeny and biogeography (Chapter 3)

In Chapter 3, I revisit the question of, “how do similar species coexist?” Often, similar organisms coexist through niche partitioning. Ecotypes of an organism are adapted to exploit often fine-scale differences in the marine environment, as defined by each type’s tolerances for biotic (Chapter 1) and abiotic (Chapter 2) conditions. These themes have been explored by other researchers in the Rocap Lab, beginning with identification of ecotypes of the cyanobacterium *Prochlorococcus* (Rocap et al., 2003) which occupy different depths of the water column by virtue of different light-harvesting capacities. Similar niche differentiation has been suggested for *Prochlorococcus’* sister taxon, *Synechococcus* (Ahlgren & Rocap, 2006). Furthermore, two clades of *Synechococcus* alternate in their seasonal dominance (Tai & Palenik, 2009).

In contrast, likely because of the focus on DA production and prediction, the ecological niches of broad groups of *Pseudo-nitzschia* have been largely overlooked. Instead, the genus is often described simply as cosmopolitan (e.g. Hasle (2002)), and distributions of individual species are examined (Trainer et al. 2012; Lelong et al. 2012) without a synthesis of broader trends. Similarly, although multiple previous studies of *Pseudo-nitzschia* phylogeny existed, much of this work is potentially outdated, or it focuses on cryptic species complexes (e.g. Lundholm et al. (2003)), rather than on the entire genus. The first aim of this chapter is to identify broad clades of the genus that reflect patterns in phylogeny, morphology, and physiology. I construct new phylogenies of *Pseudo-nitzschia*, and I integrate them with existing knowledge of major characters of the genus.

Furthermore, this chapter builds upon the observations of *Pseudo-nitzschia* species composition in the northern Benguela Upwelling Zone (Chapter 2), to ask the question, “why do we observe certain species during certain times of the year?” Based upon seasonal trends in putative *Pseudo-nitzschia* clades, I propose the hypothesis that two major *Pseudo-nitzschia* clades vary in geographic distribution as a result of fundamental niche differentiation.

Together, these three chapters investigate the degree to which *Pseudo-nitzschia* is linked with other organisms and its abiotic surroundings. This research holds implications for both *Pseudo-nitzschia* toxigenicity and broader marine microbial distribution, highlighting the importance of integrating physiology, genetics, and morphology to address ecological and evolutionary questions.

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CHAPTER ONE*

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

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 six 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 one year 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 one *P. multiseries* (toxigenic) and one *P. delicatissima* (nontoxigenic) culture. Both cultures hosted members of the *Roseobacter* clade, Gamma-Proteobacteria, 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.

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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, three 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, Trainer et al. 2009b).

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 pg DA/cell (Bates 1998), and 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) (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 during stationary phase, when cell growth is limited by silicic acid

(Bates et al. 1991, Pan et al. 1996b, Pan et al. 1996c), 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 two orders of magnitude upon reintroduction of multiple bacterial strains isolated from *P. multiseries* or the diatom *Chaetoceros* 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 exponential 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 one field study has examined bacterial abundance in regards to DA levels and 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 Gamma-Proteobacteria, and a Bacteroidete

(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 one 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, or 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 six 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 one *P. multiseries* culture (toxigenic) and one *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.

METHODS

Pseudo-nitzschia *isolations.* Net tow samples were collected from three stations in Puget Sound during cruises in June, October, and December 2006 aboard the *R/V 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 four days 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 PCRs were performed using primers 18SF-euk (5′-CTTATCATTTAGAGGAAGGTGAAGTCG-3′) and 5.8SR-euk (5′-CTGCGTTCTTCATCGTTGTGG-3') and cycling conditions as 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-HM138910.

Domoic acid production. We determined *Pseudo-nitzschia* strain toxigenicity during stationary growth phase in f/2 (Guillard 1975) and K (Keller et al. 1987) media used for both regular culture maintenance and the experiments in this 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 stationary phase, as determined by daily fluorometer readings (10-AU fluorometer, Turner Designs). For four *Pseudo-nitzschia* strains (two strains maintained in f/2 medium; two strains maintained in K medium), nutrient add-back experiments were conducted to confirm silicic acid as the limiting nutrient. Replicate

cultures were maintained through 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, or ELISA (Biosense Laboratories; (Kleivdal et al. 2007)). After one to five days in stationary growth 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 -20ºC in the dark. PDA was extracted from filters into 10 mL of ultrapure distilled water (MilliQ®; MilliPore). Sample analysis was performed according to the Biosense protocol. DA in particulate DA extracts and dissolved DA fractions was quantified according to the working range of the ELISA kit, which corresponded to approximately 10 to 300 pg per mL of *Pseudo-nitzschia* culture. In our cultures, the working limit of detection was below 1.4 fg DA/cell for both PDA and DDA. This value is more than 25 times lower than the lowest DA/cell 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 4ºC in the dark. Cell counts were performed using a Sedgewick Rafter Cell S50 (Pyser-SGI, UK) and a Nikon Labophot-2 compound microscope. Either 30 fields or 300 cells were counted, excluding empty frustules. For each preserved sample, three subsamples were counted and averaged.

Laboratory culturing and DNA extraction of bacterial communities coexisting with **Pseudo-nitzschia*.*** Eighteen *Pseudo-nitzschia* isolates were utilized in this study, including strains obtained from collaborators and previously identified as *P. australis*, *P. pungens*, *P. granii*, and *P. multiseries* (Table 1.1). In addition, one cryptic strain, PNW H2O 233a, was included. Scanning electron microscopy (SEM) performed on this strain confirmed that this strain did not belong to any other species examined in this study (K. Hubbard, unpublished data). We therefore described this strain as a different species, *P*. sp. 233. *Pseudo-nitzschia* strains were cultured in 20-27 mL volumes in replicate glass culture tubes transferred from the same parent culture. Cultures were incubated at 13ºC under a 16:8 hour light:dark cycle, except for *P. multiseries* CLN-47, which was incubated at 20ºC under 24 hours of light. Light levels ranged between 30 – 115 μmol quanta^{-1}m^{2}. The same culture conditions were used for regular culture maintenance over the course of this study and for specific sampling time points described here.

Bacterial communities were sampled from attached and free-living size fractions, from exponential and stationary growth 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 – 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 and stationary growth phases (data not shown). AB and FLB fractions were collected for all cultures and analyzed for 11 strains (Table 1.1). Eight cultures, including strains of both typically toxigenic and nontoxigenic species, were sampled at least twice during the

diatom growth phase: once during exponential phase and once during stationary phase (Table 1.1). One strain of toxigenic *P. multiseries*, CLN-47, was sampled more extensively, at days three and six (exponential growth phase), and days eight, 10, 14, and 17 (stationary growth phase) in culture. Ten other strains, not sampled during exponential phase, were sampled between two and 15 days after the beginning of stationary growth phase, 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 two or three time points separated by repeated transfers in culture (Table 1.1). Two strains of *P. delicatissima* (commonly a nontoxigenic species; (Lundholm et al. 2006)) each were sampled at three time points: PNWH2O 604 was sampled after six, nine, and 14 months following initial isolation, and PNWH2O 605 was sampled after four, six, and nine months following isolation. Two other strains, including the commonly toxigenic species, *P. multiseries*, were sampled at two different time points in culture. *P. multiseries* strain PNW H2O A4 was sampled at 22 and 26 months following isolation, and strain PNW H2O 233a was sampled at 12 and 14 months following isolation. Filters were frozen at -80ºC 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 lysozyme) using a bead-beater (BioSpec Products) without beads for 60 seconds at maximum speed, and then incubated at 37°C for 30 minutes. Cells were lysed further by incubating at 70°C for 30 minutes 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, Inc.) measured using a SpectraMax M2 microplate reader (Molecular Devices Corporation), as described by Ahn et al. (1996).

ARISA of bacterial communities. Automated Ribosomal Intergenic Spacer Analysis, or ARISA (Fisher & Triplett 1999), was conducted to amplify the bacterial internal transcribed spacer (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 bacterial primer 23S 125R (5′-GGTTBYCCCATTCRG-3′; (Fisher & Triplett 1999) with additional degeneracies as per Hunt et al. (2006) . Each 20 µL PCR mixture contained 1X 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 initial 4-minute denaturation at 94ºC, followed by 32 cycles of each of three steps: 94ºC for 1 minute, 52ºC for 1 minute, and 72ºC for 2 minutes. Extension of the final cycle occurred at 72ºC for 10 minutes. PCR products were stored at 4ºC in the dark.

Quadruplicate PCR products were pooled and quantified using PicoGreen and the Spectramax M2 microplate reader. 10 ng DNA was precipitated from pooled PCR product (0.5 – 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 fluorescently-labeled internal size standard, CST ROX 60-1500 bp (BioVentures, Inc.). Samples were analyzed on a MegaBACE 1000 automated capillary sequencer (Sequencing Mode; injection voltage = 3 killivolts; injection time = 60 seconds; run voltage = 5 killivolts; run time = 370 minutes).

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 – 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 (accessed at 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 700-1000 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 OTUs shared among two 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 1.1), we analyzed AB and FLB samples (11 strains), samples collected during exponential and stationary phases (eight strains), and samples collected at two or three time points separated by repeated transfers in culture (four 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 to compare bacterial communities across *Pseudo-nitzschia* strains according to 1.) species (for all species represented by two or more strains, thus excluding *P.* 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 Nova Scotia). 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 the R, rather than p value, 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 two 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 one toxigenic or only one nontoxigenic culture.

Bacterial clone library construction and sequencing. We created 16S-23S rDNA clone libraries from FLB coexisting with two Puget Sound *Pseudo-nitzschia* cultures: one *P. multiseries* strain (PNWH2O A4, sampled after 22 months in culture) and one *P. delicatissima* strain (PNWH2O 604, sampled after nine months in culture). Genomic DNA was amplified using the bacterial-specific primers 16S 27F (5′-AGAGTTTGATCMTGGCTCAG-3′; (Lane 1991)) and 23S 125R (described earlier). Quadruplicate PCRs (Polz & Cavanaugh 1998) were prepared for each sample. Each 20 µL PCR mixture contained 2 ng genomic DNA, 1X 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-minute

denaturation at 94ºC, followed by 22 cycles of each of three steps: 94ºC for 1 minute; 52ºC for 1 minute; and 72ºC for 3 minutes. Extension of the final cycle occurred at 72ºC for 10 minutes. Following this, a reconditioning step (Thompson et al. 2002) was performed using 3 cycles of additional amplification. For each of the two samples, the four 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 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, one to four clones were sequenced. High-quality plasmid DNA was extracted using a Spin Miniprep Kit (Qiagen) according to 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 six primers: 27F and 1492R (described earlier) as well as internal bacterial primers 907R (5′-CCGTCAATTCCTTTGAGTTT-3′), 926F (5′-AAACTCAAAGGAATTGACGG-3′), 515F (5′-GTGCCAGCAGCCGCGGTAA-3′), and 519R (5′-GTATTACCGCGGCTGCTG-3′) (Lane 1991).

Contigs were assembled from reads using all six 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 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-HM140680.

RESULTS

Pseudo-nitzschia isolate characterization

We obtained seven *Pseudo-nitzschia* isolates during three 2006 Puget Sound cruises, as well as from sampling conducted at two other Washington sites in 2005 and 2006 (Table 1.1). New *Pseudo-nitzschia* isolates were putatively identified as four isolates of *P. delicatissima* and three 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 Pacific Northwest waters (Hubbard et al. 2008). Specifically, all four *P. delicatissima* isolates represented *P. delicatissima* genotype 11, which has been previously detected in Pacific Northwest waters and other regions (Hubbard et al. 2008). These isolates exhibited 100% ITS1 identity to genotype 11 reference strains verified by scanning electron microscopy (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 two ITS1 genotypes. First, the ITS1 sequence of PNWH2O LB2 was 100% identical to a previously identified Washington *P. pungens* isolate included in this study, PNWH2O 101WB (DQ996020) (Hubbard et al. 2008), as well as isolate NA177 clone 7 (FM207594) obtained from the Juan de Fuca eddy region offshore from the Washington Coast (Casteleyn et al. 2009), both confirmed using SEM. Second, the other two new isolates, PNWH2O 607 and PNWH20 608, both differed from PNWH2O LB2 by three 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 two 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 seven new *Pseudo-nitzschia* isolates, as well as 11 isolates provided by collaborators, together representing a total of five species from four geographic origins (Table 1.1). The majority of these cultures were from coastal waters, with the exception of two strains of *P. granii* from the Northeast Pacific. Cultures from Washington waters included two isolates representing two different species, obtained from the same Puget Sound water sample (*P. multiseries* strain PNWH20 A4 and *P. pungens* strain PNWH20 C1).

The toxigenicity of these cultures was determined by measuring DA production during stationary growth 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 *P. australis* and *P. multiseries*, as well as the cryptic species *P.* sp. 233, produced detectable levels of DA (Table 1.1). Particulate DA (PDA) levels ranged between 0.20 and 21.85 pg/cell, and dissolved DA (DDA) levels varied between 0.04 and 3.78 pg/cell. 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 Nova Scotia waters, and only nontoxigenic cultures were obtained from the Northeast Pacific. Because the culture conditions used to conduct these DA measurements are the same as those used to maintain the cultures routinely in the lab, 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 1.1), we therefore conducted the following three 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 and stationary growth phase following the same transfer; and (3) between AB and FLB fractions. First, bacterial community composition did not differ significantly over time in culture for both nontoxigenic (*P. delicatissima*) and toxigenic (*P. multiseries*, *P*. sp. 233) species. Specifically, the bacterial community composition from *P. delicatissima* strain PNWH20 604 was similar across six, nine, and 14 months in culture, and bacterial community composition from *P. delicatissima* strain PNW H20 605 was similar across four, six, and nine months in culture. Toxigenic cultures *P. multiseries* PNWH2O A4 and *P.* sp. PNWH2O 233a did not differ in bacterial community composition across two time points separated by approximately two months and repeated transfers in culture. Second, for all eight *Pseudo-nitzschia* strains tested, bacterial community composition did not differ between

exponential and stationary growth phases. In particular, five toxigenic strains of *Pseudo-nitzschia* strains did not exhibit significantly different bacterial community composition during conditions correlated with both low DA levels (exponential phase 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 three days during exponential and stationary growth phases of a single transfer. Third, composition of the AB and FLB communities did not differ significantly for nine of the 11 *Pseudo-nitzschia* strains assessed. One toxigenic strain of *Pseudo-nitzschia* (PNWH2O 233a) and one nontoxigenic strain of *Pseudo-nitzschia* (PNWH2O 101WB) exhibited significantly different AB and FLB community composition (SIMPROF; $p < 0.05$). Because bacterial community composition coexisting with a single diatom strain was comparable over time and between the AB and FLB size fractions, the remainder of our analyses included one representative FLB sample for each strain, collected during the diatom's stationary growth phase.

We assessed patterns of bacterial community composition among 18 *Pseudo-nitzschia* cultures representing five identified species and one unknown species (Table 1.1). 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 both toxigenic as well as nontoxigenic types of each of the species, from each of the three geographic origins, we were unable to statistically separate three factors from one another. Therefore, we separately tested each of three 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

Northeast Pacific waters), using one-way ANOSIM and interpreted the resulting p values using the Bonferroni correction for multiple comparisons.

Bacterial community composition differed significantly among *Pseudo-nitzschia* species (Table 1.2), as observed in the clustering in the NMDS plot (Figure 1.1). Pairwise species comparisons conducted along with global ANOSIM statistics indicated that four of the five total possible pairwise comparisons across toxigenic versus nontoxigenic cultures contributed most strongly to the species differences (as indicated by higher R values) (Table 1.2). Furthermore, bacterial community composition in nontoxigenic cultures of *Pseudo-nitzschia* was significantly different relative to the bacterial communities in toxigenic cultures (Table 1.2, Figure 1.1). When all 18 cultures were compared (Table 1.2, 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 Northeast Pacific) versus coastal strains (from Washington or California). When only the 16 coastal cultures were compared (Table 1.2, Dataset B), bacterial communities from coastal *Pseudo-nitzschia* strains did not differ according to origin from Washington versus California waters (Table 1.2, 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 one 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 versus 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 one of the seven toxigenic or 11 nontoxigenic cultures.

Next, we examined the distribution of the 33 OTUs that were detected in two 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 cultures (Figure 1.2). Notably, two cultures obtained from the same Puget Sound water sample (toxigenic strain *P. multiseries* PNWH2O A4 and nontoxigenic strain *P. pungens* PNWH20 C1) only shared one ARISA OTU, of the total of six and eight 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 two or more cultures, 22 OTUs contributed less than 3% to the dissimilarity 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 seven OTUs, which together contributed a total of 39% to the overall dissimilarity. These seven OTUs included six OTUs that were found exclusively or predominantly among toxigenic cultures and one OTU that was found only among nontoxigenic cultures (Figure 1.2).

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 non-singleton OTUs had an equal probability of occurring in the seven

toxigenic or 11 nontoxigenic cultures. These three OTUs (Figure 1.2) 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 three OTUs was detected with more than one *Pseudo-nitzschia* species and with isolates obtained from more than one 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 two *Pseudo-nitzschia* cultures, both obtained from Puget Sound: nontoxigenic *P. delicatissima* strain PNWH2O 604 and 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 eight distinct OTUs). Of the 177 clones screened by ARISA, only five 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 (Figure 1.3) demonstrated that the two clone libraries were sampled close to saturation in terms of unique ARISA OTUs.

Both ARISA profiles and 16S rDNA clone libraries differed between the two *Pseudo-nitzschia* cultures. First, the bacterial communities coexisting with *Pseudo-nitzschia delicatissima* and *P. multiseries* did not share any ARISA OTUs (Figure 1.4), despite both cultures originating from Puget Sound. To further assess differences in bacterial community composition between the two cultures, the 16S rDNA was sequenced for unique ARISA OTUs detected in the clone libraries. In total, 36 clones were sequenced, including 21 clones (representing 12 unique

ARISA OTUs) derived from *P. delicatissima* and 15 clones (seven ARISA OTUs) derived from *P. multiseries*. Thus, 19 unique ARISA OTUs were identified by 16S rDNA sequencing (Figure 1.4), including 14 of the 33 OTUs shared among two or more *Pseudo-nitzschia* cultures (Figure 1.2). The number of clones representing each ARISA OTU generally corresponded to the intensity (relative fluorescent units, or RFUs) of each peak in the ARISA profile (Figure 1.4). In particular, OTU 755, a type that contributed strongly to the differences across all toxigenic and nontoxigenic cultures (Figure 1.2), was identified as *Winogradskyella*, a Flavobacterium. This bacterium produced the highest-intensity ARISA peak in the *P. multiseries* PNWH2O A4 culture (Figure 1.4).

The 16S rDNA sequences revealed that, although generally the same phyla (Flavobacteria and Alpha- and Gamma-Proteobacteria) were represented in the two *Pseudo-nitzschia* cultures, the specific bacteria in the two communities differed on a finer taxonomic scale (Figure 1.5). Only the *P. multiseries* strain hosted a member of Beta-Proteobacteria. The two highest-intensity ARISA peaks in each profile, OTU 755 (*P. multiseries*) and OTU 770 (*P. delicatissima*), were both Flavobacteria (Figure 1.4). However, the bacteria coexisting with the two *Pseudo-nitzschia* strains belonged to different clades (Figure 1.5). For example, among the Gamma-Proteobacteria identified in the two cultures, *P. multiseries* hosted *Neptunomonas* sp. (order Oceanspirillales), whereas *P. delicatissima* hosted Alteromonads (order Alteromonadales). These two strains hosted similar species richness, with five species (as defined by >99% 16S rDNA similarity) coexisting with the *P. multiseries* culture, and six different species coexisting with the *P. delicatissima* culture.

We evaluated whether a single ARISA peak in our cultures represented one 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 16S rDNA sequence but exhibited a

range of finer-scale variability. For two 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 two ARISA peaks from which we sequenced four clones (*Winogradskyella* OTU 755 and *Percisivirga* OTU 770), each of the four 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 per bp) would result in less than 0.3 error in each rDNA sequence reported in this 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 more than one ARISA peak. Some clones associated with different ARISA OTUs clustered together in the 16S rDNA tree (Figure 1.5), exhibiting sequence identities within the same range observed for clones associated with the same ARISA peak. For each of four 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).

DISCUSSION

This study characterized bacteria coexisting with cultures from several geographic origins, including five *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 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, Bates 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 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 four months in culture), the presence of bacterial ARISA types did not differ significantly over a time frame of several months to one year for both nontoxigenic (*P. delicatissima*) and toxigenic (*P.*

multiseries and *P*. sp. 233) cultures of *Pseudo-nitzschia*. 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 and stationary growth phases with the reasoning that bacterial responses to DA (or other physiological differences) could manifest 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 and stationary growth phases for five toxigenic cultures, as well as for three nontoxigenic cultures. Within a single toxigenic culture sampled six 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 others (Grossart et al. 2005, Sapp et al. 2007a). In these latter cases, 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 this 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 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 one 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 Gamma-Proteobacteria, 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. Nine of ten ARISA peaks sequenced in replicate contained multiple non-identical 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 two *Pseudo-nitzschia* strains that had been in culture for a substantial period of time - nine months for *P. delicatissima* strain and two years 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.

Across *Pseudo-nitzschia* cultures, differences in both ARISA OTU composition and richness therefore both 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, three 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* produce higher levels of DA as a chemical defense to 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 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 stationary phase and low-DA conditions following each transfer, eventually favoring 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.

This 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 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), argue for the existence of algal-specific bacterial communities.

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Figure 1.1. 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.

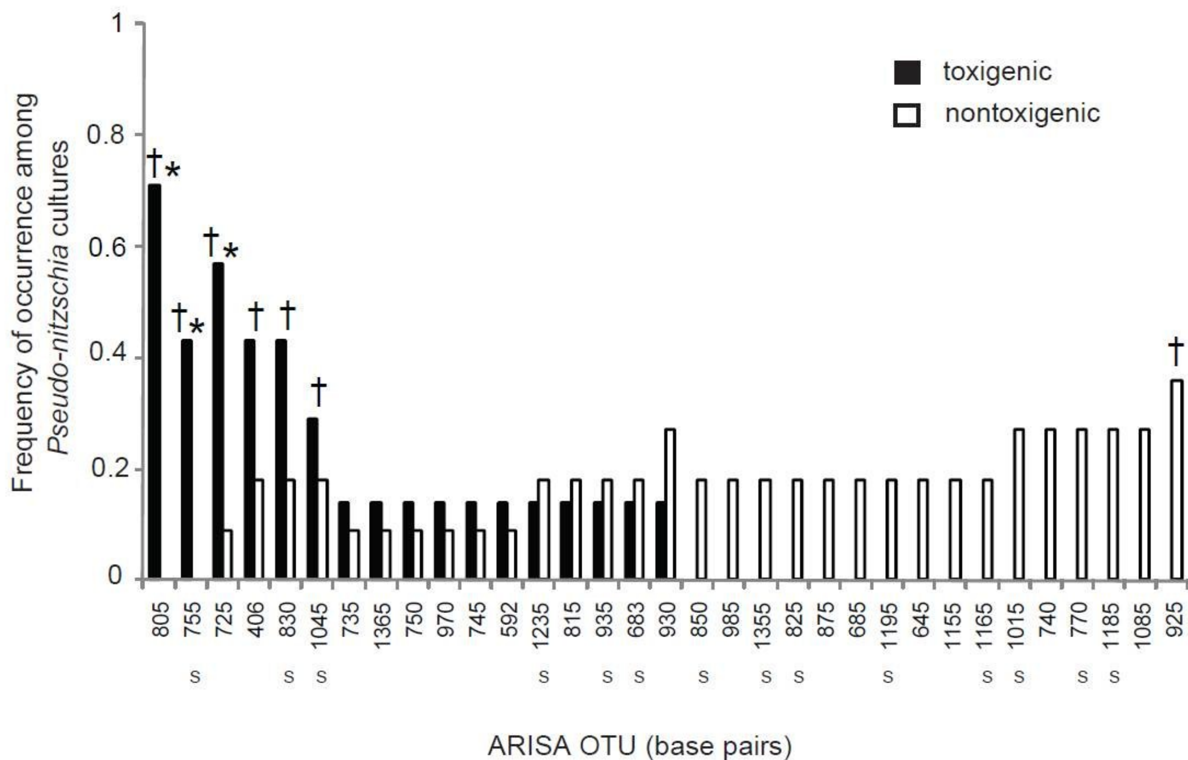

Figure 1.2. Distribution of Automated Ribosomal Intergenic Spacer Analysis (ARISA) peaks, or operational taxonomic units (OTUs), among toxigenic and nontoxigenic *Pseudo-nitzschia* cultures, for OTUs shared among 2 or more cultures. Notations indicate 7 OTUs that together accounted for 39% of the dissimilarity 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 (*). The letter 'S' below the ARISA fragment length indicates an OTU identified by 16S rDNA sequencing.

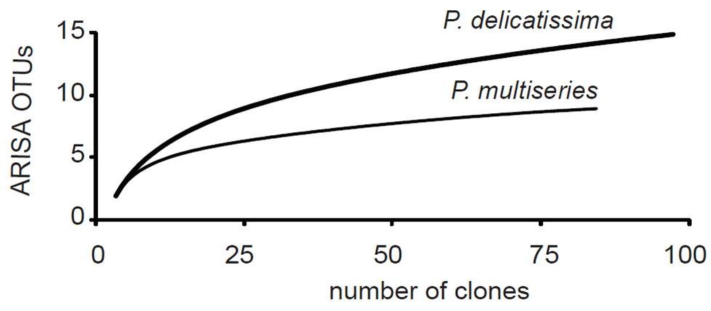

Figure 1.3. Rarefaction curves for 16S–23S rDNA clone libraries constructed for bacterial communities coexisting with *Pseudo-nitzschia delicatissima* (Strain PNWH2O 604) and *P. multiseries* (Strain PNWH2O A4). Curves demonstrate the number of distinct automated ribosomal intergenic spacer analysis (ARISA) peaks, or operational taxonomic units (OTUs), represented by bacterial clones.

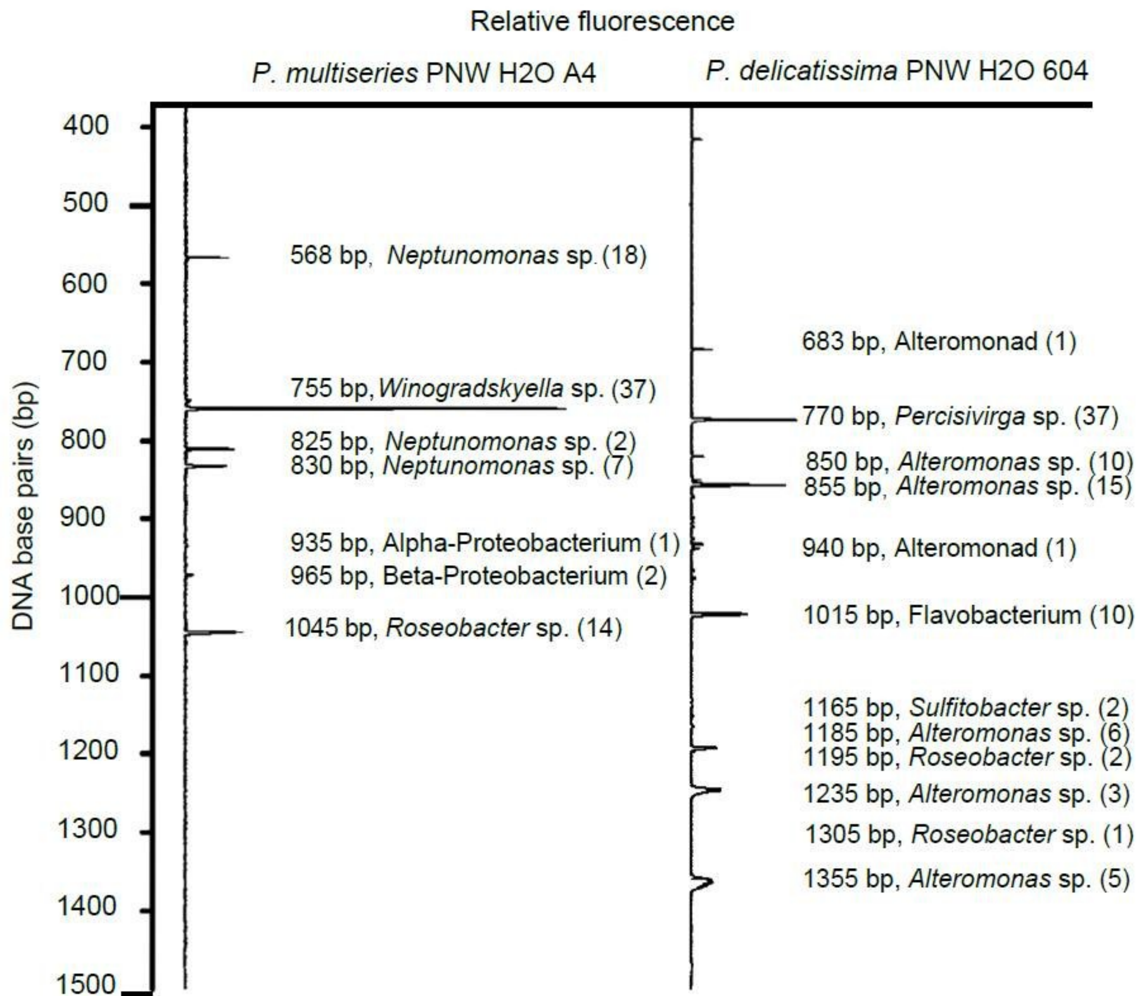

Figure 1.4. Automated Ribosomal Intergenic Spacer Analysis (ARISA) of bacterial community profiles from *Pseudo-nitzschia multiseries* Strain PNWH2O A4 and *P. delicatissima* Strain PNWH2O 604. Individual ARISA peaks were identified by 16S rDNA sequencing. Numbers in parentheses indicate number of clones representing each ARISA peak in the clone libraries.

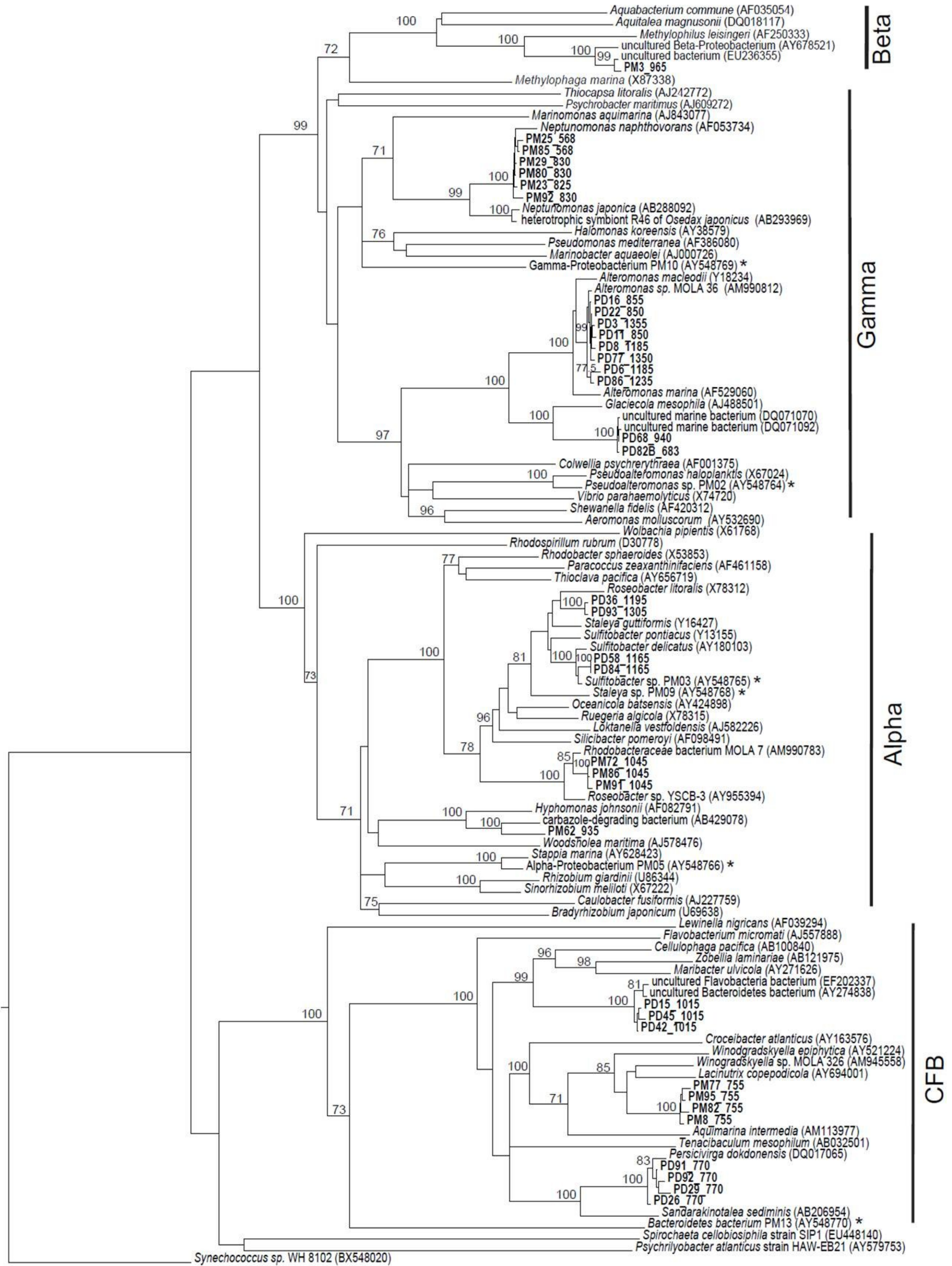
Aquabacterium commune (AF035054)
Aquitalea magnusonii (DQ018117)
Methylophilus leisingeri (AF250333)
uncultured Beta-Proteobacterium (AY678521)
uncultured bacterium (EU236355)
PM3_965
Methylophaga marina (X87338)
Beta
Thiocapsa litoralis (AJ242772)
Psychrobacter maritimus (AJ609272)
Marinomonas aquimarina (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)
Gamma-Proteobacterium 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)
Alpha-Proteobacterium 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)
0.1

Figure 1.5. 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 PNWH20 A4 [PM] and *P. delicatissima* Strain PNWH20 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.

Table 1.1. *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, source, 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: ARISA profiles were compared across exponential and stationary growth 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.

Species	Strain	PDA (pg/cell)	DDA (pg/cell)	Exp/Sta	AB/FLB	Time in Culture	Origin	Isolated by	Reference
P. australis†	03199_1B	21.85	1.63		X		Monterey Wharf, CA	G. Jason Smith	Hubbard et al. (2008)
P. australis†	04063_3C	3.15	1.26	X	X		Bodega/Tomales Bay, CA	G. Jason Smith	Hubbard et al. (2008)
P. australis†	03184_5D	9.00	3.78				Monterey Wharf, CA	G. Jason Smith	Hubbard et al. (2008)
P. australis†	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		Nova Scotia	Claude Léger	Mafra Jr et al. (2009)
P. multiseries†	PNWH2O A4	5.84	1.32	X	X	X	Puget Sound, WA (Main Basin)	Katherine Hubbard	Hubbard et al. (2008)
P. sp. 233‡	PNWH2O 233a	13.48	0.85	X	X	X	Sequim Bay, WA 48°03′ N, 123°01′ W	Karie Holtermann	This study
P. delicatissima	PNWH2O 604	BD	BD		X	X	Puget Sound, WA 47° 44.1′ N, 122° 45.7′ W	Michele Guannel	This study
P. delicatissima	PNWH2O 605	BD	BD	X	X	X	Puget Sound, WA 47° 44.1′ N, 122° 45.7′ W	Michele Guannel	This study
P. delicatissima	PNWH2O 609	BD	BD				Puget Sound, WA 47°16.6′ N, 122°42.5′ W	Michele Guannel	This study
P. delicatissima	PNWH2O K2	BD	BD				Sequim Bay, WA 48°03′ N, 123°01′ W	Karie Holtermann	This study
P. granii	UWOSP22	BD	BD				NE Pacific 50° N, 145° W	Adrian Marchetti	This 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	This study
P. pungens	PNWH2O 608	BD	BD				Puget Sound, WA 48°1′ N, 122° 18.2′ W	Michele Guannel	This study
P. pungens	PNWH2O LB2	BD	BD				Long Beach, WA 46°42′ N, 123°58′ W	Karie Holtermann	This study
P. pungens	PNWH2O C1	BD	BD	X	X		Puget Sound, WA (Main Basin)	Katherine Hubbard	Hubbard et al. (2008)
P. pungens†	PNWH2O 101WB	BD	BD	X	X		Willapa Bay, WA	Katherine Hubbard	Hubbard et al. (2008)

Table 1.2. Results of analysis of similarity tests performed on bacterial communities (Automated Ribosomal Intergenic Spacer Analysis data) coexisting with *Pseudo-nitzschia* cultures, analyzed for 2 datasets: all cultures and only cultures isolated from coastal waters. 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 '–' 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. An asterisk (*) denotes statistical significance (p < 0.0167) after Bonferroni correction.

	---------Dataset A-------- All cultures (n=18), except as noted			---------Dataset B--------- All coastal cultures (n=16), except as noted		
TESTS	R	p	permutations	R	p	permutations
SPECIES[†]						
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	---	---	---	---	---	---
ORIGIN[‡]						
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	---	---	---

CHAPTER TWO

Community composition and toxigenicity of the diatom *Pseudo-nitzschia* across distinct environmental regimes in the South Atlantic Ocean

ABSTRACT

We surveyed the community composition and toxigenicity of the diatom *Pseudo-nitzschia* in the open South Atlantic Ocean and the northern Benguela Upwelling Zone in austral spring. Multiple morphological types of *Pseudo-nitzschia* were detected by light microscopy in coastal waters. Partial internal transcribed spacer 1 (ITS1) clone libraries identified the most frequently-detected species as similar to *P. inflatula*, *P. subpacifica*, *P. heimii*, and *P. galaxiae*. *Pseudo-nitzschia* community composition, as measured by the DNA fingerprinting technique Automated Ribosomal Intergenic Spacer Analysis (ARISA), comprised a total of 37 ARISA types distributed among 17 stations in both open ocean and coastal regions. Here we report multiple novel ARISA types and expanded ranges for several *Pseudo-nitzschia* species. *Pseudo-nitzschia* community composition varied significantly according to six distinct environmental regimes across the region surveyed. Local similarity analysis (LSA) was used to form hypotheses about potential environmental drivers of *Pseudo-nitzschia* community variability. Domoic acid, the toxin produced by *Pseudo-nitzschia*, was detected for the first time throughout the northern Benguela Upwelling Zone and approached levels that could pose harm to higher trophic level organisms (near 200 ng DA/L seawater and up to 4.5 pg DA/cell). Two types of *P. galaxiae*, a species previously described as very weakly toxigenic, were correlated significantly with particulate DA concentrations, indicating that this species may have been the causative

organism of the DA outbreaks. Our findings suggest that *Pseudo-nitzschia* species believed previously to produce only low levels of DA may indeed be capable of high levels of toxin production.

INTRODUCTION

Members of the marine diatom genus *Pseudo-nitzschia* are ecologically successful and important to human and ecosystem health due to their toxigenic properties. *Pseudo-nitzschia* species are ubiquitous in coastal waters (Hasle 2002). These diatoms are also present in low numbers in open ocean waters, where they respond to fertilization experiments using iron and other nutrients (De Baar et al. 2005, Marchetti et al. 2008, Trick et al. 2010). *Pseudo-nitzschia* produces domoic acid (DA), a toxin that impacts marine ecosystems and human health. DA, similar in structure to the amino acid glutamate, acts as a neurotoxin by interfering with glutamate receptors (Teitelbaum et al. 1990), causing Amnesic Shellfish Poisoning (ASP) in humans and Domoic Acid Poisoning (DAP) in other organisms (Bates et al. 1998; Lelong et al. 2012; Trainer et al. 2012). Indeed, DA has been detected in vast array of organisms (Trainer et al. 2012) and can be transferred via multiple routes throughout the food web. For example, DA can be transferred through planktivorous fish such as anchovies and sardines, which are then preyed upon by organisms such as sea lions (Scholin et al. 2000) and birds (Work et al. 1993); through shellfish to humans (Bates et al. 1989, Wright et al. 1989); and through krill (Bargu & Silver 2003) to finfish and whales (Lefebvre et al. 2002). Despite the potentially severe impacts of DA on marine organisms and humans, some regions, such as open oceans and coastal waters in the Southern Hemisphere, remain poorly described in terms of *Pseudo-nitzschia* community composition and toxigenicity.

Laboratory studies have shown that DA production is influenced by genetic variability among *Pseudo-nitzschia* types, as well as environmental conditions, yet DA outbreaks in the field

remain unpredictable. Among approximately 37 defined *Pseudo-nitzschia* species, at least 14 species are capable of producing DA (Lelong et al. 2012; Trainer et al. 2012). Highly toxigenic species such as *P. australis* (Scholin et al. 2000), *P. multiseries* (Bates et al. 1989), and *P. cuspidata* (Trainer et al. 2009) often produce DA on the order of pg DA / cell in comparison to weakly toxigenic species or species that appear to be nontoxigenic, such as *P. galaxiae* (measured to produce 0, $7.8 * 10^{-7}$, and $3.6 * 10^{-4}$ pg DA/cell; Cerino et al. (2005)), *P. heimii* (no detectable DA; Marchetti et al. (2008)) and *P. turgiduloides* (no detectable DA; as reviewed by Lelong et al. (2012)). Generally, open ocean *Pseudo-nitzschia* produce far lower levels of DA in comparison to *Pseudo-nitzschia* inhabiting coastal waters (Marchetti et al. 2008, Trick et al. 2010). Some work has identified caustative organisms of DA outbreaks by correlating species abundance with DA levels (Scholin et al. 2000), suggesting that the presence of characteristically toxigenic species may serve as a warning sign for monitoring efforts. However, for many species, DA production levels vary across strains (Bates 2000; Thessen et al. 2009). Perhaps most strikingly, *P. turgidula* has been reported to produce between $5.2 * 10^{-6}$ pg DA / cell (Trick et al. 2010) and $9 * 10^{-2}$ pg DA / cell (Bill 2011). Even highly toxigenic species cannot be assumed to be producing DA continuously, as DA production in laboratory conditions can be triggered by various environmental conditions including limitation by silicic acid (Bates et al. 1991; Pan et al. 1996a; Pan et al. 1996b), phosphate (Pan et al. 1996c), and iron (Maldonado et al. 2002), as well as exponential growth on urea (Howard et al. 2007) and the addition of bacteria (Bates et al. 1995). In contrast, most field studies have not been able to correlate environmental conditions such as nutrient levels or ratios (Marchetti et al. 2004; Trainer et al. 2009a; Trainer et al. 2009b), or bacterial abundance (Trainer et al. 2009b) with DA levels. However, in Southern California waters, correlations between DA and nutrient levels have suggested that P- or Si-limitation may have triggered DA outbreaks (Anderson et al. 2006, Schnetzer et al. 2007). Finally, in addition to bloom composition and potential environmental triggers of DA, physical processes are critical to the ultimate fate of toxigenic *Pseudo-nitzschia*

blooms (MacFadyen et al. 2005), determining whether onshore or offshore organisms are most likely to be impacted.

While all types of *Pseudo-nitzschia*, like many planktonic organisms, can be broadly dispersed by currents (Finlay 2002, Fenchel & Finlay 2004), the geographic ranges of individual *Pseudo-nitzschia* species vary from cosmopolitan to restricted distributions (Hasle 2002). Many *Pseudo-nitzschia* species, such as *P. australis*, *P. delicatissima*, *P. fraudulenta*, *P. multiseries*, *P. pseudodelicatissima*, and *P. pungens* are detected around the world and are believed to be cosmopolitan in coastal waters (Hasle 2002; Lelong et al. 2012; Trainer et al. 2012). Species typical of the open ocean include *P. granii*, *P. turgidula*, *P. heimii*, *P. inflatula*, and *P. prolongatoides* (Lelong et al. 2012). Interestingly, some open ocean species, such as *P. turgidula*, *P. heimii*, and *P. inflatula* have also been detected in coastal waters (Hubbard 2010, Ribalet et al. 2010, Lelong et al. 2012). Other species appear to be limited to colder (e.g. *P. obtusa*; Hasle and Lundholm (2005)) or warmer (e.g. *P. subfraudulenta*; Hasle (2002)) waters. Emerging data are rapidly revising our understanding of *Pseudo-nitzschia* biogeography, toxigenicity, and diversity, describing toxin production by species once believed to be nontoxigenic (Silver et al. 2010, Trick et al. 2010), and novel genotypes (e.g. Churro et al. (2009)). Biogeographical patterns of *Pseudo-nitzschia* also exist within currently-defined species, likely due in part to cryptic species (Hubbard et al. 2008); indeed , multiple new species have been described in the past several years (Lundholm et al. 2012; Quijano-Scheggia et al. 2009). Increasingly, work on *Pseudo-nitzschia* biogeography is revealing links between species distribution and specific environmental parameters (Almandoz et al. 2007, 2008, Kaczmarska et al. 2007, Schnetzer et al. 2007), including seasonal patterns (Fryxell et al. 1997; Fehling et al. 2006; Hubbard 2010; Klein et al. 2010).

Situated along the southwestern African coast, the Benguela Current is one of four global eastern boundary currents that result in seasonal upwelling and high biological productivity,

supporting known vectors of domoic acid (DA) such as sardines, anchovies, oysters, and sea lions (Hutchings et al. 2009). Upwelling zones, and eastern boundary currents in particular, are frequent hot-spots for DA outbreaks (Pitcher et al. 2010; Trainer et al. 2010). However, in countries bordering the Benguela Upwelling Zone, monitoring efforts for DA are sporadic, and most of the research related to harmful algal blooms along the western African coast has focused on dinoflagellates within the southern Benguela system (Pitcher & Calder 2000, Fawcett et al. 2006). Of the likely toxigenic *Pseudo-nitzschia* species in the Benguela Upwelling Zone, *P. australis* were confirmed in Lambert's Bay, South Africa, in March 2001, but isolates of this species did not produce detectable levels of DA (Marangoni et al. 2001). Subsequently, *Pseudo-nitzschia* communities in this bay in March 2006 and April 2007 were discovered to be toxigenic (Fawcett et al. 2007), in blooms that included *P. australis* and two morphologically distinct types of very small, unidentified cells (Seeyave et al. 2009). DA has also been reported in phytoplankton and bivalve samples in Luanda Bay, northern Angola, in connection with two separate blooms that occurred in September and November of 2007 (Blanco et al. 2010), although *Pseudo-nitzschia* were not identified to the species level. DA levels in these blooms measured between approximately 0.05 - 3 µg/L, in the range of other toxigenic blooms that have resulted in harmful effects of DA throughout higher trophic levels (Scholin et al. 2000; Trainer et al. 2000). Thus, the presence of *Pseudo-nitzschia* throughout upwelling zones, and the specific detection of DA near Angola and South Africa, suggests that *Pseudo-nitzschia* could pose a serious ecological threat throughout the Benguela and Angola systems.

More broadly, throughout greater western African coastal waters, *Pseudo-nitzschia* communities likely include common coastal *Pseudo-nitzschia* species (Hasle 2002). *P. australis* is the species most consistently reported in the Benguela Upwelling Zone (Marangoni et al. 2001; Hasle 2002; Seeyave et al. 2009). As summarized in Hasle (2002), the western African coast has reportedly harbored *P. australis*, *P. delicatissima*, *P. fraudulenta*, *P. multiseries*, *P.*

pseudodelicatissima, *P. pungens*, and *P. subfraudulenta*, although it is possible that some of these species types would now be reclassified as more recently established species. For regions offshore of the west African coast (the open South Atlantic Ocean), no previous studies to date have characterized *Pseudo-nitzschia* community composition (Lelong et al. 2012).

In this study, we assessed the toxigenicity and distribution of *Pseudo-nitzschia* across the South Atlantic Ocean, with a focus on the northern Benguela Upwelling Zone, testing the central hypothesis that *Pseudo-nitzschia* biogeography is coupled with environmental conditions. Further, we tested links among DA, *Pseudo-nitzschia* community composition, and environmental conditions, in order to explore potential environmental drivers of *Pseudo-nitzschia* biogeography and to identify potential toxigenic types. At four sites in the South Atlantic, we characterized *Pseudo-nitzschia* community composition using clone libraries of the partial internal transcribed spacer region 1 (ITS1), a highly variable region that detects intraspecific diversity. We characterized *Pseudo-nitzschia* communities across a broader expanse of the South Atlantic by utilizing Automated Ribosomal Intergenic Spacer Analysis (Hubbard et al. 2008), which describes communities in terms of the length of the partial ITS1 region. We document the detection of toxigenic *Pseudo-nitzschia* blooms within the northern Benguela Upwelling Zone. Our findings suggest that *Pseudo-nitzschia* display biogeographic patterns that are tightly tied to environmental conditions, and that species believed previously to produce only low levels of DA may indeed be capable of high levels of toxin production.

METHODS

Study area and environmental data collection overview. From November 18 - December 11, 2007, aboard the *R/V Knorr* (cruise 192-05), we conducted a transoceanic survey across the South Atlantic Ocean, followed by more intensive sampling in the northern Benguela Upwelling Zone (Figure 2.1). For reference to ocean conditions at the time of the cruise, we plotted the

categorized sites against chlorophyll a data (averaged over November and December 2007; Figure 2.1) produced with the Giovanni online data system, developed and maintained by the National Aeronautics and Space Administration's Goddard Earth Sciences Data and Information Services Center (NASA GES DISC; Acker and Leptoukh (2007)) and visualized with MODIS (Moderate Resolution Imaging Spectroradiometer)-Aqua data (9 km resolution).

Twenty-seven stations were sampled using two Niskin rosettes coupled with Conductivity-Temperature-Density (CTD) sensors, for surface, deep chlorophyll maxima (DCM), and several additional depths. Temperature, salinity, and fluorescence data were collected by the CTD at all 27 stations. DCM depths were determined based on the location of subsurface peaks in chlorophyll a fluorescence measured by the CTD. Water collected from Niskin bottles was sterile-filtered through 0.2-µm mesh, frozen at -20 °C, and analyzed for macronutrient concentrations using a Technicon AutoAnalyzer II™ (phosphate, ammonium) or an Alpkem RFA 300™ (silicic acid, nitrate plus nitrite) at Oregon State University. Total dissolved iron, total and labile cobalt, and total dissolved manganese concentrations were determined (details below) after sampling water from a trace-metal-free rosette.

To obtain an overview of physical oceanographic conditions over the cruise duration, surface current data were obtained from OSCAR (Ocean Surface Current Analyses – Real time; www.noaa.oscar.gov) and averaged over the time period of November 25 – December 15, 2007. The OSCAR project utilizes satellite altimeter (ocean level) and scatterometer (ocean wind) data to determine surface current speed and direction. Results were used to infer upwelling/downwelling conditions across the study region.

Cobalt analyses. Total dissolved and labile cobalt concentrations were determined using a previously described cathodic stripping voltammetry (CSV) method with a hanging mercury drop

electrode (Saito and Moffett 2001; Saito et al. 2004). Concentrations were determined by the standard additions using a programmed dosing procedure (Noble et al. 2008; Noble et al. 2012). Further details on the analyses and instrumentation are described in Noble et al. (2012).

Iron and manganese analyses. Total dissolved iron and manganese concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS), as described by Saito and Schneider (2006). Briefly, 13.0 mL aliquots of acidified seawater were weighed into cleaned polypropylene centrifuge tubes and ^{57}Fe was added for isotope dilution analysis and equilibrated overnight. Concentrated ammonium hydroxide (Seastar) was added to induce $Mg(OH)_2$ and trace metal co-precipitation, followed by centrifugation and decanting the supernatant. The isolated pellet was then dissolved in 5% nitric acid (Seastar) containing 1 ppb indium prior to analysis. Further details on the method and dataset are described in Saito and Schneider (2006) and Noble et al. (2012), respectively.

Phytoplankton community composition. For size-fractionated chlorophyll determination, duplicate samples were collected at each of four depths (surface, DCM, above mixed layer, below mixed layer), at most odd-numbered stations (3, 9, 13, 15, 17, 19, 21, 23, 25, and 27). For each depth, 500 mL of water were filtered in duplicate, sequentially through three filters: 10 µm (polycarbonate; Millipore), 1.2 µm (polycarbonate; Millipore), and GF/F (an approximate pore size of 0.7 µm, Whatman). Filters were extracted in 6 mL of 90% acetone in the dark at -20˚C for a minimum of 24 hours, and samples were analyzed using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) without acidification. The mean chlorophyll concentration was calculated for duplicate samples prepared at each depth.

From the surface waters of most stations (1, 3, 5, 7, 9, 11, 13, 15-27), qualitative net tow samples were collected using a 10-µm mesh phytoplankton net, and whole water samples were collected from Niskin bottles. Net tow samples were preserved (final concentration of 0.04%

buffered formalin phosphate) using 7 mL concentrated sample, and whole water samples were preserved similarly using 30 mL seawater (at stations 1-17 and station 27) or 7 mL seawater (at stations 18-26). All samples were stored in the dark prior to processing. Samples were settled in an Utermöhl settling chamber for a minimum of 24 hours. For samples containing 300 or more cells, diatom and dinoflagellate cells were counted and identified to the genus level when possible, using a Nikon Eclipse TS100 inverted microscope. Apical and transapical axes of *Pseudo-nitzschia* cells were measured under the light microscope. Individual cells were assigned to one of three morphological groups (Bill et al. 2006), based upon aspect ratio (apical axis : transapical axis) calculated from the range of dimensions for individual species (Hasle and Syvertsen 1997; Horner 2002) . The three morphological groups included the *P. pseudodelicatissima / P. delicatissima* group (40 – 140 μm by 1.1 – 3.5 μm; aspect ratio near 40); the *P. australis / P. fraudulenta* / *P. heimii* group (64 – 144 μm by 4.0 – 8.0 μm; aspect ratio near 16), and the *P. pungens / P. multiseries* group (68 – 174 μm by 3.0 – 5.0 μm; aspect ratio near 26). *Pseudo-nitzschia* cells that could not be unambiguously assigned to one of these three morphological groups were identified simply as *Pseudo-nitzschia sp*.

Domoic acid analysis. Water collected from Niskin bottles at stations 15 and 17-27 was used for particulate and dissolved domoic acid (PDA and DDA, respectively) determination at the surface and DCM, as well as at additional depths for stations 18, 19, 24, and 25. Underway samples were collected at 37 discrete points, via surface seawater inflow. Surface water from all underway sites was processed for PDA analyses.

Samples for particulate domoic acid (PDA) determination were filtered from 0.5 – 2 L of seawater through 0.45 μm HA filters (Millipore) or GF/F filters (Whatman; approximate pore size of 0.7 μm). DDA samples were collected by filtering several milliliters of whole water through a 0.45 μm HA filter enclosed in a Swinnex filter cartridge and reserving 1 mL of the filtrate. All filters and filtrate samples were stored at -80°C until extraction. Filters were extracted with 2.5

mL or 5.0 mL aqueous methanol (10%) prior to centrifugation for 1 min at 6000 *x g*, followed by filtration through a 0.22 µm syringe filter if liquid chromatography/mass spectrometry (LC/MS) was performed (Bates et al. 1991). All extracts were stored at -20°C until analysis by LC/MS and enzyme-linked immunosorbent assay (ELISA).

All PDA and DDA samples were analyzed according to manufacturer's instructions using direct competitive ASP ELISA (Biosense Laboratories; Kleivdal et al. (2007)). PDA was extracted into 10 ml of ultrapure distilled water (Milli-Q; MilliPore), and subsequent analysis was conducted according to manufacturer's instructions. The detection limit for this method is approximately 10 pg/mL in PDA extracts and DDA filtrates.

Selected samples (n=18), representing a range of DA levels as measured by ELISA, were analyzed for the presence of DA using tandem mass spectrometry coupled with liquid chromatographic separation (LC/MS) (Wang et al. 2007). Briefly, this method utilized reversed phase chromatography using an Agilent 1100 HPLC coupled to an ABI-SCIEX API-4000 triple quadrupole mass spectrometer. Chromatographic separation was performed on a Phenomenex Luna C18, 5µ, 150 x 2 mm column and retention time of DA in samples was compared to a certified reference standard (NRC Canada, Halifax, Canada). The DA fragments monitored were (in *m/z*): 266, 248, and *m/z* 193. The detection limits (dl) for this method were ~200 ng/L seawater (dissolved) and ~0.2 ng/L seawater (particulate) with a signal to noise ratio above ten.

Characterization of environmental regimes. We analyzed environmental data to identify significantly different environmental regimes across the South Atlantic Ocean. First, we examined 11 environmental variables (temperature, salinity, chlorophyll *a* fluorescence, depth of DCM, nitrate, nitrite, ammonium, silicic acid, phosphate, iron, and manganese), for 49 depths across stations 1-27, at both the surface and DCM. Nutrient samples were not collected from surface waters, yet at all stations, the surface mixed layer extended below 10 m, likely resulting

in similar nutrient concentrations across this upper layer. Consequently, we used nutrient data from 10 m along with the surface dataset (which included temperature, salinity, and chlorophyll a fluorescence from 3 m). For each station/depth sample, outliers were determined using IBM Statistical Package for the Social Sciences (SPSS) Statistics 19 and excluded from further analyses. For the 11 variables examined, we identified variables that were strongly correlated based on values above 0.85 for either Pearson linear or Spearman's non-parametric rank correlations, using SPSS. For each pair of strongly correlated variables, only one variable was retained in further analyses. Data were square-root-transformed and relativized across variables.

We conducted both qualitative and quantitative analyses of the resulting environmental data, in order to identify regions that differed significantly in terms of environmental conditions. First, we performed Principal Components Analysis (PCA), using the statistical package PC-ORD version 5.10 (McCune 2011). A clustering dendrogram was constructed in order to quantitatively compare resemblance among samples (based on Euclidean distance), using PRIMER-E version 6 (Clarke & Warwick 2001). Significantly different samples ($p = 0.05$) were determined by applying a Similarity Profile (SIMPROF) test to the dendrogram. This test allowed us to identify significantly different environmental regimes and to categorize stations according to these regimes. Second, we constructed temperature-salinity plots in Ocean Data View 4 (Schlitzer 2012), plotting CTD values for each meter of the water column between surface and a maximum bottom depth of 200 m. All station sites were categorized according to significantly different environmental regimes.

Pseudo-nitzschia *species and community composition: partial ITS1 clone library construction*. Four samples were selected for ITS1 clone library construction, each corresponding to a different environmental regime: station 13 surface, station 13 DCM, station 19 surface, and station 25 surface. First, PCR was performed using non-fluorescent PnAll

primers (PnAll-F: 5'-TCTTCATTGTGAATCTGA-3' and PnAll-R: 5'-CTTTAGGTCATTTGGTT-3'; Hubbard et al. (2008)), for a total of 32 cycles, using 10 ng of genomic DNA for the stations closest to the African coast (stations 19 and 25). In order to successfully amplify more dilute samples from the open ocean (station 13), 30 ng of genomic DNA were amplified, as determined by PCR optimization experiments utilizing a range of DNA concentrations. PCRs were then reconditioned for 3 cycles using 5 µL of initial PCR product and 15 µL fresh PCR reagents (Thompson et al. 2002). This reconditioning step was conducted using three replicates of the coastal samples (stations 19 and 25) and using 9 replicates of the open-ocean samples (station 13). The resulting PCR products were pooled and purified using a MinElute PCR Purification kit (Qiagen). Transformations were performed using ~2 ng DNA, with PCR products cloned into One Shot TOP 10 *Escherichia coli* cells, using the TOPO TA Cloning Kit (Invitrogen). Clone library sequencing was conducted at the University of Washington's High Throughput Genomics Unit (Seattle, WA), using an ABI 3730xl DNA analyzer (Applied Biosystems) and the universal M13 vector forward primer (M13F (-21), 5'-TGTAAAACGACGGCCAGT-3') to sequence up to 96 clones per clone library.

***Clone library data analysis*.** Sequences were trimmed to the PnAll regions, including the PnAll primers in order to identify ARISA fragment length, using Sequencher 4.8 (Gene Codes). Trimmed sequences with lengths less than 100 bp or greater than 300 bp were excluded from further analysis, on the grounds that these sequences represented primer-dimers or other sequencing artifacts. Within each clone library, identical sequences were grouped and counted.

Unique clone sequences were compared (tblastn) to nucleotide files downloaded from GenBank on October 19, 2011. The top 5 BLAST (Basic Local Alignment Search Tool) hits (hereafter referred to as "reference sequences") were collected for each query sequence. Subsequent analyses included unique clone sequences that were similar to known organisms in GenBank. All 72 of these unique sequences were checked for chimeras, using the uchime command in the

bioinformatics software package mothur v.1.23.0 (Schloss et al. 2009). To identify *Pseudo-nitzschia* clone sequences to the species level, we used the cluster command (with method set to “average”) to group the unique clone sequences with unique reference sequences from BLAST hits (n=67), where >18% nucleotide divergence distinguished unique “species-groups.” This level of nucleotide divergence was chosen to define distinct *Pseudo-nitzschia* species groups, based on the previously-reported divergence levels in PnAll regions of the ITS1 among species (up to 16%; Hubbard et al. (2008)), as well as to choose a manageable number of clusters to further align. Both unique clone sequences and reference sequences were trimmed to exclude the PnAll primer regions, and an initial alignment was created using BioEdit version 7.1.3 (Hall 1999) and ClustalX version 2.1 (Larkin et al. 2007). A distance matrix was calculated in PAUP* 4.0 (Swofford 2003), accounting for nucleotide divergence, using the number of nucleotide polymorphisms as unique characters, relative to the number of total characters (Page & Holmes 1998) but not the presence of insertions or deletions (in/dels). Each cluster was aligned separately in BioEdit version 7.1.3 (Hall 1999) and ClustalX version 2.1 (Larkin et al. 2007) and a distance matrix was calculated for each cluster, in PAUP* 4.0(Swofford 2003) as before (accounting for nucleotide divergence but not in/dels). Finally, for each clone library, rarefaction curves were constructed using EstimateS version 8.2.0 (Colwell 2006), in three forms, each of which compared number of clones with 1) number of unique genotypes, 2) number of ARISA OTUs, or 3) number of species-groups, as defined by <18% average nucleotide divergence.

Pseudo-nitzschia *community composition: Automated Ribosomal Intergenic Spacer Analysis (ARISA)*. For each ARISA profile, one liter of seawater was filtered onto 0.2 µm Supor filter, which was then placed in 1 mL sucrose lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris-HCl, pH 8.4), flash-frozen in liquid nitrogen, and stored at -80 °C. DNA extractions were conducted using a modified DNeasy Mini Protocol for Plant Tissue

(Qiagen). Briefly, filters stored in sucrose lysis buffer were thawed, and then 200 μL of 10% SDS (sodium dodecyl sulfate) and 4 μL of RNase A stock solution (Qiagen, 100 mg/mL) were added. During subsequent steps of the extractions, we added three-fold the volumes of reagents stated in the protocol, to scale up the reactions appropriately for 1.2 mL, rather than a typical starting suspension volume of 0.4 mL. Because of the increased sample lysate volume, each DNeasy mini spin column was reused five times, rather than the typical two-fold reuse. Finally, the purified DNA was eluted in two separate volumes of 30 μL of buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0). DNA extracts were stored at -20 °C until further processing.

ARISA was performed to amplify the variable-length portion of the ITS1 region of species of the diatom *Pseudo-nitzschia* (Hubbard et al. 2008). Briefly, in 20 μL triplicate reactions, 2.5 mM of MgCl2, 1X buffer (Promega), 0.15 uL *Taq* polymerase (GeneChoice), 0.4 mM dNTPs, and 0.5 μM of the PnAll-F (5'-TCTTCATTGTGAATCTGA-3') and PnAll-R (5'-CTTTAGGTCATTTGGTT-3') primers. To conduct ARISA, the PnAll-R primer was labeled with the fluorescent tag FAM added to the 5' end of the primer sequence. For coastal stations (stations 16 – 27), 10 ng of genomic DNA were amplified. As done for clone library construction, 30 ng of genomic DNA were amplified from more oligotrophic stations (stations 1 – 15). In order to successfully amplify more dilute samples from open ocean stations (stations 1 – 15), 30 ng of genomic DNA were amplified, as determined from PCR optimization experiments utilizing a range of DNA concentrations. For all PCR, 32 cycles were performed, as done for clone library construction (see subsection '*Pseudo-nitzschia* species and community composition: partial ITS1 clone library construction').

Following the procedure of Hubbard (2010), each single PCR product was purified using MultiScreen PCRμ96 filter plates (Millipore) and eluted with 25 uL ultrapure distilled water. Triplicate PCR products were pooled and quantified using Qubit High-Sensitivity DNA

Quantitation Kit (Invitrogen). All purified PCR products were diluted to a final DNA concentration of 0.1 ng/L, and 10 ng DNA were further purified using ethanol precipitation (Sambrook & Russell 2001). Each purified, precipitated PCR product was resuspended in a dilute Tween solution with an internal size standard fluorescently-labeled with Et-ROX 550 (GE Healthcare), such that each well contained 0.078 µL 10% Tween, 9.77 µL sterile water, and 0.15 µL fluorescent size standard. Fragment analysis was conducted on a MegaBACE 1000 automated sequencer (Amersham Biosciences).

ARISA electropherograms were analyzed using the software DAx 7.0 (Van Mierlo software, Eindhoven, the Netherlands), following application of a spectral matrix correction. Low-intensity electropherograms (with total single peak height below 500 relative fluorescence units) were excluded from further analyses. Data were binned at a resolution of 0.1 bp, using dakster version 4.4, a Perl binning tool (http://rocaplab.ocean.washington.edu/cgi/dakster/index.html). To account for variability in peak-calling across plate runs and sample wells, we applied an Excel Macro that binned data multiple times, each time beginning with a different base pair starting point, to create several different “frames” of data (after Hewson and Fuhrman (2006)). This Macro binned the ARISA peaks into 2 bp bins, utilizing a 0.5 bp frame shift for a total output of four frames of data. Only peaks that were detected across all four frames were included in further analyses; peaks that occurred only in a single frame were considered to be binning artifacts and were therefore excluded from further analyses. We then calculated Sorensen (presence-absence analysis) and Bray-Curtis (semi-quantitative analysis) similarity coefficients for each of four frames of ARISA data. The maximum Sorensen and Bray-Curtis coefficients were calculated across all frames, in order to create the most conservative test of the null hypothesis that communities do not differ significantly (Hewson & Fuhrman 2006).

We applied several statistical analyses to test the hypothesis that *Pseudo-nitzschia* community composition differed significantly across different environmental regimes, using the statistical

software PRIMER-E version 6 (Clarke & Warwick 2001). First, *Pseudo-nitzschia* community data were analyzed by quantitative clustering in two configurations: grouping communities with other similar communities, and grouping together OTUs with similar distributions across whole communities. Similarity profile (SIMPROF) tests were applied to both data configurations, thereby identifying significantly different ($p = 0.05$) samples. Second, we tested these *Pseudo-nitzschia* community patterns with analysis of similarity (ANOSIM) statistics, to determine whether communities differed significantly across the defined environmental regimes. Third, we conducted similarity percentage (SIMPER) analysis to identify specific ARISA OTUs that were important to *Pseudo-nitzschia* biogeography. Using semi-quantitative ARISA data, we determined the top OTUs that contributed up to 70% of similarity among communities in each significantly different environmental regime.

Correlations among* Pseudo-nitzschia *types, environmental parameters, and domoic acid. Statistically significant correlations among ARISA OTUs and environmental parameters were calculated as both linear correlations (Pearson Correlation Coefficient, or PCC) and nonlinear correlations (Local Similarity Analysis, or LSA; Ruan et al. (2006)). These correlations were determined from a dataset that included 19 environmental parameters and relative peak height ARISA data, for 29 station/depth combinations. The environmental data were square-root-transformed, as conducted earlier for PCA and SIMPROF analyses. The matrix of environmental and ARISA data was submitted to the Galaxy server of the Sun Lab (University of Southern California) at http://meta.cmb.usc.edu/. The function “LSA Compute” was used to calculate all correlations among variables, without a time delay and using one replicate per station/depth combination. Correlations for which the LSA p-value ≥ 0.025 (corresponding to PCC p-values ≤ 0.05) were visualized using Cytoscape version 2.8.2 (Shannon et al. 2003), open-source network visualization and analysis software downloaded from http://www.cytoscape.org.

We explored whether environmental conditions could have predicted DA production by *Pseudo-nitzschia*. In particular, we tested the hypothesis that nutrient limitation induces DA production, as suggested by laboratory studies (as reviewed by Lelong et al. (2012) and Trainer et al. (2012)). Pearson (linear) and Spearman's (nonparametric) correlations were calculated between PDA values (as determined by ELISA) and each of the environmental parameters included in the subsection 'Characterization of environmental regimes,' as well as nutrient ratios, using SPSS.

RESULTS

Characterization of environmental regimes in the South Atlantic

Across the South Atlantic Ocean and northern Benguela Upwelling Zone in November and December, 2007, we measured fourteen environmental parameters. Broadly, lower nutrient and fluorescence levels distinguished oligotrophic, open ocean sites (stations 1-15, 25-27) from more eutrophic, coastal sites (stations 16-24) (Figure 2.1). As determined by data from OSCAR, surface water currents were moving offshore (westward) along the entire cruise track. The Benguela Current appeared to flow outside and south of the cruise track, indicated by northward-moving currents.

We quantitatively determined environmental differences among stations using 13 environmental variables collected from CTD data. We excluded the variables of DCM depth, nitrate, phosphate, and total cobalt from statistical analyses because they were highly correlated with other variables under consideration (Pearson or Spearman correlation values > 0.85). Furthermore, we excluded labile cobalt from these analyses because comparatively few data points were available. Station 23 was determined to be a statistical outlier (SPSS), mainly due to anomalously high silicic acid values, and data from both depths at this station were excluded from the initial analyses to determine environmental regimes. Nutrient data were not available

for station 25, and therefore environmental data from this station were also excluded from initial analyses.

In total, for 25 of the 27 stations, eight environmental variables were analyzed by PCA and cluster analysis: temperature, salinity, silicic acid, ammonium, nitrite, fluorescence, iron, and manganese (Figure 2.2A). Axis 1 of the PCA was significant (eigenvalue greater than the broken-stick eigenvalue) and represented 56.0% of variance in the environmental data, but Axis 2 was not significant and represented 13.9% of variance in the environmental data. Axis 1 was driven primarily by temperature, nitrite, and salinity. The ordination of stations along Axis 1 represents a transition from coastal stations (left) to open ocean stations (right).

Six environmental regimes within the South Atlantic represented a continuum from open ocean to coastal sites, including one group of stations that signified a transition between these conditions (Figure 2.2A). The six regimes differed significantly ($p=0.05$) from one another, according to the SIMPROF test applied to the clustering dendrogram, and each regime included stations that exhibited a Euclidean distance similarity of less than two. Because stations 23 and 25 were not included in the PCA and clustering analyses, we utilized temperature-salinity plots (Figure 2.2B) to assign these stations to appropriate environmental regimes: station 23 clustered with stations 22 and 24, and station 25 clustered with stations 26 and 27. Thus, the final environmental regime determination encompassed three coastal groups ("North Coastal," "Mid-Coastal," and "South Coastal"), two northern open ocean groups ("North Open Ocean Surface" and "North Open Ocean Deep" (DCM)), and one group including southern open ocean stations and northern DCM samples ("Transition") (Figure 2.1, Figure 2.2). These six environmental regimes were used to test the hypothesis that *Pseudo-nitzschia* community composition differed significantly according to distinct environmental conditions.

Phytoplankton community composition

The distribution of phytoplankton size classes in the surface waters (Figure 2.3) differed across open ocean and coastal sites, as well as across the northern and southern coast. Total chlorophyll levels were low (< 0.4 µg/L) at stations 3, 9, 13, 15, 17, 25, and 27, whereas total chlorophyll ranged between 4.7 and 5.8 µg/L at stations 19, 21, and 23 (Figure 2.3A). Furthermore, the relative chlorophyll a size fractionation (Figure 2.3B) revealed a trend of three different types of communities in the northern open ocean, northern coast, and southern sites. These three different groups of sites included communities with mostly large (>10 µm fraction) phytoplankton (stations 19 and 21), communities comprising predominantly >1.2 µm phytoplankton (stations 23, 25, 27), and communities with a substantial (30% or greater) proportion belonging to the 0.7 – 1.2 µm size class (stations 3, 9, 13, 15, 17). For each station, the distribution of chlorophyll across size fractions across deeper depths was similar to the size-fractionation observed for the surface waters (data not shown).

According to light microscopy classifications of net tow and whole water samples, *Pseudo-nitzschia* were detected across multiple distinct environmental regimes, where this genus co-existed with other widespread diatom and dinoflagellate taxa. Net tow data should not be considered as representative of the whole community, because some small cells likely passed through the 10µm mesh. For example, at station 18, markedly fewer *Pseudo-nitzschia* were detected in net tows (Figure 2.4A), compared to the whole water analysis (Figure 2.4B). Therefore, phytoplankton community composition in net tow samples is likely a conservative estimate of the total proportion of *Pseudo-nitzschia* and other small cells.

Qualitatively, the net and whole water data revealed the presence of *Pseudo-nitzschia* at diverse sites in the South Atlantic. Specifically, at seven stations, *Pseudo-nitzschia* cells were

detected, and their abundance comprised between 2.8 and 44.5% of the total preserved communities of diatoms and dinoflagellates in net tow samples (Figure 2.4A). In addition, we detected the harmful algal bloom-causing dinoflagellate genera *Alexandrium* and *Dinophysis* (Figure 2.4A), as well as other dinoflagellate genera (*Protoperidinium*, *Ceratium*, *Pyrophacus*, *Prorocentrum*). The diatom community included *Rhizosolenia*, *Chaetoceros*, *Skeletonema*, centric diatoms (either *Thalassiosira* or *Coscinodiscus*), and unidentified types (pennates and other morphologies).

Quantitatively, *Pseudo-nitzschia* were present at stations 18 and 19 (up to $6.5 * 10^4$ cells/L; Figure 2.4B). Furthermore, among *Pseudo-nitzschia* cells detected in whole water samples (Figure 2.4B), the extent of morphological diversity (measured by aspect ratio) indicated that three or more species were present in the South Atlantic. Specifically, *Pseudo-nitzschia* communities included short, narrow *P. pseudodelicatissima / P. delicatissima* type cells, long, wide *P. australis / P. fraudulenta / P. heimii* type cells, and long, narrow *P. pungens / P. multiseries* type cells. Small cells of the *P. pseudodelicatissima*/*P. delicatissima* type were the predominant morphological type at stations 18 and 19 (Figure 2.4B).

Domoic acid in the northern Benguela Upwelling Zone

Particulate domoic acid was detected across a large expanse of the northern Benguela Upwelling Zone. We detected PDA in the surface waters of three regions: the northern coast (stations 18, 19), the mid-coastal region (station 21), and the southern coast (stations 22-27) (Figure 2.5). The high correlation between ELISA and LC-MS values (Figure 2.5 inset, r^2 = 0.95) indicated that ELISA data did not substantially reflect false positive measurements. PDA levels in the surface waters ranged between 1.05 and 184 ng/L according to ELISA, with highest DA levels recorded at station 19. In addition to the data shown here for surface waters (Figure

2.5), moderately high PDA levels (85.0 ng/L) were detected at 50 m depth at station 24. Detectable DDA levels were found only at stations 18 and 19, measuring between 0.6 – 0.8 ng/L. When normalized for total number of *Pseudo-nitzschia* cells enumerated in whole water samples (Figure 2.4B), PDA measured 1.5 pg/cell at station 18, 3.0 pg/cell at station 19, and 4.6 pg/cell at station 21.

To address the hypothesis that specific environmental conditions predict DA production by *Pseudo-nitzschia* in the field, we tested 17 environmental parameters, including nutrient ratios, for correlations with PDA levels for 21 data points for which all of these environmental parameters were measured. For either Pearson (linear) or Spearman (nonparametric) correlations, three environmental parameters exhibited significant correlations with PDA. We found positive correlations with PDA for chlorophyll a fluorescence (Pearson correlation, $p < 0.01$), and total dissolved manganese (Spearman's rho, $p < 0.05$), and we detected negative correlations with PDA for DCM depth (Pearson correlation, $p < 0.05$ and Spearman's rho, $p < 0.01$). The correlations with high fluorescence and shallow DCM depth likely reflect the occurrence of DA production within coastal waters.

***Pseudo-nitzschia* species and community composition: partial ITS1 clone libraries**

To describe *Pseudo-nitzschia* communities in terms of species composition, we constructed partial ITS1 clone libraries for four communities representing four different environmental regimes: station 13 surface (North Open Surface), station 13 70 m (North Open Deep), station 19 surface (North Coastal), and station 25 surface (South Coastal). First, our clone library data validated the specificity of the PnAll primers for *Pseudo-nitzschia*. For clone libraries except for station 13 70 m, the majority of clones per library were determined to be valid ARISA fragments (100-300 bp in length). In total, 227 clones, represented by 72 unique sequences, were identified as *Pseudo-nitzschia* based on most similar sequences in GenBank. None of these 72

sequences were predicted to be chimeras, as determined by the uchime command in the program mothur. These 72 sequences were included in subsequent analyses.

Pseudo-nitzschia community composition was generally well-sampled by clone libraries and revealed that community richness varied considerably across the South Atlantic (Figure 2.6). In terms of ARISA OTUs (Figure 2.6B) and species groups (Figure 2.6C), the community at station 19 was the richest, whereas the station 13 community was the least rich. ARISA OTU richness, which reflects intraspecific diversity (Hubbard et al. 2008), correlated well with species-group richness at approximately twice the magnitude, suggesting that ARISA OTU richness could be a good proxy for relative species richness among communities. Among all the communities, station 25 exhibited the highest genotypic richness (Figure 2.6A), whereas this community had relatively low richness on the levels of ARISA OTUs and species groups. However, because genotypic richness did not plateau for any community (Figure 2.6A), we conclude that we sampled a subset of ITS1 genotypes in these communities. In clone libraries constructed from station 13 70 m and station 25 communities, fewer *Pseudo-nitzschia* clones (e.g. 12 clones at station 13 70 m) and multiple sequences < 100 bp were detected, compared to the other clone libraries. We interpret this finding as suggestive of nonspecific amplification, such as primer-dimers, after the target DNA had been amplified. These results further indicate that *Pseudo-nitzschia* cells may have been in lower relative abundance among these communities, compared to Stations 13 and 19 surface water communities.

In addition, when characterized according to likely species composition, these four South Atlantic *Pseudo-nitzschia* communities chosen were markedly distinct (Figure 2.7). The *Pseudo-nitzschia* types were similar in partial ITS1 sequence (PnAll fragment) to a total of 11 previously-described species and one unidentified species, and they clustered into eight species-groups based on a nucleotide divergence of <18%. In order of the most clone-rich

species-groups, these eight groups included clone sequences most similar to 1) *P. inflatula / P. micropora*, 2) *P. galaxiae*, 3) *P. subpacifica* / *P. heimii*, 4.) *P. turgiduloides / P. turgidula,* 5.) *P. cuspidata*, 6.) *P. sp*., 7.) *P. caciantha*, and 8.) *P. pungens* (Figure 2.7). The *Pseudo-nitzschia* community in station 19 surface waters was the most diverse and species-rich, comprising six of the eight species-groups. Strikingly, only two species-group types were detected in more than one community*: P. inflatula / P. micropora* (station 13 surface and 70 m, station 19 surface) and *P. subpacifica* / *P. heimii* (stations 19 and 25 surface). Major species represented in the South Atlantic communities included *P. inflatula* (84 clones), *P. galaxiae* (63 clones), and *P. heimii* (27 clones), with other detected species represented by fewer clones.

We detected a substantial amount of new diversity within the *Pseudo-nitzschia* genus, as evidenced by novel predicted ARISA OTU fragment lengths. The majority of the predicted ARISA OTU fragment lengths differed from predicted lengths of the closest reference sequence (Table 2.1). By pairing predicted ARISA OTU fragment lengths with clone library sequence data, we discovered potentially new types of *Pseudo-nitzschia* within six species-groups. Most notably, these include a putative *P. heimii* type (ARISA = 217-221 bp), two new *P. galaxiae* types (ARISA = 138, 156 bp), *P. inflatula* (163-164 bp, 170-171 bp), *P. micropora* (188 bp, 197 bp), *P. turgidula* (155 bp, 179 bp), *P. caciantha* (230 bp), *P. cuspidata* (220 bp, 226 bp). In certain cases, it is possible that these clones represent previously undiscovered species; for example, both *P. micropora*-like ARISA OTUs differ greatly in fragment length between our study (188 bp, 197 bp) and the predicted fragment length (146 bp) of the most similar reference strain. Furthermore, some level of genotypic richness is apparent among *Pseudo-nitzschia* types of the same species-group and ARISA OTU length. Many commonly detected clone types included different genotypes (as define by unique nucleotide sequences) within the same community, such as *P. heimii* 196 bp (5 genotypes among 19 clones from station 25 surface), *P. galaxiae* 138 bp (4 genotypes among 22 clones from station 19 surface), *P. galaxiae* 149-152

bp (8 genotypes among 40 clones from station 19 surface), *P. inflatula* 163-164 bp (9 genotypes among 44 clones from station 13 surface). However, for individual ARISA OTUs (e.g. *P. cf. subpacifica*, 195 bp), one genotype tended to dominate the clone sequences. Unique clone sequences identified as *Pseudo-nitzschia* (Table 2.1) were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers JX441017-JX441088.

In contrast to the clones that varied considerably from previously reported *Pseudo-nitzschia* strains, we discovered some cases of South Atlantic clones that were similar to reference sequences from geographically distant regions. For example, one genotype of *P. subpacifica* OTU 195 (detected in station 19 surface community) was similar to the reference strain *P. cf. subpacifica* AY257858, isolated from Costa Nova, Portugal (Lundholm et al. 2003), and *P. inflatula* OTU 154/157 (station 13 surface) was similar to the sequence of *P. inflatula* from Phuket, Thailand (Priisholm et al. 2002). *P. turgidula* OTU 176-179 (station 25) possessed the same or similar ARISA fragment length of 176 bp, with little sequence divergence, compared to *P. sp.* environmental clone EU068696 detected at Ocean Station Papa in the Northeast Pacific (Marchetti et al. 2008). Furthermore, the overall species composition of the station 25 surface community (*P. heimii* and *P. turgiduloides / P. turgidula*) resembled the species composition reported from iron-enrichment experiments conducted as part of the Subarctic Ecosystem Response to Iron Enrichment Study (SERIES) at Ocean Station Papa, where the two most abundant types of clones were similar to *P. heimii* and *P. turgidula* (Marchetti et al. 2008).

***Pseudo-nitzschia* community composition and geographic distribution: Automated Ribosomal Intergenic Spacer Analysis**

As determined by ARISA, *Pseudo-nitzschia* were distributed widely throughout the South Atlantic Ocean (Figure 2.8). *Pseudo-nitzschia* were detected at 17 of the 20 stations examined,

including several stations towards the western portion of the South Atlantic Gyre (7, 9, 11, and 13, 15) as well as all of the stations closer to the African coast (16-27). In total, 37 distinct *Pseudo-nitzschia* ARISA OTUs were found across the region.

Pseudo-nitzschia ARISA community composition differed significantly according to the six distinct environmental regimes, as determined by quantitative clustering (Figure 2.8) and analysis of similarity, or ANOSIM (Table 2.2). A substantial number of rare *Pseudo-nitzschia* types were found: 16 OTUs occurred among three or fewer communities (Figure 2.8). Few *Pseudo-nitzschia* types were distributed broadly across the open South Atlantic Ocean (stations 7-13) or even within the northern Benguela Upwelling Zone (stations 15-27). Of the 37 total OTUs detected (Figure 2.8), eight OTUs were identified as contributing strongly to similarity among communities at each of the six environmental regimes: 135, 149, 153, 161,195, 219, 221, and 225 (Table 2.3).

To investigate *Pseudo-nitzschia* species ranges, we matched putative species identifications (Table 2.3) with the distributions of the ARISA OTUs determined to be characteristic of the significantly different environmental regimes (Table 2.3). A single OTU, 195, was detected in 20 of 32 communities examined (Figure 2.8), and this type was a predominant community member of diverse environments: North Open Deep, Transition, and South Coastal regimes (Table 2.3). However, based upon comparison of predicted *Pseudo-nitzschia* ARISA fragments and clone library sequence data, this OTU was likely represented by two different species-groups: *P. subpacifica / P. heimii* at coastal and transition stations, and *P. micropora* in the open ocean. Other OTUs, although also detected in many communities, exhibited specific ranges. For example, two types of *Pseudo-nitzschia* were predominant among more oligotrophic sites: *P. inflatula / P. turgidula* OTU 153 (North Open Surface and Transition regimes) and unidentified OTU 135 (North Open Deep and Transition regimes). In contrast, OTUs 149 and 225 tended to co-occur among communities near the coast (Figure 2.8). The more broadly-distributed OTU

225, putatively identified as *P. cuspidata* or *P. caciantha*, was detected in 12 near-coastal communities (Figure 2.8), and this type was predominant among all coastal regimes but none of the other regimes (Table 2.3). In contrast, *P. galaxiae* OTU 149 was found mainly in communities where DA was also detected (e.g. Stations 18, 19, 21, and 24; Figure 2.5). Four OTUs were characteristic of single regimes: *P. inflatula* OTU 161 (North Open Surface), *P. heimii / P. cuspidata* OTU 221 (North Open Deep), and *P. cuspidata / P. heimii* OTU 219 (North Coastal), and *P. galaxiae* OTU 149 (North Coastal). Most importantly, none of the species were distributed broadly across the study region.

Potential environmental drivers of *Pseudo-nitzschia* biogeography in the South Atlantic

We used Local Similarity Analysis, or LSA, to generate hypotheses about the dominance of certain *Pseudo-nitzschia* types under specific environmental conditions. Overwhelmingly, both nonlinear correlations (LSA) and linear correlations (Pearson Correlation Coefficient, or PCC) identified the same or similar variables as being correlated significantly with *Pseudo-nitzschia* types. Fifteen of the 37 ARISA OTUs, including six of the biogeographically important OTUs (as identified by SIMPER analysis), exhibited significant nonlinear or linear correlations with specific environmental variables (Figure 2.9). In the Pearson Correlation analysis (data not shown), *P. cuspidata / P. caciantha* OTU 225 was correlated with the greatest number of environmental variables, particularly N sources, suggesting that this type is predominant in high-N environments. This result is consistent with the finding that OTU 225 was predominant among all three coastal regimes (Table 2.3). We also identified several *Pseudo-nitzschia* types as potentially preferring relatively higher salinity waters across the cruise track (*P. cuspidata / P. heimii* OTU 221, *P. turgidula* OTU 179, *P. cuspidata / P. heimii* OTU 215, and unidentified OTU 107), in contrast with one lower-salinity type, *P. subpacifica / P. heimii / P. micropora* OTU 195. Other types showed positive correlations with Co: *P. cuspidata / P. heimii* OTU 219, *P. galaxiae*

OTU 137, *P. micropora*-like OTU 187, and *P. galaxiae* OTU 151. Although these two *P. galaxiae* OTUs were positively correlated with cobalt, *P. galaxiae* OTU 149 was correlated with different environmental variables. Positive correlations with DCM depth indicated that *P. cuspidata / P. heimii* OTU 221, *P. turgidula* OTU 179, and unidentified OTU 107 were specific to open-ocean sites (with deep DCM), whereas a negative correlation with DCM depth suggested that *P. galaxiae* OTU 149 was specific to coastal sites (shallow DCM).

We also employed LSA in order to address a second hypothesis that a single *Pseudo-nitzschia* species was responsible for DA production throughout the northern Benguela Upwelling Zone. Our results found that two OTUs, both identified as *P. galaxiae,* were the only ARISA OTUs that were correlated significantly with PDA levels (Figure 2.9). Among the 11 sites at which DA was detected and *Pseudo-nitzschia* ARISA was performed, *P. galaxiae* OTU 149 was found at seven sites, whereas *P. galaxiae* OTU 137 was found at five of these sites. In contrast, *P. galaxiae* OTU 149 was detected at only one of the 11 sites where DA was not detected; *P. galaxiae* OTU 137 was detected at two of the sites where DA was not detected.

DISCUSSION

We detected highly diverse *Pseudo-nitzschia* communities including novel types, as reflected by novel ARISA OTUs and species composition of these communities. In total, we detected 37 *Pseudo-nitzschia* ARISA OTUs, suggesting a great richness and diversity of these communities within the South Atlantic alone. Interestingly, we detected three ARISA types of *P. inflatula*, whereas only a single isolate has been reported previously in the literature (Priisholm et al. 2002). These three *P. inflatula* types co-existed with one another in at least one water parcel (station 13 surface waters), suggesting either intraspecific niche differentiation or nonfunctional

microdiversity. Intraspecific niche differentiation was further suggested by the correlation of different *P. galaxiae* ARISA types with different environmental conditions. Similarly, for the Gulf of Naples, McDonald et al. (2007) reported three types of *P. galaxiae*, where a fairly ubiquitous *P. galaxiae* type likely co-existed with either one of two apparently seasonal *P. galaxiae* types. It is possible that some of the diversity we report here may actually represent previously undiscovered species of *Pseudo-nitzschia*. Indeed, as for many diatoms (Mann 1999), previous determination of *Pseudo-nitzschia* "species" designations may be too inclusive, masking ecologically significant diversity. The microdiversity within the *Pseudo-nitzschia* genus is likely a key to understanding physiological differences. Specifically, variability in toxin production among strains of the same species (Bates 2000; Thessen et al. 2009) suggests that some of this microdiversity could explain the apparent disconnect between environmental DA levels and reported toxigenicity of *Pseudo-nitzschia* cultures.

Our results support the hypothesis that individual communities of the cosmopolitan *Pseudo-nitzschia* genus exhibit distinct biogeographical patterns. These diatoms, therefore, shared some similarities with distributions of heterotrophic and photosynthetic marine bacterial communities, sampled during the same cruise and which varied across open ocean and coastal regions (Morris et al. 2010; Morris et al. 2012). However, in contrast to these South Atlantic bacterial studies, we report finer-scale spatial variability in coastal *Pseudo-nitzschia* community composition, whereby significantly different *Pseudo-nitzschia* communities were detected within three environmentally distinct coastal regimes of the northern Benguela Upwelling Zone. Furthermore, in the South Atlantic, both coastal and open ocean bacterial communities differed between surface and deep chlorophyll maximum waters, with a more pronounced difference at open ocean sites (Morris et al. 2012). By comparison, for coastal regions, we discovered that *Pseudo-nitzschia* community composition did not vary with depth across the northern Benguela Upwelling Zone. However, *Pseudo-nitzschia* community composition varied with depth in the

open ocean, a finding that has not been previously reported for this diatom. This result suggests that *Pseudo-nitzschia* communities could be driven by parameters that vary with depth, such as light and nutrients, much like ecotypes of the photosynthetic cyanobacteria *Prochlorococcus* (Moore & Chisholm 1999, Rocap et al. 2003) and *Synechococcus* (Ahlgren & Rocap 2006). Here, we also describe spatial variability in *Pseudo-nitzschia* communities that seems to parallel the change in environmental conditions between open ocean and coastal waters, such that a distinct type of *Pseudo-nitzschia* community was detected at the environmental transition between open ocean and coastal regimes. Similarly, Ribalet et al. (2010) described a very similar *Pseudo-nitzschia* community which appeared to thrive at an ecotone where coastal and open ocean waters mixed, near Ocean Station Papa.

Although *Pseudo-nitzschia* as a genus is commonly described as cosmopolitan, our clone library data, in addition to the variability in ITS1 type community composition (ARISA), highlight the spatial variability in individual species distribution. We present our clone library data in terms of the most similar species, which should be understood as an estimation of the actual or closely related species, because the intraspecific variability in the partial ITS1 region varies greatly depending on the species (between <3% and 16% including in/dels; Hubbard et al. (2008)). Although *Pseudo-nitzschia* species identifications are most conclusively solidified using electron microscopy on cultured isolates in combination with genetic characterization and mating studies, our putative identifications are powerful in that they allow us to compare the genetic similarity between South Atlantic *Pseudo-nitzschia* and EM-verified isolates from other regions. In our clone libraries, we did not detect types similar to species commonly described as cosmopolitan, such as *P. australis*, *P. delicatissima*, *P. fraudulenta*, and *P. multiseries,* even though our study spanned a large geographic region. We detected only a single clone of the "cosmopolitan" species, *P. pungens*, and it is possible that historic reports of *P. pseudodelicatissima* actually represent the similar species *P. cuspidata* (Lundholm et al. 2003),

as we report the detection of *P. cuspidata* here. Overall, our study demonstrates a high level of variability in *Pseudo-nitzschia* community composition, with neither any species nor any ARISA OTUs displaying a cosmopolitan distribution across this region. For example, *Pseudo-nitzschia* species group composition varied considerably across the four clone libraries, with only two of the species groups being detected in more than one clone library. These findings suggest that some *Pseudo-nitzschia* types may possess limited environmental tolerances. However, some types are likely distributed widely across the worlds' oceans if these preferred environmental conditions are present, as suggested by the high similarity between the South Atlantic clones at station 25 and those at Ocean Station Papa in the Northeast subarctic Pacific (Marchetti et al. 2008, Ribalet et al. 2010). We present correlations with environmental conditions, which should be tested as hypotheses for factors that influence the relative dominance of individual *Pseudo-nitzschia* species in a single region.

Here we found that *Pseudo-nitzschia* types similar to *P. turgidula*, *P. heimii*, and *P. inflatula* were distributed in both open ocean and coastal waters, a finding that parallels the description of the ranges of these species given in Lelong et al. (2012). These species may possess flexible physiologies, enabling the species to persist despite common differences between open ocean and coastal waters, in parameters such as temperature, salinity, and nutrients. Given the prospect for global environmental change, such as warming ocean waters, we might expect that species with such flexible physiologies could thrive and expand their ranges, outcompeting species that possess more restricted ranges (and presumably, more restricted environmental tolerances). Ultimately, analysis of the environmental constraints upon *Pseudo-nitzschia* species distribution, rather than a focus on geographic distribution alone, will provide insights into environmental niches of different *Pseudo-nitzschia* types. In turn, an understanding of niche preferences can inform our understanding of the global distribution of *Pseudo-nitzschia* species, as well as predictions of *Pseudo-nitzschia* community responses to environmental

change. In addition to the abiotic environmental drivers of community composition investigated in this study and others, biotic drivers (interactions with other organisms) also have great potential to influence biogeographical patterns of *Pseudo-nitzschia.*

Compared to previous reports of *Pseudo-nitzschia* species composition in west African coastal waters, we detected markedly different species assemblages. Previously-reported species for this region included common coastal *Pseudo-nitzschia* species such as *P. australis*, *P. multiseries*, and *P. pungens* (Hasle 2002). In contrast, we did not detect either *P. australis* or *P. multiseries*, and we detected only a single clone of *P. pungens*. Thus, it is possible that either previously reported species do not dominate the northern Benguela Upwelling Zone, or that different species assemblages exhibit seasonal patterns throughout the Angola and Benguela systems, as shown for other ocean regions (Fryxell et al. 1997; Hubbard 2010; Klein et al. 2010) (Fryxell et al. 1997; Hubbard 2010; Klein et al. 2010). One piece of evidence supporting potential seasonal succession in the northern Benguela Upwelling Zone is the fact that published reports for the detection of *P. australis* within this region come from blooms in March or April, in contrast to the November-December sampling conducted as part of our study. Furthermore, based on *Pseudo-nitzschia* distribution near this region (Hasle 2002; Fawcett et al. 2007; Seeyave et al. 2009; Blanco et al. 2010) and current understanding of species toxigenicity (Lelong et al. 2012; Trainer et al. 2012), we expected to find toxigenic species such as *P. australis, P. multiseries, and P. pseudodelicatissima.* In contrast, the detection of *P. galaxiae* was somewhat unexpected, as was as its association here with high domoic acid levels. First, our detection of *P. galaxiae* was novel for African waters, as this species has previously been detected near Mexico, Australia, and in the Mediterranean (Lundholm and Moestrup 2002; Cerino et al. 2005; McDonald et al. 2007; Quijano-Scheggia et al. 2010), in addition to an unpublished report from the Sargasso Sea (as described in Hasle (2002)). Furthermore, *P. galaxiae* has not been described elsewhere as a highly toxigenic species. Previous publications

have reported either no DA (at a detection level of $9.6 * 10^{-4}$ pg DA/cell; Quijano-Scheggia et al. (2010)) or very low levels of DA produced by this species (10^{-7} to 10^{-4} pg DA/cell; Cerino et al. (2005)), many orders of magnitude lower than the DA measured for communities at stations 18, 19, and 21 (1.5 – 4.6 pg DA / cell, a calculation that presumes that all *Pseudo-nitzschia* cells were producing DA, not only *P. galaxiae* types). Clearly, there is a large disparity in reported DA production, for *P. galaxiae* cultures, in comparison to the field measurements at station 19 for a community likely dominated by *P. galaxiae*. We attribute this disparity to the likelihood that cells in the field are experiencing different environmental conditions compared to culturing conditions (i.e. different environmental triggers of DA production), in addition to strain-to-strain variability in DA production (i.e. different genetic potential for DA production).

Here for the first time, we publish evidence of potentially ecologically harmful levels of domoic acid (Scholin et al. 2000; Trainer et al. 2000) in the northern Benguela Upwelling Zone, a region that includes organisms known to be vulnerable to DA and to act as vectors of the toxin to humans. Combined with previous reports of DA outbreaks off the coasts of South Africa and Angola (Fawcett et al. 2007; Seeyave et al. 2009; Blanco et al. 2010), our findings suggest that the entire Benguela Upwelling Zone is susceptible to toxigenic *Pseudo-nitzschia* blooms. During the time frame of our study, surface currents indicated upwelling conditions throughout the sampling region, suggesting that these specific DA outbreaks would have impacted offshore organisms more heavily relative to humans consuming shellfish such as the oyster fisheries of Namibia. In contrast, downwelling-favorable conditions would likely have advected toxigenic cells towards coastal shellfish beds (Adams et al. 2000; MacFadyen et al. 2005; Adams et al. 2006). Because of the residence of offshore organisms that commonly act as vectors of DA (anchovies, sardines) or are sensitive to DA (sea lions, sea birds), the occurrence of DA in the northern Benguela Upwelling Zone is a concern for impacts on marine ecosystems. Furthermore, the moderately high levels of DA, as well as its widespread occurrence throughout

our cruise track, argue for the importance of regular shellfish monitoring in this region to safeguard human health, even if toxigenic *Pseudo-nitzschia* cells in this case were likely moving offshore away from shellfish beds.

Two hypotheses could explain the production of DA by these *Pseudo-nitzschia* communities in the northern Benguela Upwelling Zone. First, it is possible that a single species, *P. galaxiae*, caused DA outbreaks throughout the Benguela Upwelling Zone. This hypothesis is supported by our Local Similarity Analysis results, which revealed significant correlations between PDA and *P. galaxiae* OTUs 137 and 149. Indeed, given the predominance of *P. galaxiae* types at the high-DA station 19, we believe that it is likely that *P. galaxiae* was indeed producing toxin. Alternatively, different *Pseudo-nitzschia* types (and potentially more than one species per community) could have produced DA detected at different sites. This alternative hypothesis is supported by the absence of *P. galaxiae* from station 25, where low levels of DA were detected in association with two other species groups: *P. heimii* and *P. turgiduloides / P. turgidula*. However, according to previous lab studies, neither *P. heimii* (Marchetti et al. 2008) nor *P. turgiduloides* (Lelong et al. 2012) have produced detectable levels of DA. Cultures of *P. turgidula* have been reported to produce a range of DA from $5.2 * 10^{-6}$ pg DA / cell (Trick et al. 2010) to $9 * 10^{-2}$ pg DA / cell (Bill 2011), orders of magnitude lower than highly toxigenic species *P. multiseries*, *P. australis*, and *P. seriata* (as reviewed by Trainer et al. (2012). Other assessments of DA production by *P. cf. turgidula* did not detect the toxin (Marchetti et al. 2008). Depending on the number of *Pseudo-nitzschia* cells at station 25, our data could suggest that in fact, these “open-ocean/coastal” types could be capable of moderately high toxin production. Certainly our findings highlight the fact that the toxigenicity of known *Pseudo-nitzschia* species has not been fully described. Consequently, if HAB monitoring efforts were to depend on the current understanding of species toxigenicity to predict bloom severity, potentially harmful DA outbreaks would likely be overlooked.

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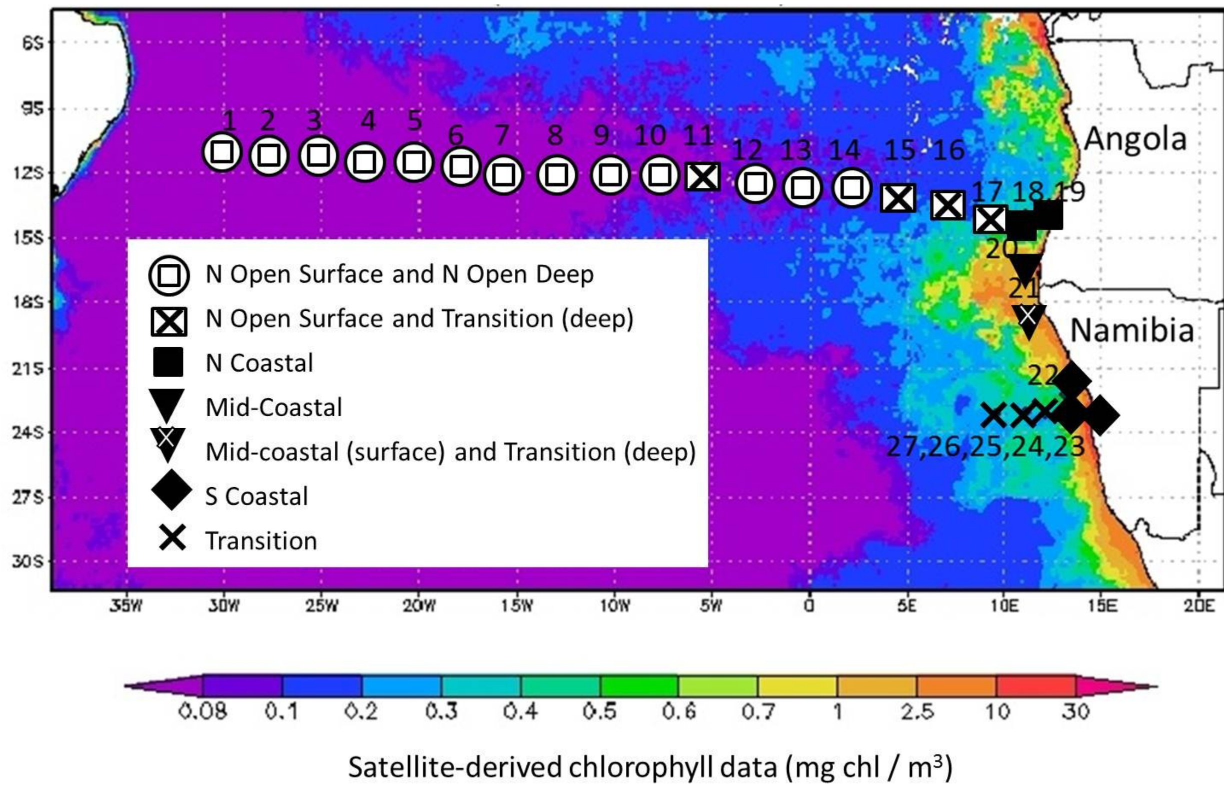

Figure 2.1. *R/V Knorr* 192-05 cruise stations overlain on satellite-derived chlorophyll data (mg chlorophyll / m^3) obtained using the Giovanni online data system, averaged for the period of November – December 2007. Surface and deep waters at each station are categorized according to significantly different environmental regimes based upon data shown in Figure 2.2. In the legend above, "N" signifies "North" and "S" signifies "South."

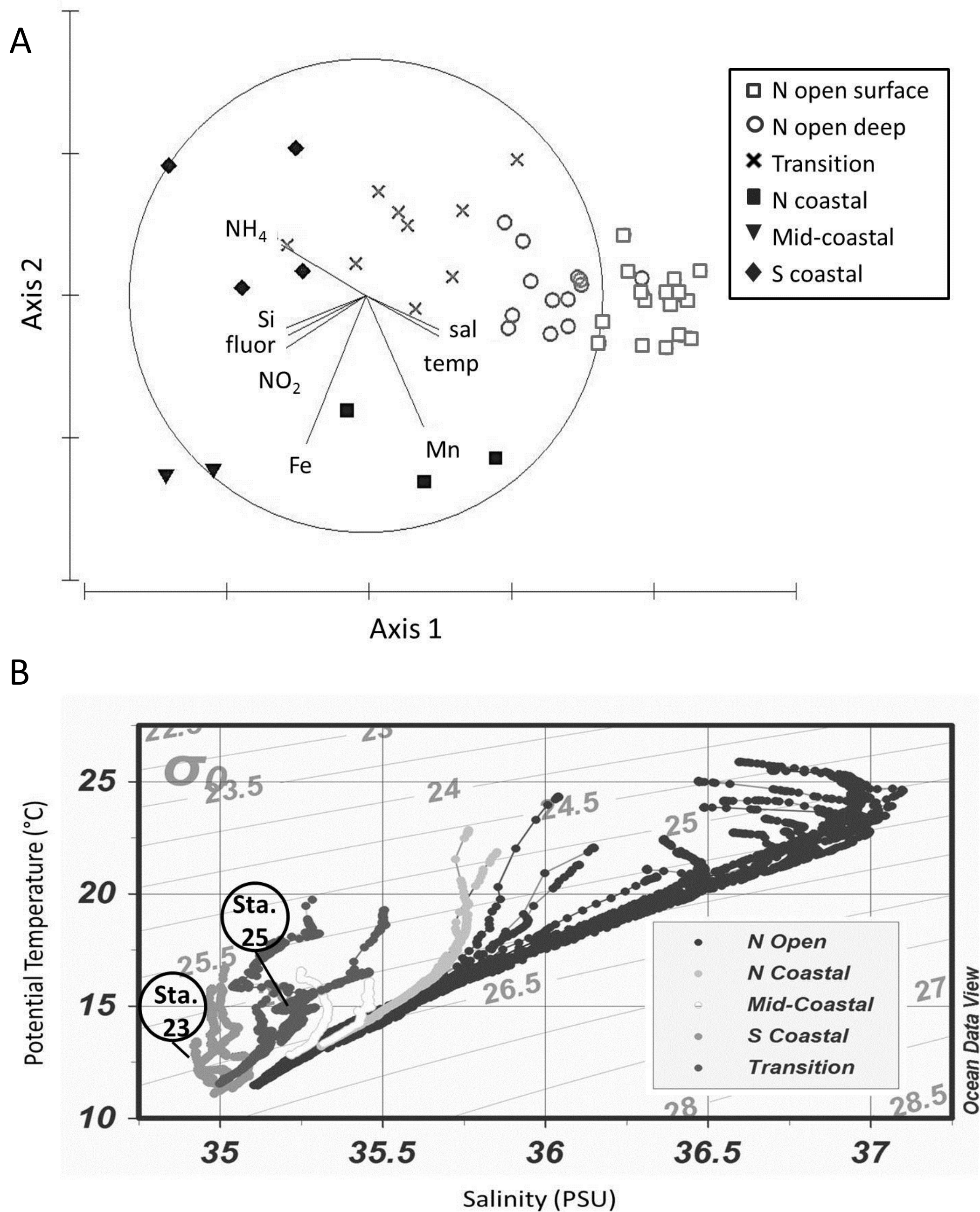

Figure 2.2. Identification of six significantly different environmental regimes in the South Atlantic Ocean; in regime names, "N" signifies "North" and "S" signifies "South." Panel A shows quantitative clustering and similarity profile (SIMPROF) test (p=0.05) overlain on Principal Components Analysis (PCA), for surface and deep chlorophyll maxima (DCM) samples from stations 1-22, 24, 26-27. In addition to nutrients and metals represented by standard symbols, abbreviations include "sal" (salinity), "temp" (temperature), and "fluor" (chlorophyll a fluorescence). Panel B shows temperature-salinity plots for the upper 200 meters of the water column at stations 1-27, in which individual dots represent data points and density isopycnals are shown in gray behind the data. Here, Station 23 ("Sta. 23") and Station 25 ("Sta. 25") were categorized as belonging to "S Coastal" and "Transition" regimes, respectively.

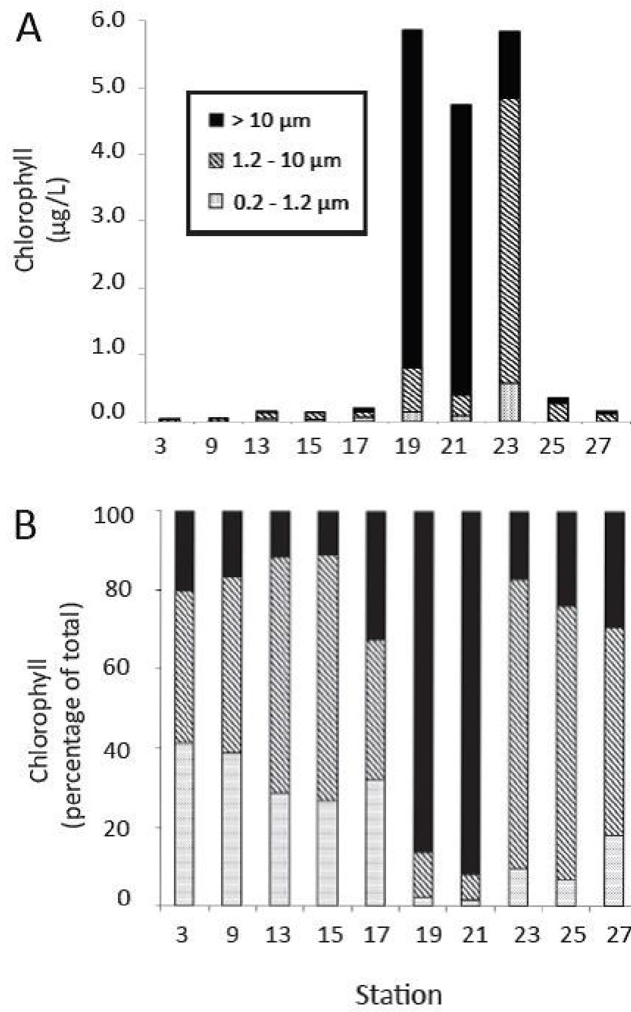

Figure 2.3. Surface water size-fractionated chlorophyll a data, as determined by acetone extractions, in each of three fractions (> 10 µm, 1.2 – 10 µm, and 0.7 – 1.2 µm), expressed as total chlorophyll (µg/L; Panel A) and relative chlorophyll (Panel B).

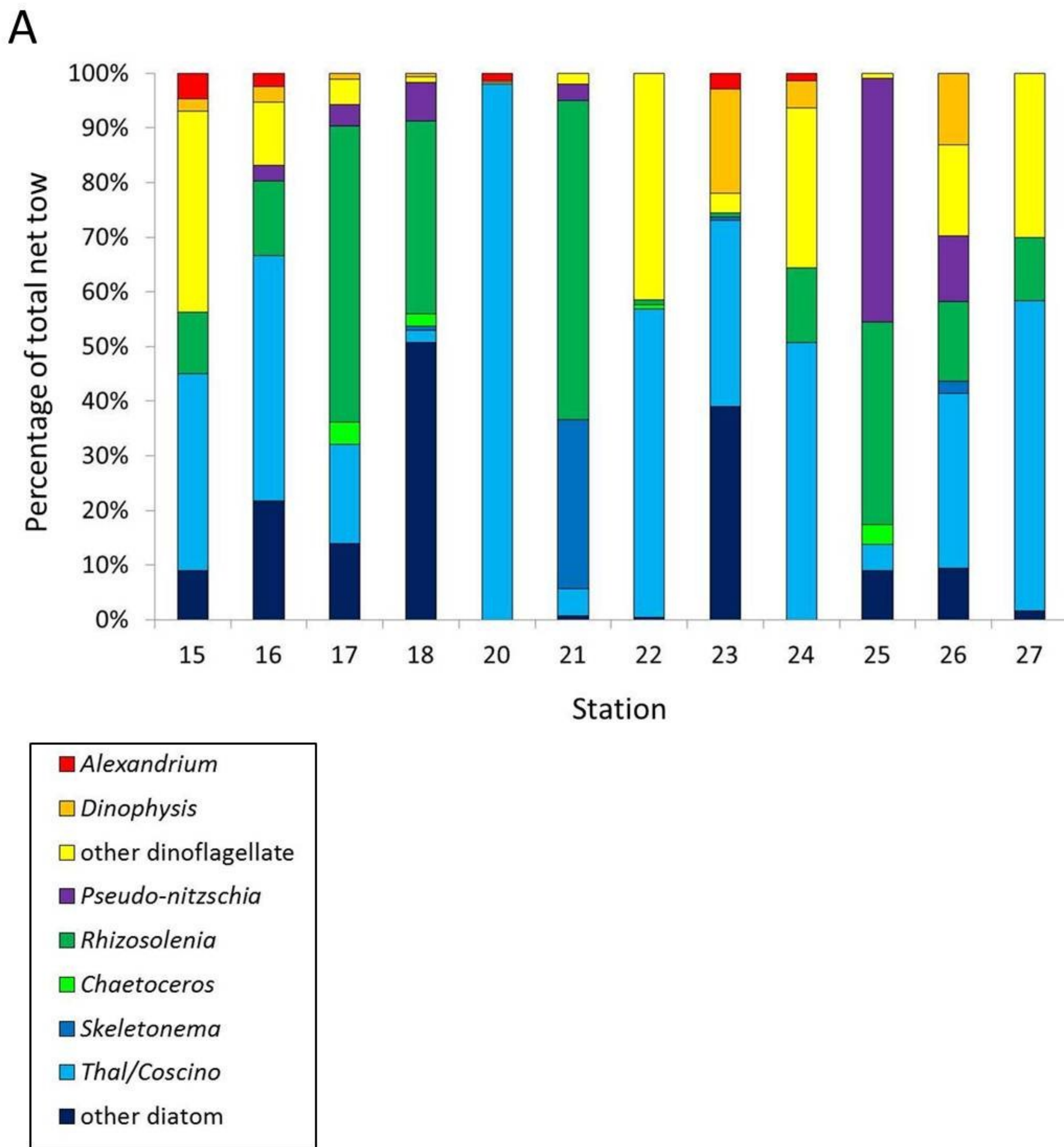

Figure 2.4. Phytoplankton community composition in South Atlantic surface waters, as determined by light microscopy. Panel A shows net tow data for twelve stations, showing proportions of total dinoflagellate and diatom cells identified to genus level. Panel B shows whole water data for three stations, showing cells per liter of seawater identified as dinoflagellates, distinct genera of diatoms, and three morphological types of *Pseudo-nitzschia* (*P. pseudodelicatissima/P. delicatissima* type, or "*P. pseudo/deli*;" *P. australis/P. fraudulenta/P. heimii* type, or "*P. australis/fraud*;" and *P. pungens/P. multiseries* type, or "*P. pungens/multi*"). In both panels, "*Thal/Coscino*" describes cells that were centric diatoms belong to the genera *Thalassiosira* or *Coscinodiscus*.

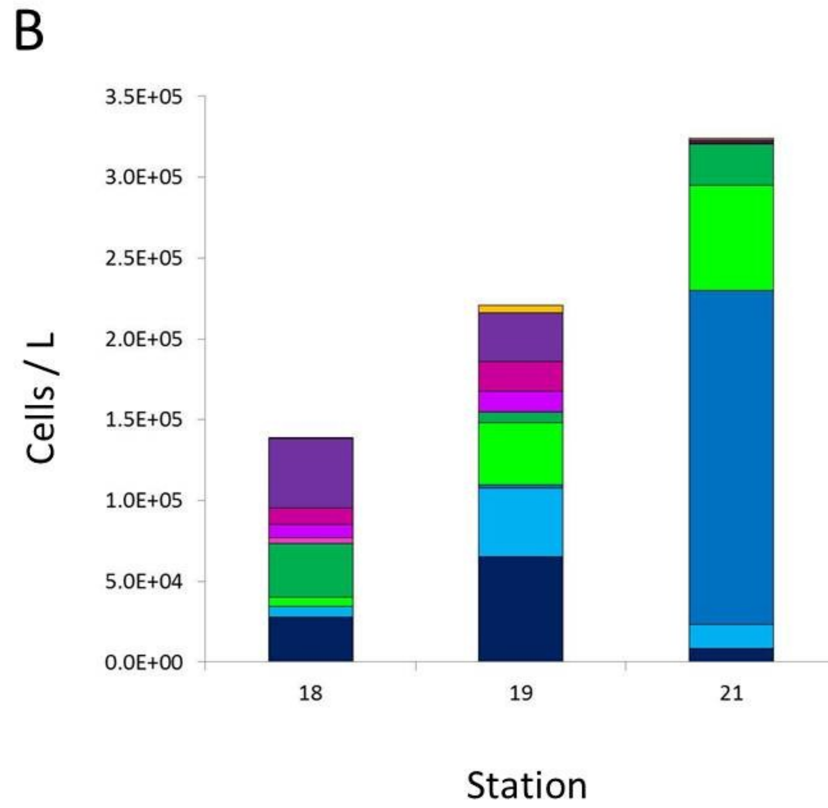

Figure 2.4, continued.

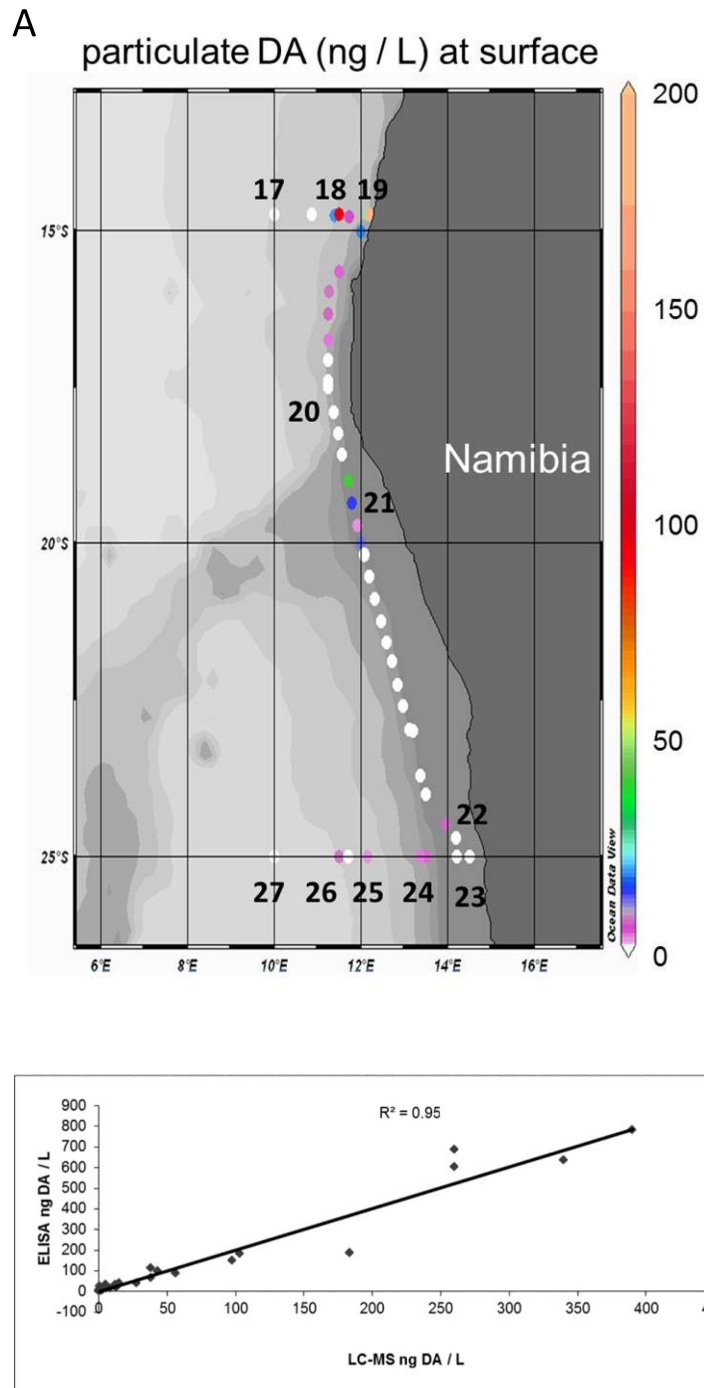

Figure 2.5. Particulate domoic acid (DA) levels detected in surface waters at numbered cruise stations and underway points, as analyzed by Enzyme-Linked ImmunoSorbent Assay (ELISA) (Panel A). Panel B shows correlation between ELISA and liquid chromatography – mass spectrometry (LC-MS) data for subset of samples.

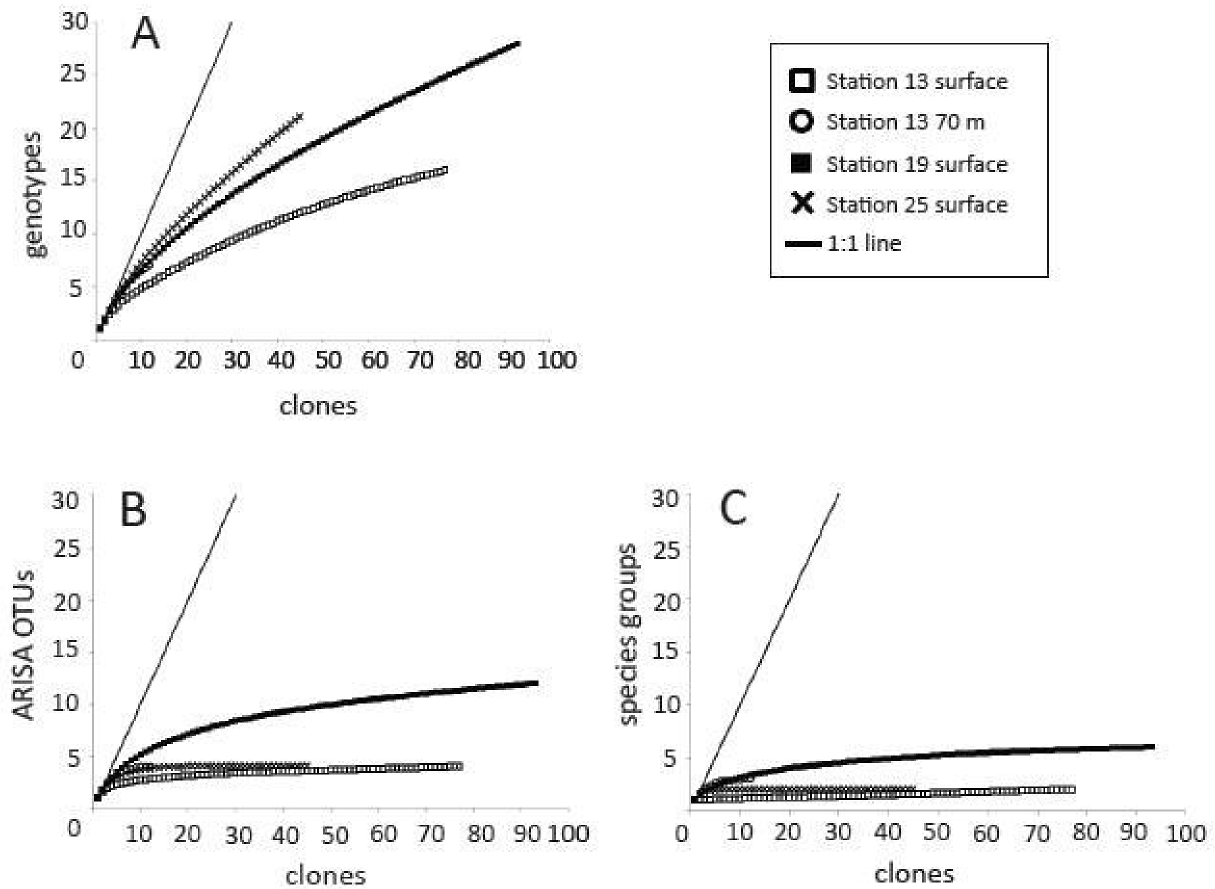

Figure 2.6. Rarefaction curves constructed for four partial internal transcribed spacer 1 (ITS1) clone libraries, plotted according to increasing level of genotypic difference, including number of distinct genotypes (Panel A); number of Automated Ribosomal Intergenic Spacer Analysis (ARISA) operational taxonomic units (OTUs), or distinct ARISA peaks (Panel B), and number of species-groups, as defined by <18% nucleotide divergence (Panel C).

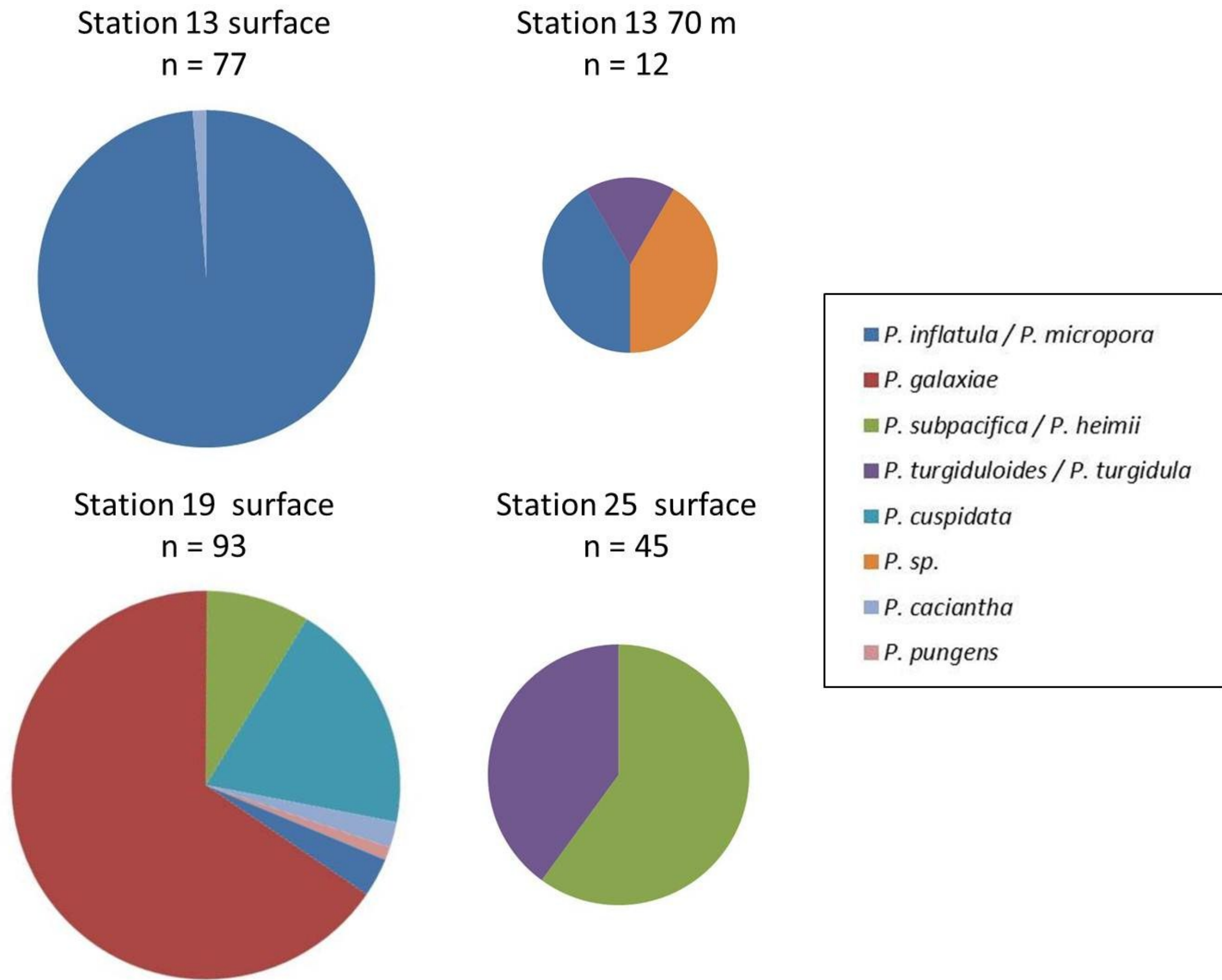

Figure 2.7. Species composition of four partial internal transcribed spacer 1 (ITS1) clone libraries, plotted according to number of clones per library (n) that were identified according to eight different *Pseudo-nitzschia* species-groups (as defined by < 18% nucleotide divergence).

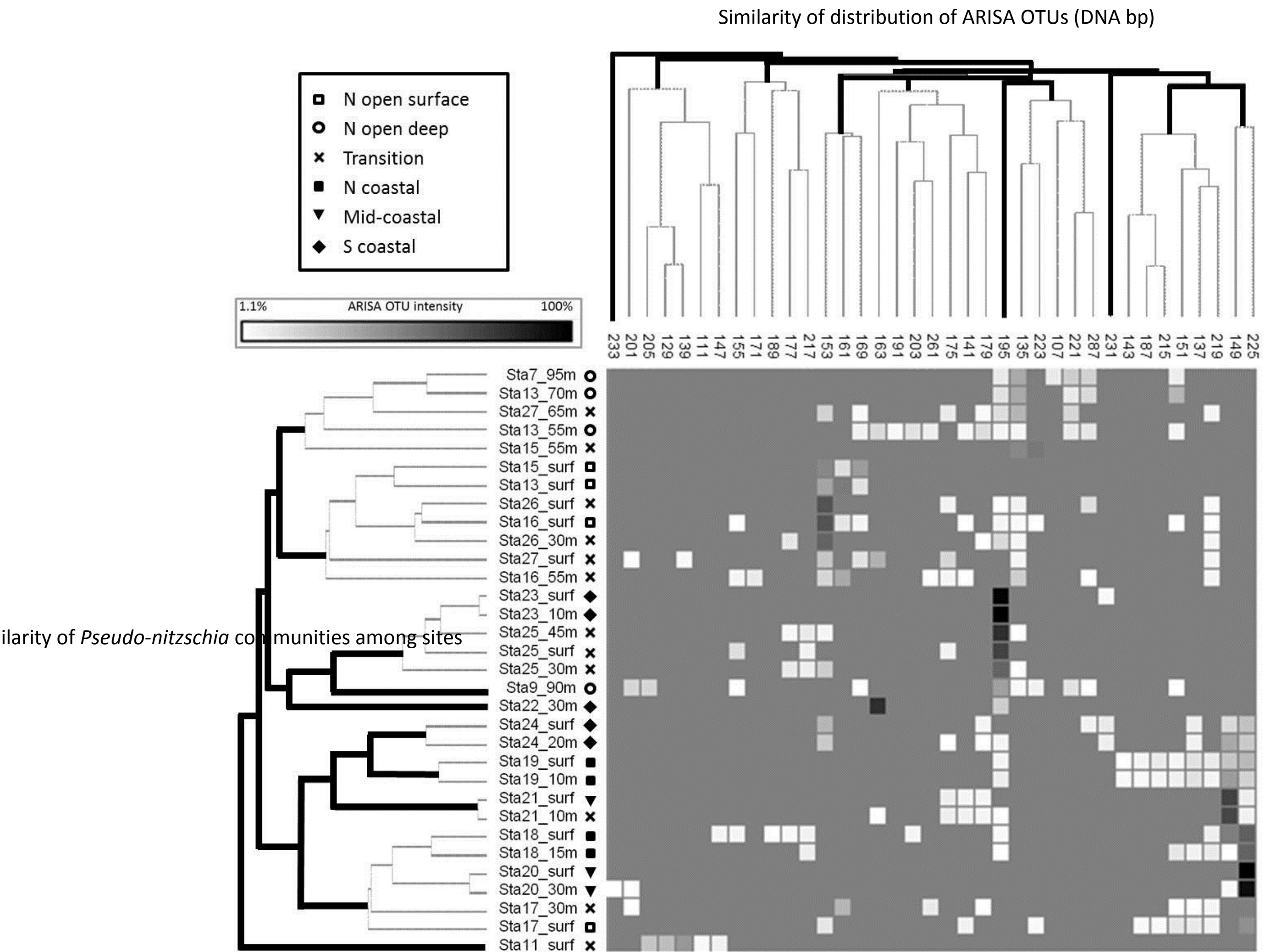

Figure 2.8. *Pseudo-nitzschia* community composition (determined by Automated Ribosomal Intergenic Spacer Analysis, or ARISA) analyzed by quantitative clustering dendrogram using Bray-Curtis similarity, for South Atlantic stations (e.g. "Sta7" = station 7) and specific depths sampled (e.g. "95m" = deep chlorophyll maximum at 95 meters depth; or "surf" = surface waters.). Light gray branches of dendrograms indicate samples that did not differ significantly, as determined by similarity profile (SIMPROF) analysis, whereas thick black branches indicate samples that differed significantly. Heat map (generated using tool at http://www.chibi.ubc.ca/matrix2png/bin/matrix2png.cgi) shows relative contribution of each ARISA fragment (DNA base pairs, or bp), such that darker coloring indicates greater relative intensity of ARISA OTU within each individual community. Communities are coded according to one of six environmental regimes: North (N) open surface, North (N) open deep, Transition, North (N) coastal, Mid-coastal, and South (S) coastal.

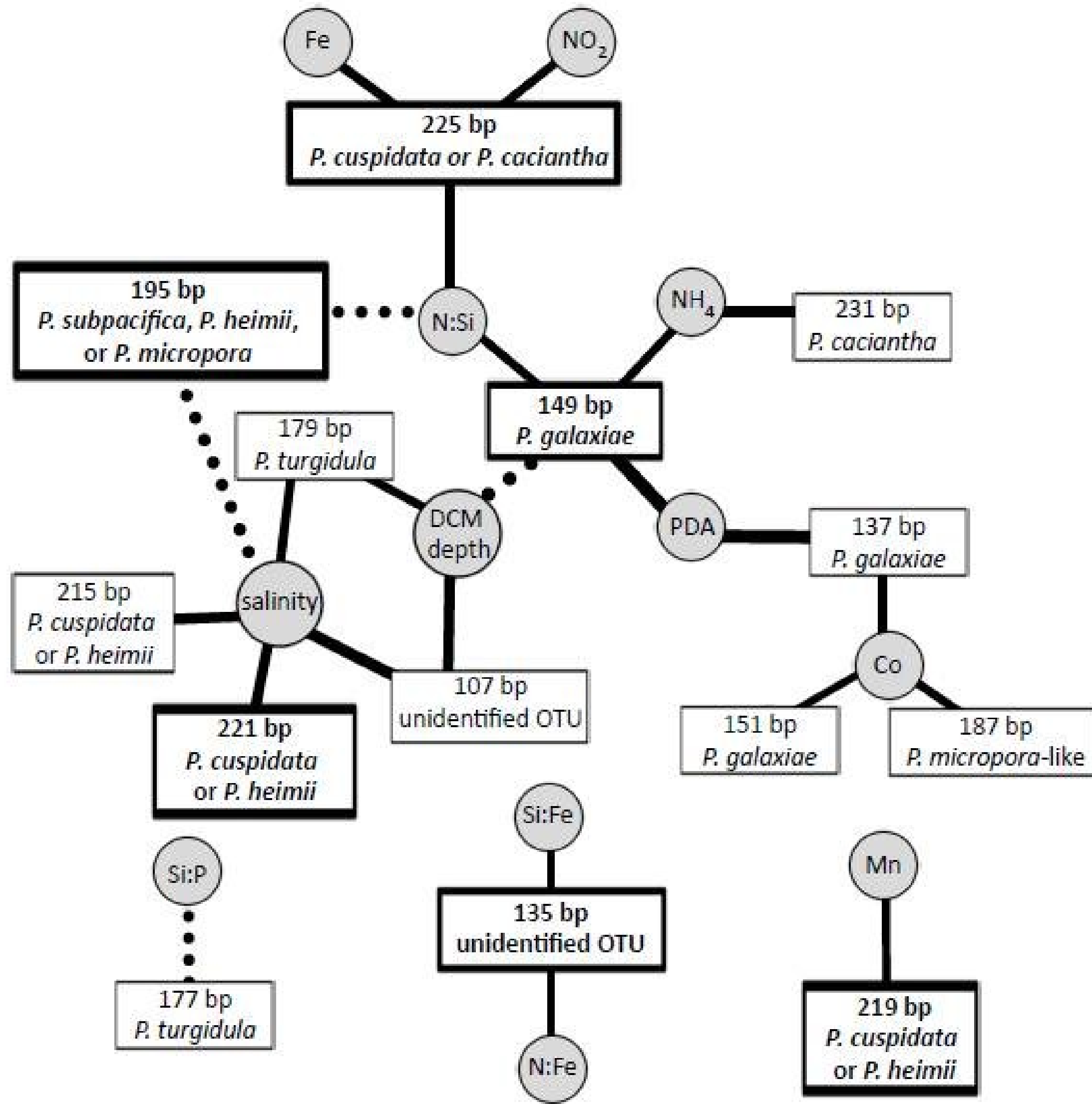

Figure 2.9. Local Similarity Analysis (LSA) results describing significant ($p < 0.025$) nonlinear correlations between environmental parameters and *Pseudo-nitzschia* types (identified by putative species identifications and Automated Ribosomal Intergenic Spacer Analysis, or ARISA, fragment length in DNA base pairs, or bp). The width of connecting lines indicates the magnitude of the LSA correlation, whereby thicker lines indicate stronger correlations. Positive correlations are denoted by solid lines; negative correlations are denoted by dotted lines. Biogeographically important *Pseudo-nitzschia* types (as determined by similarity percentage analysis, Table 2.1) are outlined here with bold rectangles. In addition to nutrients and metals represented by standard symbols, “DCM depth” denotes the depth of deep chlorophyll maximum and “PDA” denotes particulate domoic acid.

Table 2.1. Identification of putative *Pseudo-nitzschia* species in four clone libraries from Station 13 surface waters (S13), Station 13 70 meters depth (S13 70m), Station 19 surface waters (S19), and Station 25 surface waters (S25), along with predicted Automated Ribosomal Intergenic Spacer Analysis (ARISA) fragment lengths (DNA base pairs, or bp). Clones are categorized according to most similar species-group compared to closest BLAST (Basic Local Alignment Search Tool; GenBank) hit, as defined by < 18% nucleotide divergence without adjusting for the presence of insertions or deletions. Species-groups are listed in order of decreasing frequency of detection among the clone libraries. Reference sequences were derived from electron-microscopy-verified species, except for those described as “clone,” which represents a sequence from an environmental clone library. In the cases where the most similar reference sequence was an environmental clone, the most similar sequence from an isolate is also presented. Unique sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers JX441017-JX441088. Table excludes three *Pseudo-nitzschia* sequences that possessed > 18% nucleotide divergence compared to closest BLAST hit. These sequences, represented by a total of five clones, were detected at Station 13 70 meters and predicted to generate ARISA fragment lengths of 222 or 223 bp.

Species group (based on closest BLAST hits)	ARISA fragment lengths (bp)	Community (number of clones/ number of genotypes)	Closest reference sequence and predicted ARISA fragment length (range of nucleotide divergence)	Origin of reference
P. inflatula/ P. micropora (n=84)	154*, 157* 163*, 164* 170*, 171* 188* 197*	S13 surface (26/4) S13 surface (44/9) S13 70 m (2/1) S13 surface (6/2) S19 surface (3/1) S13 70 m (3/2)	*P. inflatula*, DQ329204, 156 bp (0 – 1.7%) *P. inflatula*, DQ329204, 156 bp (0 – 1.8%) *P. inflatula*, DQ329204, 156 bp (0.8%) *P. micropora*, AY257847, 146 bp (6.8%) *P. micropora*, AY257847, 146 bp (12%)	Thailand Thailand Thailand Vietnam Vietnam
P. galaxiae (n=63)	138* 149, 150*, 151, 152* 156*	S19 surface (22/4) S19 surface (40/8) S19 surface (1/1)	*P. galaxiae*, DQ336158, 143 bp (9.7 – 11.9%) *P. galaxiae*, EU327368, 149 bp (0 – 13.6%) *P. galaxiae*, EU327368, 149 bp (8.9%)	Australia Spain Spain
P.cf. subpacifica / *P. heimii* / *P. sp.* environmental samples (n=35)	195 196* 217*, 218*, 219*, 221*	S19 surface (8/2) S25 surface (19/5) S25 surface (8/5)	*P. cf. subpacifica*. AY257858, 195 bp (0 - 0.6%) *P. sp.* clone, EU068693, 195 bp (5.6 - 7.5%), or *P. heimii*, EU051655, 195 bp (6.2 - 8.1 %) *P. sp.* clone, EU068693, 195 bp (5.6 - 9.3%), or *P. heimii*, EU051655, 195 bp (6.2 – 9.9 %)	Portugal NE Pacific NE Pacific NE Pacific NE Pacific
P. turgiduloides / P. turgidula (n=20)	155* 176, 177*, 178*, 179*	S25 surface (12/5) S25 surface (6/6) S13 70 m (2/1)	*P. sp.* clone, EU068695, 176 bp (5.1 – 6.7%) or *P. turgiduloides*, AY257839, 175 bp (8.2 – 9.1%) *P. cf. turgidula*, EU051653, 151 bp (12 – 17%) or *P. sp.* clone, EU068696, 176 bp (0.7 – 19.4%)	NE Pacific Antarctica NE Pacific NE Pacific
P. cuspidata (n=18)	216, 220* 226*	S19 surface (10/5) S19 surface (8/4)	*P. cuspidata*, AY257852, 216 bp (0.6 – 2.8%) *P. cuspidata*, AY257862, 230 bp (1.6 – 2.7%)	Mexico Australia
P. caciantha (n=3)	225* 229*, 230*	S19 surface (1/1) S13 surface (1/1) S19 surface (1/1)	*P. caciantha*, AY257861, 226 bp (0%) *P. caciantha*, DQ813834, 227 bp (4.7%)	Mexico Italy
P. pungens (n=1)	144	S19 surface (1/1)	*P. pungens*, EU327366, 142 bp (5.65%)	Spain

Table 2.2. Analysis of similarity (ANOSIM) statistics testing hypothesis that *Pseudo-nitzschia* community composition (based on Automated Ribosomal Intergenic Spacer Analysis, or ARISA) differed significantly according to each of six significantly different environmental regimes. Gray-shaded panels highlight significant differences (p value > 0.15 and R value > 0.2).

	Semi-quantitative (Bray-Curtis similarity)		Presence-absence (Sorensen similarity)		Both analyses
Groups	**R value**	**p value**	**R value**	**p value**	**# permutations**
Global test (all groups)	0.288	0.001	0.471	0.001	999
Pairwise comparisons	--	--	--	--	--
N open surface, N open deep	0.575	0.024	0.434	0.040	126
N open surface, Transition	0.193	0.094	0.393	0.012	999
N open surface, N coastal	0.456	0.016	0.353	0.024	126
N open surface, Mid-coastal	0.579	0.036	0.585	0.036	56
N open surface, S coastal	0.244	0.095	0.416	0.016	126
N coastal, N open deep	0.885	0.029	0.995	0.029	35
N coastal, Transition	0.373	0.012	0.514	0.005	999
N coastal, Mid-coastal	0.278	0.114	0.972	0.029	35
N coastal, S coastal	0.288	0.079	0.503	0.040	126
Mid-coastal, N open deep	0.870	0.028	0.981	0.029	35
Mid-coastal, Transition	0.522	0.008	0.647	0.008	364
Mid-coastal, S coastal	0.282	0.054	0.451	0.036	56
Transition, S coastal	0.070	0.270	0.411	0.010	999
Transition, N open deep	0.125	0.214	0.303	0.038	999
S Coastal, N open deep	0.163	0.222	0.484	0.040	126

Table 2.3. Specific *Pseudo-nitzschia* ARISA OTUs, with putative species identifications, that were dominant among whole *Pseudo-nitzschia* communities at each of six different environmental regimes, as identified by similarity percentage (SIMPER) analysis (for OTUs contributing up to 70% or more of similarity among each community) applied to semi-quantitative data (Bray-Curtis similarity). Putative species identifications (ID) are given according to match with clone library identifications (Table 2.1). OTU 135 exhibited "no significant similarity" to existing sequences in GenBank. In regime names, "North" is abbreviated as "N" and "South" is abbreviated as "S."

Environmental regime	OTU (bp)	Putative species ID	Contribution to similarity	Cumulative contribution
N open surface	153	*P. inflatula* or *P. turgidula*	70.2	86.3
	161	*P. inflatula*	16.1	
N open deep	221	*P. heimii* or *P. cuspidata*	26.5	71.1
	135	No significant similarity	23.8	
	195	*P. micropora* or *P. subpacifica* / P. heimii	20.8	
Transition	153	*P. turgidula* or *P. inflatula*	32.8	76.7
	195	*P. heimii / P. subpacifica* or *P. micropora*	29.1	
	135	No significant similarity	14.8	
N coastal	225	*P. cuspidata* or *P. caciantha*	53.9	75.1
	219	*P. cuspidata* or *P. heimii*	12.3	
	149	*P. galaxiae*	8.9	
Mid-coastal	225	*P. cuspidata* or *P. caciantha*	96.9	96.9
S coastal	195	*P. heimii / P. subpacifica* or *P. micropora*	65.2	74.9
	225	*P. cuspidata* or *P. caciantha*	9.7	

CHAPTER THREE

Molecular phylogeny of the marine diatom *Pseudo-nitzschia:* implications for physiology, morphology and biogeography

ABSTRACT

Pseudo-nitzschia is a cosmopolitan genus of marine diatoms that is significant in its contributions to global nutrient cycling and its formation of Harmful Algal Blooms (HABs). Previous work has suggested the existence of two major clades. We sought to integrate phylogeny, morphology, physiology and biogeography of *Pseudo-nitzschia*, by re-examining the phylogeny of *Pseudo-nitzschia*, creating a synthesis with major morphological and physiological features, and interpreting species distributions in the context of putative clades. We constructed phylogenetic trees based upon nuclear- and chloroplast-encoded markers: respectively, the hypervariable D1-D3 region of the ribosomal RNA (rRNA) large subunit (LSU), or 28S rRNA (25 species) and the *rbcL* gene, which encodes the large subunit of the carbon fixation enzyme, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) (14 species). The branching order of many species could not be resolved using the highly conserved LSU marker; thus we propose further elucidation of *Pseudo-nitzschia* phylogeny utilizing the *rbcL* gene. Based upon *rbcL* phylogeny, we observed that members of two clades varied in cell size, silica frustule structures (striae to fibulae ratio and central interspace), and chlorophyll c pigments. In multiple regions of the world's oceans, these two putative clades tend to exhibit seasonal variations, alternating between Clade I (corresponding to smaller "*delicatissima*" group; spring) and Clade II

(corresponding to the larger "*seriata*" group; summer/fall). As suggested by morphological differences, we hypothesize that Clades I and II inhabit distinct environmental niches.

INTRODUCTION

The cosmopolitan marine diatom genus *Pseudo-nitzschia* is ecologically significant in terms of its contributions to global nutrient cycles and formation of Harmful Algal Blooms (HABs).Through the production of the neurotoxin, domoic acid (DA), *Pseudo-nitzschia* is responsible for Amnesic Shellfish Poisoning in humans (Bates et al. 1989) as well as severe impacts on a diverse array of marine organisms (as reviewed by Trainer et al. (2012)). In the environment, DA outbreaks likely arise from both genetic encoding of the ability to produce DA as well as environmental triggers. Despite much research into the distributions and toxigenicity of individual *Pseudo-nitzschia* species, little work has examined broader-scale patterns of *Pseudo-nitzschia* phylogeny and how such patterns might influence temporal and spatial distributions of the genus.

Pseudo-nitzschia species have been described most thoroughly in terms of cell size, silica frustule characteristics, and toxigenicity. Perhaps the most fundamental division of *Pseudo-nitzschia* species is the difference in cell size between two groups named for the species that exemplify each one. Specifically, members of the "*delicatissima*" group are smaller cells, with cell width less than 3 μm, whereas members of the "*seriata*" types are larger cells, with cell width greater than 3 μm (Hasle and Syvertsen 1997). Additionally, *Pseudo-nitzschia* species are identified based upon morphological features in the silica frustule, visible via electron microscopy (EM). For example, one species-defining character is the number of poroids, gaps in the frustule that connect the interior of the cell with the exterior surroundings (Lelong et al.

2012). The number of striae (columns of poroids or similar features) and fibulae (silica structures within the raphe) and their ratio are also diagnostic (Amato et al. 2007). In terms of physiology, most research has investigated the ability of *Pseudo-nitzschia* species to produce DA. Certain *Pseudo-nitzschia* species, such as *P. australis* (Scholin et al. 2000) and *P. multiseries* (Bates et al. 1989) have been reported to produce particularly high levels of the toxin. However, strains of the same species can vary widely in DA production (Thessen et al. 2009). Similarly, considerable intraspecific diversity exists for environmental tolerances across ranges of salinity (Thessen et al. 2005), and temperature and nutrient preferences (Thessen et al. 2009). In contrast, recent work by Zapata et al. (2011) showed cohesion in the presence or absence of three chlorophyll c pigments across strains of the same *Pseudo-nitzschia* species.

Several markers have been used for phylogeny and identifications of *Pseudo-nitzschia* species. First, the hypervariable region (D1-D3) of the large subunit (LSU) ribosomal DNA (rDNA) was initially utilized to identify species upon which molecular probes were developed (Miller & Scholin 1996). This marker has also been used to construct the most comparatively complete *Pseudo-nitzschia* phylogenetic analyses to date. Specifically, Lundholm et al. (2002a) suggested that 12 *Pseudo-nitzschia* species could be grouped into two clades: Clade I, which included *P inflatula*, *P. delicatissima*, *P. micropora*, *P. pseudodelicatissima*, *P. subpacifica*, *P. subfraudulenta*, and *P. fraudulenta* (mostly“*delicatissima*” group species) and Clade II, which included *P. pungens*, *P. multiseries*, *Nitzschia americana*, *P. australis*, *P. seriata*, and *P. multistriata* (mostly “*seriata*” group species). However, the proposed “Clade I” was not a monophyletic group. Since the proposal of these LSU clades, additional studies have constructed LSU phylogenies for newly defined species, indicating that Clade I also contains *P. galaxiae* (Lundholm and Moestrup 2002) and that Clade II contains *P. americana, P. brasiliana, and P. linea* (Lundholm et al. 2002b) In comparison to the LSU, *rbcL*, the gene encoding the large subunit of the carbon fixation enzyme, ribulose-1,5-bisphosphate carboxylase oxygenase

(RuBisCO), is likely less conserved. Among *Pseudo-nitzschia* (Ghiron et al. 2008; Casteleyn et al. 2009; Lundholm et al. 2012) and other diatoms (MacGillivary & Kaczmarska 2011), the *rbcL* marker has been used for species identifications, suggesting that it may be informative for broader-level taxonomic groupings as well. In addition, considerable work on the highly variable internal transcribed spacers (ITS1; e.g. Hubbard et al. (2008) and microsatellites (e.g. Evans et al. (2004)) have been critical to exploring microdiversity within the genus, as shown in Chapter 2 and other work (Casteleyn et al. 2009; Ribalet et al. 2010). By combining both nuclear-encoded (LSU rDNA) and chloroplast-encoded (*rbcL*) markers, we may obtain a more comprehensive understanding of diatom evolution (Alverson 2009). In addition, studies which have combined functional and nonfunctional ribosomal RNA regions by constructing phylogenies based on the ITS1-5.8S rDNA-ITS2 region (Lundholm et al. 2006) could inform finer-scale patterns among *Pseudo-nitzschia* clades.

The goal of the present study was to integrate *Pseudo-nitzschia* phylogeny, morphology, physiology, and biogeography. First, we re-examined *Pseudo-nitzschia* phylogeny, utilizing two genetic markers (LSU and *rbcL*). Second, we compared the resulting phylogeny to existing knowledge of physiological and morphological patterns within the *Pseudo-nitzschia* genus. Based upon our preliminary findings in comparison with reported geographic distributions of *Pseudo-nitzschia*, we further propose the hypothesis that two major *Pseudo-nitzschia* clades possess specialized physiological capabilities, which are manifested in distinct temporal and spatial patterns.

METHODS

***Pseudo-nitzschia* clades: construction of LSU and *rbcL* phylogenies.** *Pseudo-nitzschia* and outgroup member sequences for the LSU hypervariable D1-D3 region and the *rbcL* gene were downloaded from NCBI GenBank on June 6, 2012. For both *rbcL* and LSU trees, outgroup members included raphid and araphid pennate diatoms, with members of the same species or genus in both analyses.

To construct the LSU tree, 823 characters were examined from the hypervariable D1-D3 region, for 25 species. Sequences were aligned using a reference alignment kindly provided by Nina Lundholm, utilized for analyses published in Lundholm et al. (2012). For *rbcL* analyses, amino acid sequences were aligned using ClustalX and backtranslated to nucleotides in BioEdit. Primary *rbcL* analyses employed 1215 characters using all nucleotide positions, for 14 species. Additional analyses were done to include species with shorter sequence coverage, including *P. linea* (692 characters) and *P. subcurvata* (539 characters). We also analyzed a second version of the full length *rbcL* alignment, using only first and second positions in each codon (810 characters).

Phylogenetic trees were constructed in PAUP (Swofford 2003). Distance trees were inferred using minimum evolution (ME) as the objective criterion and paralinear (logdet) distances. Maximum likelihood (ML) analyses used the HKY85 model of nucleotide substitution with rate heterogeneity and empirical nucleotide frequencies. The gamma shape parameter and the transition/transversion ratio were initially estimated from a distance topology and refined by iterative likelihood searches. Distance bootstrap analyses (100 resamplings) were performed with heuristic searches utilizing random addition and tree-bisection reconnection (TBR) branch-swapping methods. Likelihood bootstrap analyses (100 resamplings) were performed with

heuristic searches and tree-bisection reconnection (TBR) branch-swapping methods starting from neighbor joining tree. Phylogenetic trees were visualized with FigTree v1.3.1.

***Pseudo-nitzschia* clades: synthesis of phylogeny, morphology, and physiology.** We conducted a literature survey to identify characters that could inform the physiological and ecological significance of the clade designations based on trees conducted in this study. Of the existing literature on *Pseudo-nitzschia*, we chose eight characters which have been described for most of the 26 *Pseudo-nitzschia* species included in our phylogenetic analyses. First, we reviewed *Pseudo-nitzschia* phylogeny described elsewhere, for the LSU (Lundholm and Moestrup 2002; Lundholm et al. 2002a) and for the ITS1-5.8S-ITS2 region (Lundholm et al. 2006). Morphological characters included categorization into two morphological groups, based on Hasle and Syvertsen (1997) as summarized in Lelong et al. (2012): the "*delicatissima"* group (small cells with width <3 μm) and the "*seriata*" group (large cells with width >3 μm). We also considered two major features of the silica frustule which are widely reported as part of species identifications: the relative abundance of striae to fibulae, and presence/absence of a central interspace in the raphe. To survey known physiology of *Pseudo-nitzschia*, we considered known toxigenicity (as presence/absence (Lelong et al. 2012) and highest reported DA value (Trainer et al. 2012)) and chlorophyll c pigments (Zapata et al. 2011). Characters that varied across *Pseudo-nitzschia* species included in the primary *rbcL* trees were then mapped onto the topology of the *rbcL* tree based upon all nucleotide positions.

RESULTS

Pseudo-nitzschia clades: LSU and *rbcL* phylogenies

The LSU phylogenetic analysis, constructed from 823 characters of the D1-D3 hypervariable region, comprised 25 species, including nine species that were not previously identified as such or analyzed in Lundholm et al. (2002a) or Lundholm and Moestrup (2002). These nine species included *P. turgiduloides*, *P. heimii*, *P. lineola*, *P. linea* (Lundholm et al. 2002b), *P. decipiens* and *P. dolorosa* (previously categorized within the cryptic *P. delicatissima* complex; (Lundholm et al. 2006)), and *P. calliantha*, *P. caciantha,* and *P. mannii* (all previously categorized within the cryptic *P. pseudodelicatissima* complex; Lundholm et al. (2003); Amato and Montresor (2008)).

The LSU tree (Figure 3.1) did not support the existence of large *Pseudo-nitzschia* clades. Although the species *P. delicatissima*, *P. micropora*, and *P. dolorosa* clustered together, we did not find evidence for a larger Clade I, which was previously proposed (Lundholm and Moestrup 2002; Lundholm et al. 2002a) to also include *P. subpacifica*, *P. galaxiae*, *P. inflatula*, *P. pseudodelicatissima*, *P. fraudulenta*, and *P. subfraudulenta* (Table 3.1). The LSU tree supported the existence of *Pseudo-nitzschia* Clade II, which includes seven species previously described by (Lundholm et al. 2002a) as Clade II members: *P. brasiliana*, *P. multistriata*, *P. seriata*, *P. australis*, *P. pungens*, *P. multiseries*, and *P. americana* (Table 3.1), as well as *P. linea* (present study). However, overwhelmingly, the LSU tree exhibited low bootstrap support throughout the deepest nodes, which did not allow us to convincingly identify clades comprising more than three species.

In contrast, phylogenetic analyses of the *rbcL* gene (Figure 3.2), which included 14 of the species analyzed for LSU phylogeny (Figure 3.1), supported the existence of two *Pseudo-nitzschia* clades. Clade I members included *P. delicatissima*, *P. dolorosa*, *P. pseudodelicatissima*, *P. cuspidata*, and *P. galaxiae*. Clade II was weakly supported by the *rbcL*

tree (Figure 3.2), comprising *P. americana*, *P. brasiliana*, *P. multistriata*, *P. pungens*, *P. fraudulenta*, *P. caciantha, P. mannii*, and *P. calliantha*. Both the ME and ML trees exhibited strong support for the existence of a clade with similar membership to previously proposed Clade I members (Lundholm et al. 2002a). In addition, we found similar members of Clade II, with the exception of *P. caciantha*, *P. mannii*, and *P. calliantha* included as Clade II members in our analysis.

Additionally, we performed two additional analyses using fewer characters of the *rbcL* gene in order to determine clade affiliations of *P. linea* and *P. subcurvata (*data not shown). In both *rbcL* trees (ME and ML; 692 characters), *P. linea* always clustered with *P. americana* (Clade II) with bootstrap support of 99 (ME) or 96 (ML). In contrast, *P. subcurvata* (539 characters) could not be categorized as a member of either Clade I or Clade II, similar to placement of *P. turgiduloides*.

In another set of analyses of the *rbcL* gene, we considered nucleotides in the first and second positions. The overall topology of this tree (not shown) was similar to the tree constructed using all nucleotide positions (Figure 3.2). However, the tree based upon the first and second nucleotides exhibited very low bootstrap support at many of the deep nodes. Both versions of the *rbcL* tree included the same species in Clades I and II with bootstrap support exceeding 50, although branching order varied for some species within those clades.

The major difference between LSU and *rbcL* trees was the monophyly of Clade I, for which we only found support in the *rbcL* analysis. Secondly, the trees varied in placement of *P. caciantha*, *P. calliantha*, and *P. mannii* (Table 3.1), exhibiting mostly unresolved branching order in the LSU tree (Figure 3.1) or grouping with Clade II species in the *rbcL* tree (Figure 3.2). Henceforth in our analyses, we describe *Pseudo-nitzschia* clades in terms of the two clades determined by *rbcL* phylogeny.

Pseudo-nitzschia **clades: phylogeny, morphology, and physiology synthesis**

A comparison of phylogenies constructed in the present study (Figures 3.1 and 3.2) with other phylogenetic, physiological, and morphological studies (Table 3.1) revealed different levels of variability in major characters across *Pseudo-nitzschia*. For example, two or more ITS1-5.8S-ITS2 clades (Lundholm et al. 2006) corresponded to each of the *rbcL* clades I and II determined in the present study. Specifically, Clade I corresponded to ITS1-5.8S-ITS2 clades 2, 4, and A/B, and Clade II corresponded to ITS1-5.8S-ITS2 clades 1 and 3.

We selected four morphological and physiological characters (chlorophyll c pigments, cell width, presence/absence of a central interspace, and relative number of striae to fibulae) to compare with the *rbcL* tree topology (Table 3.1). Distributions of the four character states with the *rbcL* phylogeny generally followed the major clade groupings, for the species *P. delicatissima*, *P. dolorosa*, *P. pseudodelicatissima*, and *P. galaxiae* (Clade I) versus *P. multistriata*, *P. pungens*, and *P. fraudulenta* (Clade II). The majority of Clade I types possess chlorophyll c pigments c_2 and c_3, are small cells with width < 3 μm, exhibit a central interspace (except for *P. galaxiae*), and have a greater number of striae to fibulae (Figure 3.1). In contrast, Clade II types possess all three chlorophyll c pigments, are larger cells, lack a central interspace, and have a similar number of striae to fibulae (Figure 3.3). Larger cell types such as *P. australis* and *P. multiseries*, predicted to be Clade II members by LSU phylogeny here (Figure 3.1) and in Lundholm et al. (2002), also display a similar number of striae to fibulae (Table 3.1). In contrast, for all four characters, *P. caciantha*, *P. mannii*, and *P. calliantha* exhibited different patterns from other species that were members of *rbcL* Clade II (Figure 3.1).

DISCUSSION

We incorporated 25 of the 37 currently-described *Pseudo-nitzschia* species into a clade-level investigation of the phylogeny, morphology, and physiology of the genus. It is important to identify *Pseudo-nitzschia* clades in order to understand larger-scale environmental drivers of species distributions. Due in part to the critical focus to understand toxigenic *Pseudo-nitzschia* blooms, this type of analysis has thus far largely been missing from work on the genus, when indeed a broader perspective may permit new insights into factors driving bloom formation and toxigenicity. To date, global *Pseudo-nitzschia* biogeography has been mapped according to reported species presence and isolate toxigenicty (Lelong et al. 2012; Trainer et al. 2012), yet this information has not been analyzed within the framework of a higher taxonomic level.

Based upon the phylogenetic analysis conducted in this study, as well as review of previous work, we conclude that even the hypervariable D1-D3 region of the LSU is too conserved to clearly elucidate *Pseudo-nitzschia* clades. The LSU phylogeny in this study, similar to those constructed elsewhere (Lundholm et al. 2002a; Wang et al. 2012), exhibited low bootstrap support for deep branching nodes. Indeed, we found only weak support for Clade II, comprising eight species, mostly of the "*seriata*" group. Furthermore, the present study demonstrates that putative Clade I species were not monophyletic.

In contrast, the *rbcL* trees, incorporating 14 *Pseudo-nitzschia* species, supported the existence of two major clades within *Pseudo-nitzschia*. Clade I, comprising types mainly of the "*delicatissima*" group, was strongly supported, whereas the bootstrap support for Clade II was weaker. We therefore propose the use of the *rbcL* gene, rather than the LSU marker, in future studies aimed at resolving these clade designations within the *Pseudo-nitzschia* genus, as the *rbcL* gene appears to encode enough genetic variability to identify these clades. Similarly,

Alverson (2009) suggested that genes encoded in the chloroplast and mitochondria may be better markers for differentiating diatom clades.

Interestingly, as defined by the *rbcL* phylogeny in our study, Clade II (mostly larger cell types of the "*seriata*" group) included *P. calliantha*, *P. mannii*, and *P. calliantha*, which are species of the smaller "*delicatissima*" group that were previously defined from among the cryptic *P. pseudodelicatissima* complex (Lundholm et al. 2006; Amato and Montresor 2008). Other morphological and physiological characters of these three species were also not congruent with the patterns of these characters for other Clade II species. It is possible that the entire *rbcL* phylogeny may accurately describe *Pseudo-nitzschia* evolution, but characters have been lost and gained so this cluster of species bears similarities to other species in Clade II.

Alternatively, the placement of these smaller species with Clade II may be an artifact of the fairly small proportion of "*seriata*" group species included in the *rbcL* phylogeny. To more clearly elucidate *Pseudo-nitzschia* phylogeny, *rbcL* sequencing should be conducted for more *Pseudo-nitzschia* species, particularly those of larger cell types which were underrepresented in the current analysis, such as *P. australis, P. seriata*, and *P. multiseries*. In addition, we expect that *Pseudo-nitzschia* phylogeny can be determined through phylogenetic analyses of the mitochondrial-encoded gene cytochrome c oxidase, or *cyt c*, currently done for only a few species (Kaczmarska et al. 2008; Lundholm et al. 2012).

The evolution of DA production could not be examined by this study, due to the lack of reported variability in DA production by *Pseudo-nitzschia* species included in the *rbcL* trees. With the exception of *P. mannii* (DA production not tested), species included in our *rbcL* analyses included strains that both did and did not produce detectable DA (Lelong et al. 2012) Furthermore, these species were all reportedly low DA producers, with the maximum reported DA below 1 pg per cell (Lundholm et al. 2012). By including typically high DA producers such

as *P. australis*, *P. seriata*, and *P. multiseries* in future phylogenetic analyses, we can investigate the evolution of DA production within the genus. Understanding the evolution of DA, in conjunction with full-genome analysis, may help us to generate hypotheses about the physiological or ecological function of DA, which remains unknown. Indeed, DA can account for nearly 10% of the carbon in a *Pseudo-nitzschia* cell (Fehling et al. 2004), which suggests that the molecule holds a function for the cell.

In the present study, *Pseudo-nitzschia galaxiae* was notable as a species that may have diversified significantly compared to a more ancestral state. *P. galaxiae* lacks poroids and has perforations that are hypothesized to be a derived character (Lundholm and Moestrup 2002). Furthermore, this species displays a wide range in cell size, with morphological types varying in their seasonal occurrence in the Gulf of Naples (Cerino et al. 2005). The amount of diversity within currently-defined *P. galaxiae*, in terms of both ITS1 regions (Chapter 2) and morphology (Cerino et al. 2005) suggests that *P. galaxiae* may represent another cryptic species complex, similar to *P. delicatissima* and *P. pseudodelicatissima*.

We utilized the better-supported *rbcL* tree to investigate the hypothesis that *Pseudo-nitzschia* evolution comprised two clades. We generally observed a great deal of cohesion in four major physiological and morphological characters across the two *rbcL* clades, suggesting similar evolution of these characters. Although phylogenetically similar species (e.g. *P. fraudulenta* and *P. subfraudulenta*) reportedly show variability in the absolute number of striae and fibulae (Orsini et al. 2002), we believe that a coarser determination of similar character states (such as the relative number of striae versus fibulae; presence/absence of different chlorophyll pigments; presence/absence of the central interspace) may indeed be informative characters of the evolution and ecology of *Pseudo-nitzschia*.

Patterns of morphological and physiological features across *Pseudo-nitzschia* suggest fundamental differences in the ways in which two major clades of this genus survive in their environments. The ratio between striae (which include open poroids) and fibulae (which are solid structures) could reflect the relative amount of interchange that a particular species experiences with its surroundings. Specifically, smaller cells may possess a greater number of striae in order to facilitate nutrient uptake from the surrounding ocean waters. Large cell types are likely to have high nutrient demands, given their greater size and potentially larger genomes, such as the genome of *P. multiseries*, predicted to be 300 Mb in size (Armbrust 2009) in comparison to smaller genome sizes predicted for cells of the "*delicatissima*" group (Koester and von Dassow, unpublished data), similar to patterns observed for the diatom *Ditylum brightwellii* (Koester et al. 2010). Lelong et al. (2012) noted that temperature differences alone cannot explain patterns in *Pseudo-nitzschia* species successions, and temperature tolerances appear to overlap for the two clades.

Furthermore, Clade I includes species that possessed only c_2 and c_3 pigments, whereas Clade II types all possessed c_1 and c_2 pigments (with two species also possessing c_3). In the original study in which the chlorophyll c pigment data were obtained, Zapata et al. (2011) hypothesized that chlorophyll c pigment patterns may allow for observed temporal variability between species of the "*delicatissima*" and "*seriata*" groups reported for Scottish waters (Fehling et al. 2006). The wavelengths of light absorbed by the three chlorophyll c pigments vary slightly, where chlorophyll c_1 absorbs light at shorter wavelengths (444, 577, and 626 nm*; Phaeodactylum tricornutum*) in comparison to chlorophyll c_2 (448, 579, and 627 nm; *P. tricornutum*) and chlorophyll c_3 (454, 583, 630 nm; *Pavlova gyrans*) (Fawley 1989). The light wavelengths available to diatoms in the oceans are influenced by water depth, with longer wavelengths of light (red colors; 620-750 nm) absorbed in surface waters, leaving shorter wavelengths of light (blue colors; 450 – 495 nm) available to penetrate deeper in the water column. Because each

type of chlorophyll c pigment allows absorption of light across the spectrum, the absorbance spectra first reflect the fundamental ability of *Pseudo-nitzschia,* like other diatoms, to survive a range of depths in often turbulent coastal environments. Finer-scale depth partitioning between clades may be reflected in differences in wavelength absorption across the different chlorophyll c pigments.

The initially proposed LSU Clades I and II (Lundholm and Moestrup 2002; Lundholm et al. 2002a) exhibit intriguing temporal variability throughout the world's oceans. For example, in the South Atlantic Ocean and northern Benguela Upwelling Zone, the overwhelming majority of *Pseudo-nitzschia* types present during the austral spring were proposed Clade I types (Chapter 2). In contrast, previous studies in this region reported a member of proposed Clade II, *P. australis*, during the austral fall (Marangoni et al. 2001, Seeyave et al. 2009). Similarly, the dominant members of *Pseudo-nitzschia* communities in Puget Sound, Washington, vary temporally, between species such as *P. multiseries* and *P. pungens* (Clade II) in fall and winter, compared with *P. delicatissima* (Clade I) during the spring (Hubbard 2010; Fryxell et al. 1997). In addition to seasonal variability between "*delicatissima*" and "*seriata*" groups in Scottish waters (Fehling et al. 2006), other northern European waters have exhibited alternations of small versus large *Pseudo-nitzschia* cells (Downes-Tettmar et al. 2010, Klein et al. 2010). Even in the Gulf of Naples, which is dominated by species previously described as Clade I types (*P. delicatissima*, *P. galaxiae*, *P. pseudodelicatis*sima;(Orsini et al. 2004; McDonald et al. 2007), seasonal variability exists in cell sizes of *P. galaxiae* is observed (Cerino et al. 2005). The correlation between clades and cell size, as well as seasonal nature of the temporal variability, perhaps most strongly suggests that clades differ fundamentally in nutrient requirements. The spring blooms of the *"delicatissima"* group types in Scottish waters (Fehling et al. 2006) coincided with nitrate depletion, with the bloom diminishing upon increased nitrate

concentrations. Conversely, summer blooms of *P. seriata* types in this same region correlated directly with nitrate levels.

However, the tendency of *"delicatissima*" group cells to dominate in early springtime, relative to *"seriata"* groups in the summer and fall, in fact suggests multiple alternative hypotheses for the physiological foundations of this trend. In temperate environments, environmental parameters that differ between early spring and summer include light, nutrients, temperature, physical mixing, and grazer presence. In particular, *P. delicatissima* is a particularly interesting species for physiological studies of interspecific competition with "*seriata*" group members, given *P. delicatissima*'s dominance of early springtime communities in diverse geographic regions, including Scottish waters (Fehling et al. 2006), Puget Sound (Fryxell et al. 1997; Hubbard 2010) the Gulf of Naples (Orsini et al. 2004; McDonald et al. 2007), the Skagerrak (Hasle et al. 1996), and the Adriatic Sea (Miralto et al. 2003). Such physiological studies, in addition to further elucidation of clade affiliations, should provide insight into the larger-scale foundations for biogeographic patterns of the genus.

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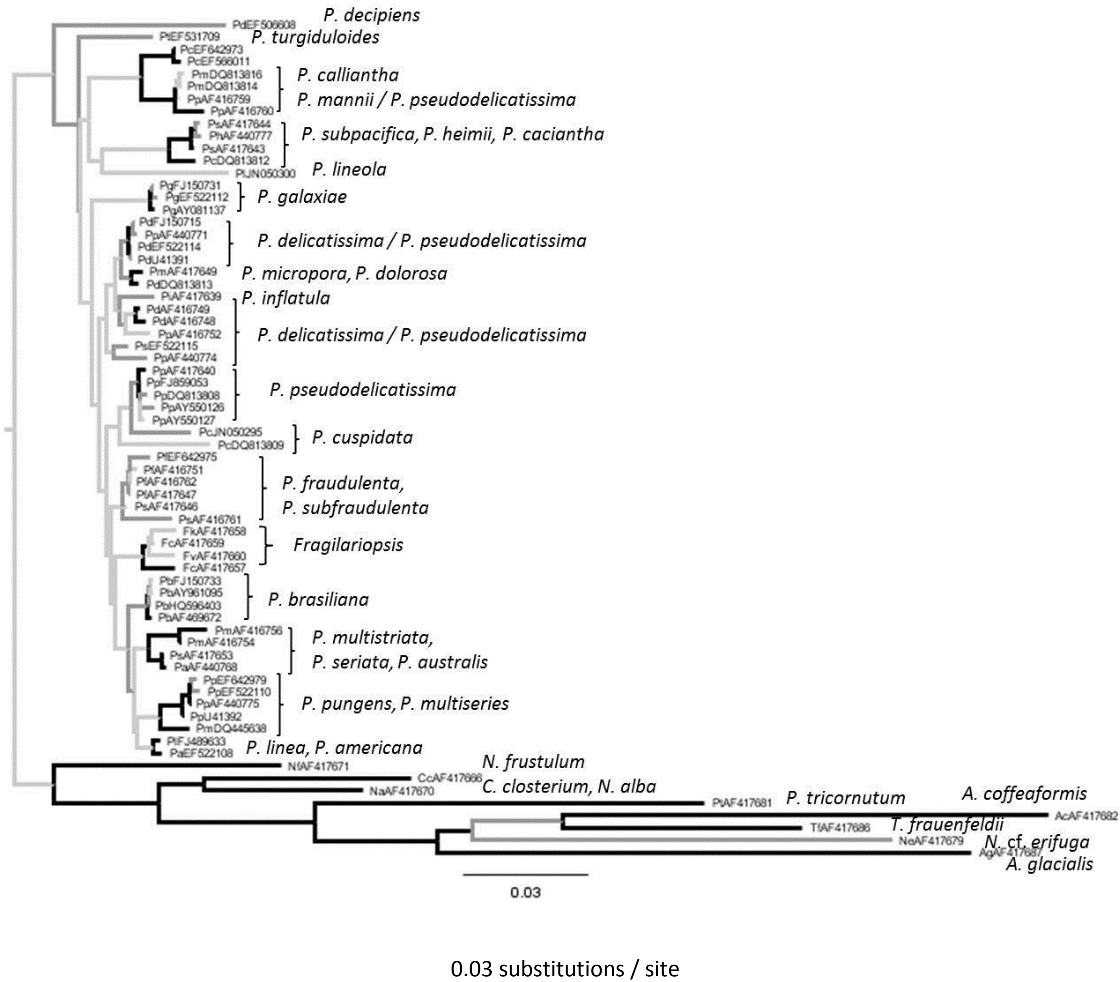

Figure 3.1. Minimum Evolution (ME) tree constructed using the hypervariable D1-D3 region of the ribosomal RNA large subunit (LSU). Bootstrap support in ME analysis is indicated by light gray (< 50), medium gray (50-75), and black (>75). Branch names indicate initials of genus and species, followed by GenBank accession number. Full species identifications, as reported in original publications, are listed for clusters of similar species.

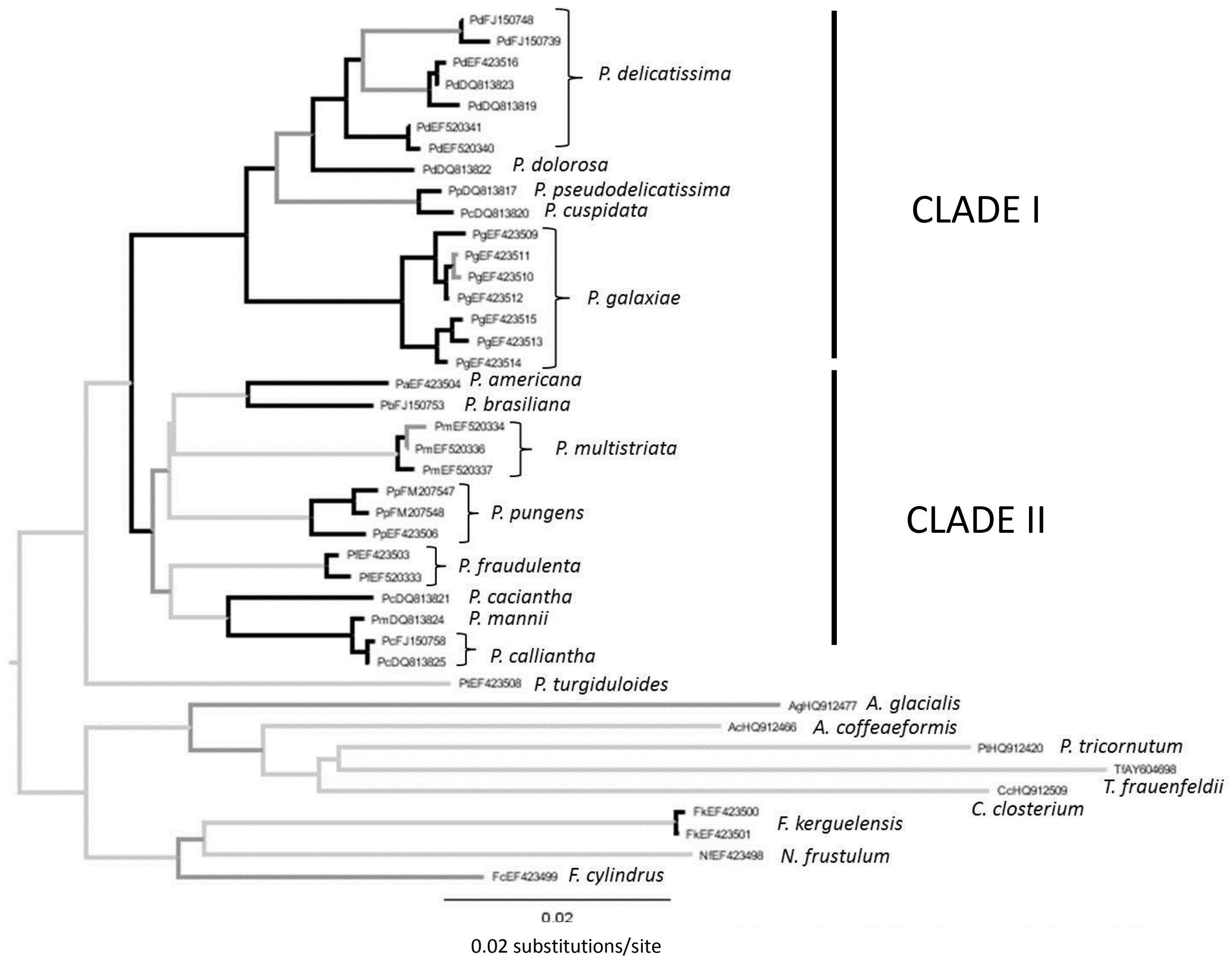

Figure 3.2. Minimum Evolution (ME) tree constructed using the gene encoding the large subunit of the carbon fixation enzyme, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), or *rbcL* gene. Bootstrap support from at least one of two analyses (either ME or Maximum Likelihood, ML) is indicated by light gray (< 50), medium gray (50-75), and black (>75). Branch names indicate initials of genus and species, followed by GenBank accession number, with designations of two major clades of *Pseudo-nitzschia*.

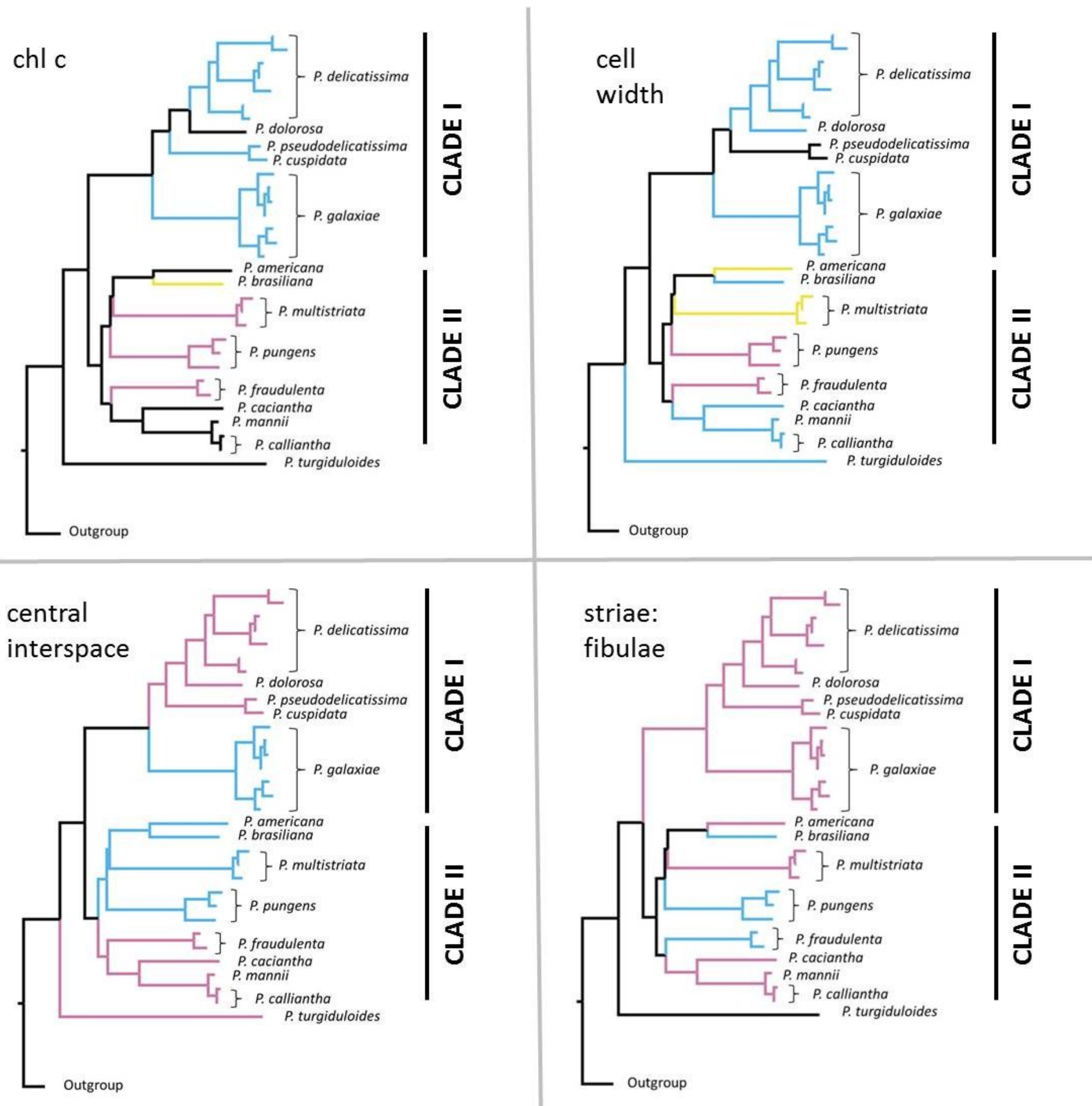

Figure 3.3. Four major physiological or morphological characters overlain on phylogenetic tree based on the *rbcL* gene, including chlorophyll c pigments (chl c), cell width, presence/absence of central interspace in the raphe, and relative number of striae to fibulae. Generally, blue indicates absence of character state (central interspace) or smaller value (cell width < 3 µm; similar number of striae and fibulae). Reddish purple indicates presence of character state (central interspace) or larger value (cell width > 3 µm; greater number of striae relative to fibulae). For chlorophyll c pigment composition, blue denotes presence of pigments c_2 and c_3, and reddish purple denotes presence of pigments c_1, c_2 and c_3 . Yellow indicates presence of chlorophyll c pigments c_1 and c_2 or cell width ≈ 3 µm. Black indicates the absence of data for the relevant character.

Table 3.1. Synthesis of phylogenetic, physiological, and morphological characters of *Pseudo-nitzschia* Column labeled “*rbcL* this study” indicate clade designations based upon LSU and RuBISCo large subunit (*rbcL*) trees constructed for the present study. References for additional data are as follows: “LSU 2002”: Lundholm et al. 2002, Lundholm and Moestrup 2002; “ITS1-5.8S-ITS2”: Lundholm et al. 2006; “chl c” (chlorophyll c pigments): Zapata et al. 2011; “DA production” (domoic acid production): multiple studies as summarized by Lelong et al. (2012); “max DA (pg/cell)” (maximum DA production reported in the literature): multiple studies as summarized by Trainer et al. (2012); “central interspace” and “striae : fibulae”: (Almandoz et al. 2008, Amato and Montresor 2007, Lundholm and Moestrup 2002, Lundholm et al. 2002, Lundholm et al. 2006, Marchetti et al. 2008, Quijano-Scheggia et al. 2009, Wang et al. 2012, Zapata et al. 2011); “cell shape”: two morphological groups defined by Hasle and Syvertsen (1997) as summarized by Lelong et al. 2012. “?” indicates that a clade of three or more species could not be defined due to low bootstrap support; “a” following a clade designation indicates species was a sister taxon to preceding clade number or letter; “-“ indicates absence of data; “n/a” denotes that character state was not applicable.

	PHYLOGENY			PHYSIOLOGY			MORPHOLOGY		
Pseudo-nitzschia species	PROPOSED LSU 2002	ITS1 - 5.8S - ITS2	*rbcL* (this study)	chl c	DA production	max DA (pg/cell)	central interspace	striae : fibulae	cell shape
delicatissima	I	A/B	I	c2, c3	yes/no	0.12	yes	>	*delicatissima*
pseudodelicatissima	I	2	I	c2, c3	yes/no	0.0078	yes	>	*delicatissima*
cuspidata	-	2	I	c2, c3	yes/no	0.031	yes	>	*delicatissima*
galaxiae	I	4	I	c2, c3	yes/no	0.00036	no	>	*delicatissima*
brasiliana	II	-	II	c1, c2	yes/no	0.0095	no	=	*delicatissima*
decipiens	-	4	-	c2, c3	no	n/a	yes	>	*delicatissima*
inflatula	I	-	-	-	no	n/a	yes	>	*delicatissima*
lineola	-	-	-	-	no	n/a	yes	>	*delicatissima*
linea	-	-	II	-	-	n/a	no	>	*delicatissima*
micropora	I	A/B a	-	-	-	n/a	no	>	*delicatissima*
subcurvata	-	-	?	c2, c3	no	n/a	no	>	*delicatissima*
turgiduloides	-	3a	?	-	no	n/a	yes	>	*delicatissima*
caciantha	-	-	II	-	no	n/a	yes	>	*delicatissima*
mannii	-	-	II	c2, c3	-	n/a	yes	>	*delicatissima*
calliantha	-	3	II	c2, c3	yes/no	0.221	yes	>	*delicatissima*
americana	II	-	II	-	no	n/a	no	>	neither
multistriata	II	1	II	c1, c2, c3	yes/no	0.697	no	>	neither
pungens	II	1	II	c1, c2, c3	yes/no	0.47	no	=	*seriata*
fraudulenta	I	1a	II	c1, c2, c3	yes/no	0.03	yes	=	*seriata*
australis	II	1	-	c1, c2	yes/no	37	no	=	*seriata*
dolorosa	-	4a	I	-	no	n/a	yes	>	*seriata*
heimii	-	-	-	-	no	n/a	yes	>	*seriata*
multiseries	II	1	-	c1, c2	yes	67 or 140	no	=	*seriata*
seriata	II	1	-	c1, c2	yes/no	33.6	no	=	*seriata*
subfraudulenta	I	-	-	-	no	n/a	yes	>	*seriata*
subpacifica	I	3	-	c2, c3	no	n/a	yes	>	*seriata*

CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation describes the diatom *Pseudo-nitzschia* as an organism that is finely tuned to its environment, and which in turn greatly influences its surroundings. The observed patterns among *Pseudo-nitzschia* and cultured bacteria suggest that this particular diatom could play a strong role in structuring bacterial communities in the marine environment. Second, *Pseudo-nitzschia* community composition was tightly coupled with abiotic environmental conditions in the South Atlantic Ocean, displaying variable communities over distinct environmental regimes yet also displaying similarities to geographically distant communities in the Northeast Pacific Ocean. Third, the cohesion of morphological and physiological characters with *Pseudo-nitzschia* phylogeny and geography highlights the largely overlooked, fundamental adaptations across species of the genus. All of these perspectives hold important implications for both *Pseudo-nitzschia* toxigenicity and broader understanding of marine microbial biogeography.

Because of the HAB-forming potential of *Pseudo-nitzschia*, many research questions have been framed from this perspective. Clearly, much work still remains, in order to inform decisions that inform public health and to understand DA impacts on marine ecosystems. However, after over three decades of research into environmental conditions that may trigger DA production, the development and perpetuation of toxigenic blooms remains largely an enigma. Specifically, it has been difficult to correlate environmental conditions with DA, or to identify definite causative organisms of DA outbreaks, based on conventional research studies comprising discrete surveys at a single time point. I expect that DA outbreaks occur through the responses of toxigenic *Pseudo-nitzschia* types to a variety of environmental stimuli, including bacteria, which could be difficult to measure in the field. Furthermore, as suggested by Chapter 2, I believe that we currently possess only limited knowledge of the *Pseudo-nitzschia* species that may be more

or less likely to cause DA outbreaks. Basing public health decisions on the absence of "toxigenic" species could be disastrous. Therefore, although shellfish bed closures simply due to the presence of *Pseudo-nitzschia* (which may or may not be producing toxin) may be unnecessary, this conservative approach is critical to safeguarding human health. I suggest three major avenues of research, which will build upon the findings described here.

At the time of this writing, the complete genome of *Pseudo-nitzschia multiseries* is in the final stages of annotation. *P. multiseries* is a highly toxigenic species and causative organism of the first published DA outbreak (Bates et al. 1989). Its genome is predicted to be very large with a significant portion comprising transposons (Armbrust pers comm). As part of the genome sequencing project, expressed sequence tag (EST) libraries were constructed from *P. multiseries* grown under DA-triggering and control conditions. Comparative bioinformatics is uncovering gene expression patterns specific to DA production. Ultimately this work may elucidate the pathway of DA production. This pathway, furthermore, may offer insight into the physiological or ecological functions of DA.

However, in the age of metagenomics and bioinformatics, classic physiological studies still offer unparalleled insights. First, model systems utilizing a single *Pseudo-nitzschia* strain and a single bacterial strain can elucidate mechanisms underlying the apparently strong associations between diatom and bacteria. Research into interactions between *P. multiseries* and associated *Sulfitobacter* is investigating the chemical interchanges that may uphold associations between these organisms (Amin, Hmelo, and van Tol pers comm). The identification of specific bacteria that enhance *Pseudo-nitzschia* toxigenicity, as yet unknown (Bates et al. 1995), may suggest the mechanism underlying toxin enhancement. Additionally, interspecific competition studies between *Pseudo-nitzschia* species of "*delicatissima*" versus "*seriata*" types will be critical to identifying the factors important to niche differentiation across members of the genus.

The elusive question, “what makes *Pseudo-nitzschia* blooms become toxigenic?”, is likely best addressed by time-series data. To understand the physiological changes that accompany bloom development and DA production, the same bloom should be followed over time, as done in the Juan de Fuca Eddy, in the Northeast Pacific Ocean (Trainer et al. 2009b). Additionally, long-term monitoring efforts, which have historically surveyed *Pseudo-nitzschia* only at the genus level (Harris 2010, Sekula-Wood et al. 2011) , are beginning to incorporate species- (Downes-Tettmar et al. 2010) or clade-level (e.g. Southern California Coastal Ocean Observing System; http://www.sccoos.org/data/habs/index.php) patterns in spatial and temporal variability of *Pseudo-nitzschia*.

The united approaches of genomics, physiological studies, and time-series observations will also inform the broader biogeography of marine organisms. Genomic analyses can generate powerful hypotheses about major physiological characters that are unique to clade, species, and ecotype survival. These characters can suggest the significance of both biotic and abiotic influences upon evolution, yet the comparative influence of any single driver must be tested in controlled studies. Time-series observations, linking environmental variability with community composition, can provide further support for physiological differences across varied organismal levels, from strain or ecotype to clade to domain. Finally, the threads of these approaches are interwoven such that biogeographical patterns observed in the environment will give rise to evolutionary changes within the genome. In this era of global environmental change, it is critical to monitor ways in which current adaptations of microorganisms will permit, or disallow, their survival under changing ocean conditions, and the consequences for the larger life forms which depend on the tiny, abundant, diverse, and powerful microorganisms of the sea.

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CURRICULUM VITAE

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MOTIVATIONS

- **Nurture** hypothesis-driven learning
- **Lead** students through responsive, dynamic mentoring relationships
- **Link** learners of different levels
- **Communicate** biological and environmental sciences within broader societal contexts

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EDUCATION

University of Washington, School of Oceanography, Seattle, WA (2004-2012)

- M.S. August 2007, "Specificity of bacterial assemblages associated with the toxigenic genus *Pseudo-nitzschia* and other marine diatoms." Advised by Dr. Gabrielle Rocap.
- Ph.D. August 2012, "Marine microbial biogeography from microscopic to global scales: ecology of the diatom *Pseudo-nitzschia*." Advised by Dr. Gabrielle Rocap.

Smith College, Northampton, MA (1993-1997)

- B.A. *magna cum laude* May 1997 (Biological Sciences with minor in Marine Sciences). Honors thesis: "Effects of bioremediation on physiological responses of the macroalgae *Ascophyllum nodosum*, *Fucus vesiculosus*, and *Laminaria saccharina* exposed to No. 2 fuel oil." Advised by Dr. Paulette Peckol.

Sea Education Association (SEA) Semester, Woods Hole, MA (1996)

- Oceanography, maritime history, and nautical science courses, followed by six weeks at sea.

RESEARCH AND LABORATORY EXPERIENCE

Graduate Research Assistant, Rocap Lab, UW School of Oceanography, Seattle, WA (2004-2012)

- Investigated interactions between the diatom *Pseudo-nitzschia* and bacteria, employing molecular methods.
- Characterized biogeographic distribution of the diatom *Pseudo-nitzschia*, employing molecular methods and *in silico* analysis of publicly available data.

Analyst, Brooks Rand LLC, Seattle, WA (2002-2004)

- Analyzed aqueous, sediment, and biological samples for trace metals using spectrometry.

Research Assistant, National Council for Air and Stream Improvement, Corvallis, OR (2001)

- Conducted analyses for organic compounds, via spectrophotometry and gas chromatography / mass spectrometry.

Undergraduate Researcher, Peckol Lab, Smith College Honors Program (1996-1997)

- Designed and performed laboratory experiments to assess influence of bioremediation as oil spill response technique, as measured by physiological responses of three species of marine macroalgae.

Research Intern, Valiela Lab, Marine Biological Laboratory, Woods Hole, MA (1996)

- Conducted amphipod grazing preference experiments and nitrogen loading research.

TEACHING EXPERIENCE

Teaching Assistant for Marine Biology (OCEAN 250), University of Washington (Summer 2012)

- Undergraduate course, 40 students
- Taught two 3-hour lab sections per week, co-facilitated field trips, graded weekly labs.

Teaching Assistant Training Facilitator, Oceanography-Fisheries-Marine Affairs, University of Washington (Autumn 2011)

- Led graduate students in mock classroom discussions and provided feedback to improve their teaching.

Teaching Assistant for Biological Oceanography (OCEAN 535), University of Washington (Autumn 2011)

- Graduate course, 14 students
- Designed and graded homework assignments, presented two lecture/discussion sessions (Microbial Ecology, Genomic Methods), co-designed and facilitated wet labs, organized additional help sessions outside of class time in response to student needs, held weekly office hours.

Teaching Assistant for Survey of Oceanography (OCEAN 101), University of Washington (Spring 2011)

- Undergraduate course, 75 students
- Led two 2-hour lab sections per week, presented one class lecture (Harmful Algal Blooms) along with undergraduate students from laboratory group, introduced feedback method for students, graded lab assignments, held weekly office hours.

Teaching Assistant for The Changing Oceans (OCEAN 102), University of Washington (Winter 2010)

- Undergraduate course, 500 students
- Coordinated one distance-learning section of 90 students using web-based grading system (Catalyst), collaborated with instructors and 6 other TAs in weekly planning sessions, wrote quiz and exam questions, proctored exams.

Teaching Assistant for Biological Oceanography (OCN 430), University of Washington (Autumn 2005)

- Undergraduate course, 30 students
- Designed and presented three lecture/discussion sessions (Bacteria, Archaea, and Viruses; Microbial Ecology), graded homework assignments, and responded to student questions.

TEACHING EXPERIENCE, continued

Undergraduate Research Advisor, Rocap Lab, University of Washington (2007 - 2010)

- Diana Haring (phytoplankton microscopy and molecular methods, June 2008 – June 2010)
    - Recipient of Oceans and Human Health REU fellowship and co-author on upcoming publication.
- Gwendolyn Hannam (phytoplankton and bacterial culturing projects, January 2007 – March 2008)

Tutor, Lane Tutoring Service, Inc., Eugene OR (1999-2001)

- Supported 20 teenagers individually in various subjects.
- Developed and implemented math curricula.

Tutor, Smith College, Northampton, MA (1996-1997)

- Assisted undergraduates with biology and chemistry coursework in individual sessions.

OUTREACH AND COMMUNITY SERVICE

UW Oceanography Outreach Group Member and Independent Outreach Activities
(2007 - 2012)

- Developed and presented original activities and lab tours related to oceans and human health research, biological oceanography, and research opportunities.
- Interacted with elementary school through high school students, undergraduates, and general public, with focus on groups traditionally underrepresented in sciences.

Volunteer Mentor, Seattle Girls' School, Seattle, WA (2006-2009)

- Mentored middle school student interested in marine biology, individually and in monthly group activities.

Youth Counselor, Looking Glass Youth and Family Services, Eugene, OR (1999-2000)

- Supported and supervised youth in residential psychiatric treatment center.

Greenfield AmeriCorps Program Member, Greenfield, MA (1998-1999)

- Tutored and mentored at-risk students individually and within classrooms in a public middle school.
- Designed and facilitated activities in weekly extracurricular art and service learning groups for teenagers.

OUTREACH AND COMMUNITY SERVICE, continued

AmeriCorps*National Civilian Community Corps (NCCC) Member, Perry Point, MD (1997-1998)

- Tutored inner city middle schoolers, built homes for families in need, and completed other service projects.

PUBLICATIONS

***** denotes undergraduate author

Guannel, M.L., Twiner, M.J., Haring, D.*, Noble, A., Wang, Z., Saito, M., and Rocap, G. in prep. Community composition and toxigenicity of the diatom *Pseudo-nitzschia* across distinct environmental regimes in the South Atlantic Ocean.

Guannel, M.L., Horner-Devine, M.C., and Rocap, G. 2011. Bacterial community composition differs with species and toxigenicity of the diatom *Pseudo-nitzschia. Aquatic Microbial Ecology* 64: 117-133.

Erdner, D.L., Dyble, J., Parsons, M.L., Stevens, R.C., Hubbard, K.A., **Wrabel, M.L.**, Moore, S.K., Lefebvre, K.A., Anderson, D.M., Bienfang, P., Bidigare, R.R., Parker, M.S., Moeller, P., Brand, L.E., and Trainer, V.E. 2008. Centers for Oceans and Human Health: a unified approach to the challenge of harmful algal blooms. *Environmental Health* 7: S2.

Wrabel, M.L. and Peckol, P. 2000. Effects of bioremediation on toxicity and chemical composition of No. 2 fuel oil: growth responses of the brown alga *Fucus vesiculosus*. *Marine Pollution Bulletin* 40:2, 135-139.

Wrabel, M.L. 1997. Effects of bioremediation on physiological responses of the macroalgae *Ascophyllum nodosum*, *Fucus vesiculosus*, and *Laminaria saccharina* exposed to No. 2 fuel oil. Undergraduate Honors Thesis. Smith College Department of Biological Sciences.

PRESENTATIONS

***** denotes undergraduate author

Carlson, M., **Guannel, M.L.**, Hubbard, K.A., Port, J., and Turner, J. 2010. Connecting researchers in oceans and human health (OHH): the OHHI traineeship program of the Pacific Northwest Consortium. NOAA Oceans and Human Health Initiative National Advisory Panel Meeting, Washington, D.C. (poster)

Guannel, M.L. 2010. Unraveling genetic and environmental factors contributing to *Pseudo-nitzschia* toxigenicity. NOAA Oceans and Human Health Initiative National Review Panel Site Visit, Seattle, WA. (oral)

Haring, D.*, **Guannel, M.L.**, Twiner, M.J., Lee, P.A., DiTullio, G.R., Wang, Z., and Rocap, G. 2009. Patterns of *Pseudo-nitzschia* and domoic acid related to phytoplankton community structure in the Benguela Current. Fifth Symposium on Harmful Algae in the U.S., Ocean Shores, WA. (poster)

PRESENTATIONS, continued

Guannel, M.L. 2009. Characterization of bacterial communities co-existing with the diatom *Pseudo-nitzschia*. NOAA Oceans and Human Health Initiative Graduate Student Seminar, Seattle, WA. (oral)

Wrabel, M.L. 2008. Associations between bacteria and *Pseudo-nitzschia*: is there a link to toxin production? Oceans and Human Health Seminar Series, University of Washington, Seattle, WA. (oral)

Wrabel, M.L., and Rocap, G. 2008. Specificity of bacterial assemblages associated with *Pseudo-nitzschia* and other marine diatoms in Puget Sound, Washington. ASLO Ocean Sciences Meeting, Orlando, FL. (poster)

Wrabel, M.L., and Rocap, G. 2007. Specificity of bacterial assemblages associated with the toxin-producing diatom genus *Pseudo-nitzschia*. Fourth Symposium on Harmful Algae in the United States, Woods Hole, MA. (poster)

Parker, M.S., Newton, J., Von Dassow, P., Marohl, R.L., Holtermann, K.E., Berthiaume, C., Marchetti, A., Iverson, V., **Wrabel, M.L.**, Hubbard, K., Gilmore, B., Williams, C. M., Rocap, G., Armbrust, E.V. 2006. Phytoplankton community responses to nutrient amendments in Hood Canal, WA. ASLO Ocean Sciences Meeting, Victoria, BC. (poster)

Wrabel, M.L., and Rocap, G. 2006. Domoic acid production and diversity of bacteria associated with *Pseudo-nitzschia* species. Joint Meeting of the NOAA, NSF/NIEHS Centers for Oceans and Human Health, Seattle, WA. (poster)

AWARDS

Oceans and Human Health Initiative (OHHI) Pre-Doctoral Traineeship (2009)
Highest Honors in Biological Sciences, Smith College (1997)
Amey Randall Brown Prize for best botanical essay, Smith College (1997)
Award for most progress in German language (1997)
Howard Hughes Medical Institute undergraduate research fellowship (1996)

PROFESSIONAL MEMBERSHIPS

Association for the Sciences of Limnology and Oceanography
Phi Beta Kappa
Sigma Xi Scientific Research Society

OCEANOGRAPHIC CRUISES

R/V Thompson TN270 Oceans and Human Health cruise: mentored new students
(October 2011, 3 days)

R/V Thompson TN243 Oceans and Human Health cruise: mentored new students
(November 2009, 3 days)

R/V Knorr 192-05: South Atlantic phytoplankton and bacterial studies
(November – December 2007, 4 weeks)

R/V Thompson TN201 Oceans and Human Health cruise: Puget Sound bacterial studies
(October 2006, 3 days)

R/V Thompson TN196: Puget Sound bacterial studies
(June 2006, 4 days)

R/V Barnes Hood Canal Water Advection project cruise
(December 2005, 1 day)

R/V Melville TUIM14VM ECOHAB PNW cruise
(September 2005, 3 weeks)

R/V Thompson TN176: Puget Sound bacterial studies
(December 2004, 4 days)

R/V Thompson TN175 Oceans and Human Health cruise: Puget Sound bacterial studies
(November 2004, 1 day)

SSV Corwith Cramer C-145: North Atlantic macroalgal and invertebrate studies
(May – June 1996, 6 weeks)