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Toxigenicity and biogeography of the diatom *Pseudo-nitzschia* across distinct environmental regimes in the South Atlantic Ocean

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ABSTRACT: The community composition and toxigenicity of the diatom *Pseudo-nitzschia* in the northern Benguela Upwelling Zone and the open South Atlantic Ocean were characterized as part of a transoceanic survey conducted during the austral spring of 2007. Multiple morphological types of *Pseudo-nitzschia* were detected by light microscopy in coastal waters. Automated ribosomal intergenic spacer analysis (ARISA), a DNA-fingerprinting technique used to assess *Pseudo-nitzschia* community composition, detected 37 ARISA types distributed among 17 stations in both coastal and open-ocean regions. Through statistical analysis of abiotic factors, we identified 6 distinct environmental regimes across which *Pseudo-nitzschia* community composition varied. *Pseudo-nitzschia* were detected in open-ocean waters, where community composition differed between surface and deep chlorophyll maxima. The toxin produced by *Pseudo-nitzschia*, domoic acid (DA), was present in coastal waters both inside and outside the northern Benguela Upwelling Zone at potentially ecologically harmful levels, up to 184 ng DA l^{-1} and 4.6 pg DA $cell^{-1}$. Partial internal transcribed spacer 1 (ITS1) clone libraries putatively identified at least 10 species in the South Atlantic, including *P. inflatula*, *P. subpacifica*, *P. heimii*, and *P. galaxiae*. Previously, these species were reported to produce DA at levels several orders of magnitude lower than our field measurements. Simple correlations were not able to identify obvious environmental triggers of DA production. Our findings suggest that species commonly believed to be weakly toxigenic could pose harm to humans and marine organisms, including those inhabiting southwestern African coastal regions.

KEY WORDS: *Pseudo-nitzschia* · Domoic acid · Benguela Upwelling Zone · Harmful algal blooms

INTRODUCTION

Members of the marine diatom genus *Pseudo-nitzschia* are broadly distributed throughout the world's oceans and influence human and ecosystem health due to their toxigenic properties. Indeed, *Pseudo-nitzschia* species are ubiquitous in coastal waters (Hasle 2002) and have been studied exten-

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sively in the Northern Hemisphere (Trainer et al. 2012). These diatoms are also present in low numbers in open-ocean waters, where they increase in abundance in response to fertilization experiments using iron and other nutrients (De Baar et al. 2005, Marchetti et al. 2008, Trick et al. 2010). Knowledge about *Pseudo-nitzschia* ecology and biogeography is particularly important to understanding impacts of the metabolite domoic acid (DA) produced by some members of the genus. DA is a neurotoxin that affects marine ecosystems and humans by interfering with glutamate receptors (Teitelbaum et al. 1990) and results in amnesic shellfish poisoning (ASP) in humans and domoic acid poisoning (DAP) in other vertebrates (Bates et al. 1998, Lelong et al. 2012, Trainer et al. 2012). DA has been detected in diverse 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, to sea lions (Scholin et al. 2000) and birds (Work et al. 1993); through shellfish to humans (Bates et al. 1989, Wright et al. 1989); through krill (Bargu & Silver 2003) to finfish and whales (Lefebvre et al. 2002); and through unknown vectors to bottlenose dolphins (Twiner et al. 2011, 2012). Despite the potentially severe impacts of DA on marine organisms and humans, coastal waters in the Southern Hemisphere and the open ocean remain poorly described in terms of *Pseudo-nitzschia* community composition and toxigenicity.

Among approximately 37 defined *Pseudo-nitzschia* species, at least 14 to date have produced DA in laboratory studies (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. 2009b) often produce DA on the order of pg DA cell^{-1}. In contrast, species that are described as weakly toxigenic or nontoxigenic include *P. galaxiae* (measured to produce up to 3.6×10^{-4} pg DA cell^{-1}; 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). To date, all of the highly toxigenic species are typically found in coastal waters, whereas open-ocean species tend to produce undetectable (Marchetti et al. 2008) or exceedingly low (Trick et al. 2010) DA levels. Some work has identified causative 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 signal of potential harm.

However, even when a highly toxigenic species is detected in the field, bloom impacts remain difficult to predict. Under laboratory conditions, a toxigenic strain does not produce high levels of DA continuously, but rather does so in response to specific environmental triggers, including limitation by silicic acid (Bates et al. 1991, Pan et al. 1996b,c), phosphate (Pan et al. 1996a), 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). Most field studies have not correlated DA levels with environmental conditions such as nutrient levels or ratios (Marchetti et al. 2004, Trainer et al. 2009a,b) or bacterial abundance (Trainer et al. 2009b), although studies in southern California waters have suggested phosphate or silicic acid limitation as triggers of DA outbreaks (Anderson et al. 2006, Schnetzer et al. 2007). Finally, in addition to species composition and potential environmental triggers of DA production, 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.

Individual *Pseudo-nitzschia* species display both cosmopolitan and restricted geographic distributions (Hasle 2002). Species including *P. australis*, *P. delicatissima*, *P. fraudulenta*, *P. multiseries*, *P. pseudodelicatissima*, and *P. pungens* have been 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 species considered to be characteristic of the open ocean, such as *P. turgidula*, *P. heimii*, and *P. inflatula*, have also been detected in coastal waters (Ribalet et al. 2010, Lelong et al. 2012). Increasingly, whole-community studies, testing correlations between relative dominance of certain species and *in situ* parameters (Almandoz et al. 2007, 2008, Kaczmarska et al. 2007, Schnetzer et al. 2007), have identified environmental conditions such as temperature, salinity, nutrient concentrations, and water column depth as potentially important bottom-up drivers that allow certain *Pseudo-nitzschia* species to outcompete other species. These studies indicate that *Pseudo-nitzschia* communities differ on finer geographical scales, rather than being restricted to only forming distinct 'open-ocean' versus 'coastal' communities.

Situated along the southwestern African coast, the Benguela Current is 1 of 4 global eastern boundary currents that result in seasonal upwelling and high biological productivity, supporting known vectors of

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 recent, and most of the research related to harmful algal blooms (HABs) along the western African coast has focused on dinoflagellates within the southern Benguela system (e.g. Pitcher & Calder 2000, Fawcett et al. 2006). More recently, *Pseudo-nitzschia* has been recorded as a common component of phytoplankton communities off Lambert's Bay, South Africa. In March 2001, *P. australis* was confirmed, but isolates of this species did not produce detectable levels of DA (Marangoni et al. 2001). Subsequently, *Pseudo-nitzschia* communities off Lambert's Bay in March 2005 and April 2006 were discovered to be toxigenic at 0.1 to 3 µg l^{-1} (Fawcett et al. 2007); the blooms included *P. australis* and 2 morphologically distinct types of very small, unidentified cells (Seeyave et al. 2009). Finally, in March 2007, *Pseudo-nitzschia* spp. produced a maximum of 0.21 pg DA $cell^{-1}$ (Hubbart et al. 2012). Further north, DA has been reported in phytoplankton and bivalve samples in Luanda Bay (northern Angola) in connection with 2 separate blooms that occurred in September and November 2007 (Blanco et al. 2010), although *Pseudo-nitzschia* were not identified to the species level. DA levels in these blooms were between 0.05 and 3 µg l^{-1}, 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). DA has also been reported in sardines off the Namibian coast (Trainer et al. 2012).

Along the western African coast, previously described *Pseudo-nitzschia* communities include common coastal *Pseudo-nitzschia* species (Hasle 2002). *P. australis* (a highly toxigenic species; e.g. Scholin et al. 2000) is the species that has been reported most frequently in the Benguela Upwelling Zone (Marangoni et al. 2001, Hasle 2002, Seeyave et al. 2009). As summarized by Hasle (2002), the western African coast has also been reported to harbor *P. delicatissima*, *P. fraudulenta*, *P. multiseries*, *P. pseudodelicatissima*, *P. pungens*, and *P. subfraudulenta*. For regions offshore of the west African coast (the open South Atlantic Ocean), no studies to date have characterized *Pseudo-nitzschia* community composition (Lelong et al. 2012).

Here, we assessed the toxigenicity and distribution of *Pseudo-nitzschia* across the South Atlantic Ocean, with a focus on coastal waters inside and outside the northern Benguela Upwelling Zone. We characterized *Pseudo-nitzschia* communities across the South Atlantic by utilizing clone libraries of the partial internal transcribed spacer region 1 (ITS1) and automated ribosomal intergenic spacer analysis (ARISA) (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* in coastal African waters and demonstrate that *Pseudo-nitzschia* display biogeographic patterns that are tightly tied to environmental conditions. Further, we tested correlations across DA levels, *Pseudo-nitzschia* community composition, and environmental conditions in order to explore potential environmental drivers of *Pseudo-nitzschia* biogeography and to identify potential toxigenic types. Our findings suggest that species believed previously to produce only low levels of DA may be capable of higher levels of toxin production.

MATERIALS AND METHODS

Study area and environmental data collection overview

We conducted a transoceanic survey across the South Atlantic Ocean from November 18 to December 11, 2007, as part of the Cobalt, Iron, and Microorganisms from the Upwelling to the Gyre (CoFe MUG) cruise (see Fig. 1). In total, 27 stations were sampled at the surface and deep chlorophyll maxima (DCM) (all stations); additional depths were sampled at Stns 18, 19, 24, and 25.

Regional conditions at the time of the cruise were visualized using MODIS-Aqua data averaged from November 1 to December 31, 2007, obtained from the Giovanni online data system (NASA GES DISC; Acker & Leptoukh 2007). To infer upwelling/downwelling conditions within the Benguela Upwelling Zone, surface current data were obtained from OSCAR (Ocean Surface Current Analyses – Real time; www.oscar.noaa.gov) and averaged over November 25 to December 15, 2007.

Macronutrient and trace metal analyses

Niskin bottles mounted on a trace-metal-free rosette were used to collect water for macronutrients and trace metals. For macronutrient analyses, 60 ml were sterile-filtered through 0.4 µm polycarbonate

filters into acid-washed HDPE bottles and frozen at –20°C (Morris et al. 2010). Nutrient concentrations were determined using a Technicon AutoAnalyzer II™ (phosphate, ammonium) or an Alpkem RFA 300™ (silicic acid, nitrate plus nitrite) at Oregon State University.

Total dissolved cobalt concentrations were determined using a cathodic stripping voltammetry (CSV) method with a hanging mercury drop electrode (Saito & Moffett 2001, Saito et al. 2004), with standard additions conducted through a programmed dosing procedure (Noble et al. 2008, 2012). Total dissolved iron and manganese concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS), as described by Saito & Schneider (2006); the dataset is decribed further by Noble et al. (2012). 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 magnesium hydroxide and trace metal coprecipitation, followed by centrifugation. The isolated pellet was then dissolved in 5% nitric acid (Seastar) containing 1 ppb indium prior to analysis.

Characterization of phytoplankton communities

For size-fractionated chlorophyll analysis, 500 ml water was sequentially filtered through 3 filter mesh sizes: 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 h, and samples were analyzed using a TD-700 fluorometer (Turner Designs) without acidification.

Qualitative net tow samples were collected from surface waters by hand using a 10 µm mesh phytoplankton net, and 7 ml of concentrated sample was preserved in buffered formalin phosphate (final concentration of 0.04%). Water from Niskin bottles (30 ml at Stns 1 to 17 and 27; 7 ml at Stns 18 to 26) was preserved similarly. Samples were stored in the dark until settled in an Utermöhl settling chamber for a minimum of 24 h.

For samples containing ≥300 cells, diatom and dinoflagellate cells were counted and identified to the genus level when possible, using a Nikon Eclipse TS100 inverted microscope. We do not report phytoplankton community composition for the 'North Open Deep' regime, nor for surface open-ocean samples east of Stn 15, because samples collected in this region were too dilute for enumeration. Individual *Pseudo-nitzschia* cells were assigned to 1 of 3 morphological groups (Bill et al. 2006), based upon aspect ratio (apical axis:transapical axis) calculated from the range of dimensions for individual species (Hasle & Syvertsen 1997, Horner 2002). The 3 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 3 morphological groups were identified simply as *Pseudo-nitzschia* sp.

DA analysis

Water collected from Niskin bottles was used for particulate and dissolved domoic acid (PDA and DDA, respectively) determination at the surface and DCM at Stns 15 and 17–27 as well as at additional depths for Stns 18, 19, 24, and 25. In addition, PDA samples were collected at 37 underway sites between station locations via the shipboard surface seawater inflow system.

Samples for PDA determination were filtered from 500 to 2000 ml 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. Filters and filtrate samples were stored at –80°C until extraction. Filters were extracted using 2.5 ml or 5.0 ml aqueous methanol (10%) prior to centrifugation for 1 min at 6000 × *g*, followed by filtration through a 0.22 µm syringe filter if liquid chromatography/mass spectrometry (LC/MS) was performed (Bates et al. 1991). Extracts were stored at –20°C.

PDA and DDA samples were analyzed using direct competitive ASP enzyme-linked immunosorbent assay (ELISA) (Biosense Laboratories; Kleivdal et al. 2007). Subsequent analysis was conducted according to manufacturer's instructions, with the exception that PDA was extracted into 10 ml of ultrapure distilled water (Milli-Q, MilliPore) rather than into dilute MeOH. The detection limit for this method is approximately 10 pg ml^{-1} in PDA extracts and DDA filtrates.

To confirm the presence and level of DA, selected samples (n = 19), 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 AB SCIEX API 4000 triple quadrupole mass spectrometer (AB SCIEX). Chromatographic separation was performed on a Phenomenex Luna C18(2), 5 µm, 150 × 2 mm column, and retention time of DA in samples was compared to a certified reference standard (NRC Canada, Halifax). The DA fragments monitored were *m/z* 266, 248, 193, and 161 from protonated DA molecules (*m/z* 312) using Multiple Reaction Monitoring (MRM) scanning mode. The limit of quantitation for this method was ~0.2 ng l^{-1} seawater (particulate) with a signal to noise ratio above 10 for the primary MRM channel *m/z* 312 to 266.

Characterization of environmental regimes

We analyzed environmental data to categorize stations according to significantly different environmental regimes. First, we examined 12 environmental variables (temperature, salinity, chlorophyll (chl) *a* fluorescence, depth of DCM, nitrate, nitrite, ammonium, silicic acid, phosphate, iron, manganese, and total cobalt) for 49 sites across Stns 1 to 27, at both the surface and DCM, to exclude environmental variables that were strongly co-correlated (defined as those with r^2 values > 0.85 for either Pearson linear or Spearman's rho non-parametric correlations, calculated in SPSS® Statistics 19). For these pairs, only 1 variable was retained, such that DCM depth, nitrate, phosphate, and total cobalt were excluded.

We conducted both qualitative and quantitative analyses of 8 remaining environmental variables: temperature, salinity, chl *a* fluorescence, nitrite, ammonium, silicic acid, iron, and manganese. First, we performed principal components analysis (PCA), using PC-ORD v.5.10 (McCune & Mefford 2011). Data were not normally distributed regardless of multiple transformations applied and were therefore square-root-transformed and relativized across variables as per McCune & Mefford (2011). A clustering dendrogram was constructed to quantitatively compare resemblance among samples (based on Euclidean distance), using PRIMER-E v.6 (Clarke & Warwick 2001). Significantly different samples ($p \leq 0.05$) were determined by applying a similarity profile (SIMPROF) test to the cluster analysis. Based upon the SIMPROF results, we categorized station/depth sites according to significantly different environmental regimes. Temperature-salinity plots were used to categorize sites with missing data points (Stns 23 and 25) in Ocean Data View 4 (Schlitzer 2012).

Pseudo-nitzschia species and community composition: partial ITS1 clone library construction

To collect *Pseudo-nitzschia* DNA, 1 l of seawater was filtered onto a 0.2 µm Supor filter, placed in 1 ml sucrose lysis buffer (20 mM EDTA, 400 mM NaCl, 0.75 M sucrose, and 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). Four samples were selected for ITS1 clone library construction, each corresponding to a different environmental regime: Stn 13 surface, Stn 13 70 m, Stn 19 surface, and Stn 25 surface.

To amplify the variable-length portion of the ITS1 region, PCR was performed in 20 µl triplicate reactions, using 2.5 mM $MgCl_2$, 1X buffer (Promega), 0.15 µl *Taq* polymerase (GeneChoice), 0.4 mM dNTPs, and 0.5 µM of each of the PnAll-F (5′-TCT TCA TTG TGA ATC TGA-3′) and PnAll-R (5′-CTT TAG GTC ATT TGG TT-3′) primers (Hubbard et al. 2008). Through PCR optimization experiments utilizing a range of DNA concentrations, we determined that a total of 32 cycles was within the linear range of the PCR for the DNA amounts used from all samples (10 ng for Stns 19 and 25, 30 ng for Stn 13 surface and DCM). PCRs were then reconditioned for 3 cycles using 5 µl of initial PCR product and 15 µl of fresh PCR reagents (Thompson et al. 2002). Replicate PCR products were pooled and purified using a MinElute PCR Purification kit (Qiagen) and cloned into One Shot TOP 10 *Escherichia coli* cells, using the TOPO TA Cloning Kit (Invitrogen). Up to 96 clones per clone library were sequenced using the universal M13 vector forward primer (M13F (-21), 5′-TGT AAA ACG ACG GCC AGT-3′). Sequences were deposited in GenBank under accession numbers JX441017 to JX441088.

Clone library data analysis

Unique clone sequences were trimmed to exclude the PnAll primer regions and queried (via BLASTN) against nucleotide files downloaded from GenBank

on October 19, 2011. The top 5 hits (hereafter referred to as 'reference sequences') were collected for each clone sequence. First, clone sequences were checked for chimeras, using the uchime command in the bioinformatics software package mothur v.1.23.0 (Schloss et al. 2009); none were detected. Next, an initial alignment containing all unique clones (n = 72) and all reference sequences was created using BioEdit v.7.1.3 (Hall 1999) and ClustalX v.2.1 (Larkin et al. 2007). A distance matrix was calculated in PAUP* 4.0 (Swofford 2003), 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). Within the program mothur, the sequences were clustered using the average method to group the clones with the most similar reference sequences, 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). Each species-group cluster was aligned separately, and a distance matrix was calculated for each cluster as before (accounting for nucleotide divergence but not in/dels). Finally, for each clone library, rarefaction curves were constructed using EstimateS v.8.2.0 (Colwell 2012), in 3 forms, each of which compared the number of clones with (1) number of unique nucleotide sequences (genotypes); (2) number of predicted ARISA operational taxonomic units (OTUs; each ARISA OTU corresponded to a distinct ARISA peak as described below); or (3) number of species-groups, <18% average nucleotide divergence (within each species-group).

Pseudo-nitzschia community composition: ARISA

DNA extracts described earlier were used for characterization by ARISA, with new PCRs conducted using a FAM-labeled PnAll-R primer (Hubbard et al. 2008). As with clone library construction, 32 cycles of PCR were performed using either 10 ng (coastal Stns 16 to 27) or 30 ng (oligotrophic Stns 1 to 15) genomic DNA.

PCR products were purified using MultiScreen PCRμ96 filter plates (Millipore) and eluted with 25 ul ultrapure distilled water (Hubbard et al. 2014). Triplicate PCR products were pooled and quantified using the Qubit High-Sensitivity DNA Quantitation Kit (Invitrogen). Purified PCR products were diluted to a final DNA concentration of 0.1 ng l^{-1}, and 10 ng DNA were further purified using ethanol precipitation (Sambrook & Russell 2001) and then 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), 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 v.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 as a starting point, to create several different 'frames' of data (after Hewson & Fuhrman 2006). This macro binned the ARISA peaks into 2 bp bins, utilizing a 0.5 bp frame shift for a total output of 4 frames of data. Only peaks that were detected across all 4 frames were included in further analyses. We then calculated Sorensen (OTU presence/absence) and Bray-Curtis (relative OTU peak height) similarity coefficients for each of 4 frames of ARISA data. The maximum Sorensen and Bray-Curtis coefficients were calculated across all frames to create the most conservative test of the null hypothesis that communities did 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. First, *Pseudo-nitzschia* ARISA data were analyzed by quantitative clustering, using the statistical software PRIMER-E v.6 (Clarke & Warwick 2001). Data were visualized in 2 dendrograms, which either grouped together whole communities with other similar communities, or grouped together individual OTUs with similar distributions. Significantly different ($p \leq 0.05$) branches were identified on both dendrograms by applying similarity profile (SIMPROF) tests. To further examine the robustness of our results, we tested for significant differences among communities using 1-way ANOSIM to determine whether *Pseudo-nitzschia* communities differed according to environmental regimes, as identified in

previous analyses. ANOSIM statistics were performed separately for both Sorensen similarity (OTU presence/absence) and Bray-Curtis similarity (relative OTU peak height), whereas the clustering analysis and SIMPROF tests were performed on Bray-Curtis similarity. A heat map was generated (using a tool at www.chibi.ubc.ca/matrix2png/bin/matrix2png.cgi) to visualize the relative contributions of each ARISA OTU to each whole *Pseudo-nitzschia* community profile.

Correlations among *Pseudo-nitzschia* types and environmental parameters

Statistically significant correlations ($p < 0.05$, $q < 0.05$) among ARISA OTUs and environmental parameters (including PDA) were calculated as Pearson (linear) correlations via extended local similarity analysis (eLSA) (Xia et al. 2011, 2013). These correlations were determined from a dataset that included 19 environmental parameters and relative OTU peak height, for 29 station/depth combinations. The environmental data were square-root transformed, as conducted for PCA and SIMPROF analyses. The resulting matrix of environmental and ARISA data was analyzed as described in the eLSA wiki (https://bitbucket.org/charade/elsa/wiki/Home), using the settings -d 0 (no time delay), -s 29 (29 time spots), -b 0 (no bootstraps), -x 1000 (1000 permutations), and all other settings on default.

RESULTS

Characterization of environmental regimes in the South Atlantic Ocean

Across the South Atlantic Ocean and northern Benguela Upwelling Zone in November and December 2007, lower nutrient and fluorescence levels distinguished oligotrophic, open-ocean sites (Stns 1–15 & 25–27) from more eutrophic, coastal sites (Stns 16–24) (Fig. 1A). As determined by data from OSCAR, surface water currents were moving offshore (westward) along the entire cruise track (data not shown), in conjunction with cooler temperatures within the Benguela Upwelling Zone (Fig. 1B).

We analyzed *in situ* oceanographic conditions in order to identify significantly different environmental regimes across the South Atlantic Ocean and coastal stations inside and outside the northern Benguela Upwelling Zone. In total, 8 environmental variables were analyzed by PCA: temperature, salinity, silicic acid, ammonium, nitrite, fluorescence, iron, and manganese (Fig. 2). Within the resulting PCA (Fig. 2), Axis 1 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 open-ocean (left) to coastal (right) sites.

The individual data points within the PCA (Fig. 2) were categorized into 6 distinct environmental regimes, based upon a cluster analysis. Across the regimes, station/depth sites differed significantly from one another ($p \leq 0.05$; SIMPROF test), and each regime included stations that exhibited a Euclidean distance similarity of <2. Environmental regimes encompassed 3 coastal groups ('North Coastal,' 'Mid-Coastal,' and 'South Coastal'), 2 northern open-ocean groups ('North Open Ocean Surface' and 'North Open Ocean Deep' [DCM]), and one group that included southern offshore sites and northern DCM sites ('Transition') (Figs. 1 & 2). These 6 environmental regimes were used to test the hypothesis that *Pseudo-nitzschia* community composition differed significantly according to distinct environmental conditions.

Phytoplankton community composition

Phytoplankton size classes (Fig. 3) varied most markedly between coastal sites (multiple regimes) and sites categorized as either open ocean or transition regimes. Surface chl *a* levels were low (<0.4 µg l^{-1}) at open-ocean Stns 3, 9, 13, 15, and 17 and at Stns 25 and 27 (Transition regime), whereas total chl *a* ranged between 4.7 and 5.8 µg l^{-1} at coastal Stns 19, 21, and 23 (Fig. 3A). The relative chl *a* size fractionation (Fig. 3B) revealed a trend of 3 different types of communities: one community type dominated by large (>10 µm) phytoplankton (Stns 19 and 21); one community type comprised mainly of phytoplankton in the 1.2 to 10 µm size fraction (Stns 23, 25, and 27); and a third community type with a substantial (≥30%) proportion belonging to the 0.7 to 1.2 µm size fraction (Stns 3, 9, 13, 15, and 17).

Pseudo-nitzschia cells were detected across all 5 of the environmental regimes analyzed by microscopy, and this genus co-existed with other widespread diatom and dinoflagellate taxa. *Pseudo-nitzschia* cells

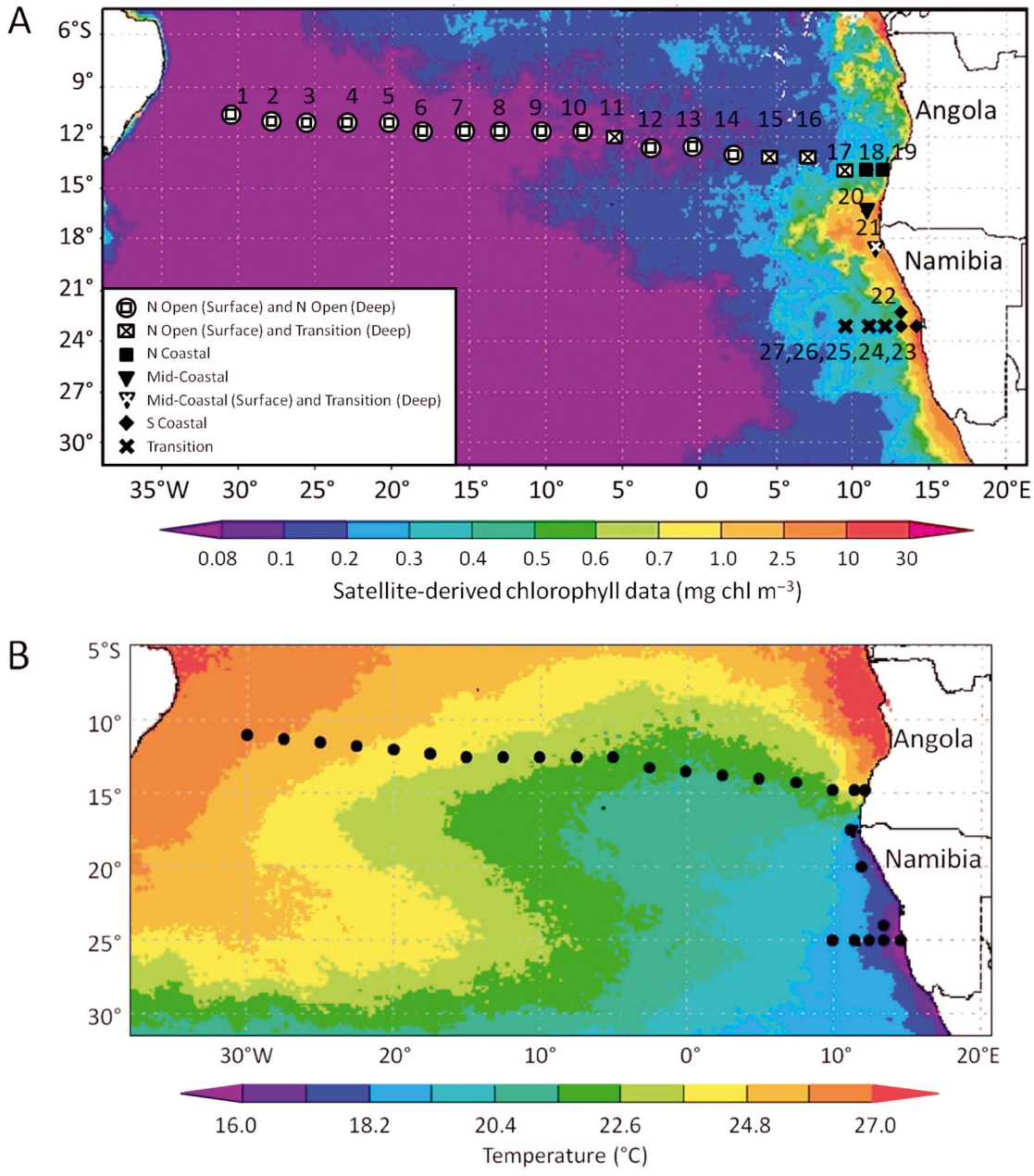

Fig. 1. Cruise stations overlain on (A) satellite-derived chlorophyll data (mg chlorophyll m^{-3}) and (B) temperature data obtained using the Giovanni online data system, averaged for the period of November to December 2007. Panel A incorporates station coding according to significantly different environmental regimes (Fig. 2), where 'N' signifies 'North' and 'S' signifies 'South'; panel B displays stations without numbers

comprised between 2.8 and 44.5% of the total preserved communities of diatoms and dinoflagellates at 7 stations (net tows; Fig. 4A) and 28.0% of Niskin sample at Stn 19 (Fig. 4B; net tow not available). In addition, we detected the HAB-forming genera *Alexandrium* and *Dinophysis* (Fig. 4A) and other dinoflagellates including *Protoperidinium*, *Ceratium*, *Pyrophacus*, and *Prorocentrum*. The diatom community included *Rhizosolenia*, *Chaetoceros*, *Skeletonema*, centric diatoms (either *Thalassiosira* or *Coscinodiscus*), and unidentified types (pennates and other morphologies). At Stn 18, a lower proportion of *Pseudo-nitzschia* was detected in the net tow (Fig. 4A), compared to the Niskin sample (Fig. 4B), perhaps because some of the small cells passed through the 10 µm mesh.

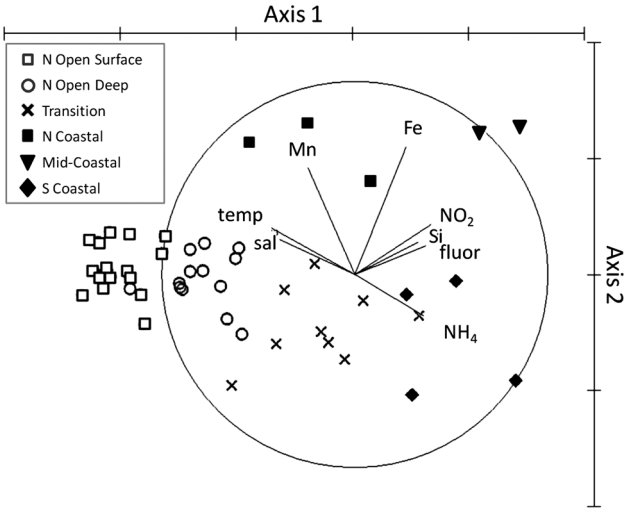

Fig. 2. Identification of 6 significantly different environmental regimes in the South Atlantic Ocean, where quantitative clustering and the similarity profile (SIMPROF) test results ($p \leq 0.05$) were overlain on principal components analysis (PCA), for surface and deep chlorophyll maxima (DCM) samples from Stns 1–22, 24, 26–27. Axis 1 was significant (eigenvalue greater than the broken-stick eigenvalue) and represented 56.0% of variance in the environmental data. In addition to nutrients and metals represented by standard symbols, abbreviations include 'Si' (as silicic acid), 'sal' (salinity), 'temp' (temperature), and 'fluor' (chl *a* fluorescence). In regime names, 'N' signifies 'North', and 'S' signifies 'South'

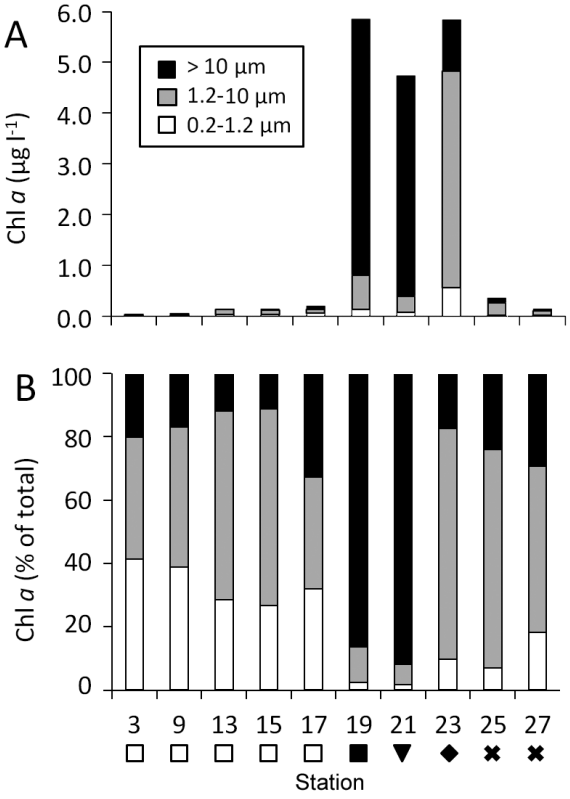

Fig. 3. Surface water size-fractionated chl *a*, as determined by acetone extractions, in each of 3 size fractions (>10 µm, 1.2–10 µm, and 0.7–1.2 µm), expressed as (A) absolute concentrations (µg l^{-1}) and (B) percentage of total chl *a*. Sites are categorized according to environmental regimes, including North Open Ocean (□), North Coastal (■), Mid-Coastal (▼), South Coastal (◆), and Transition (✖)

As enumerated from Niskin samples, *Pseudo-nitzschia* cells were present at North Coastal Stns 18 and 19 at concentrations of 6.5×10^4 cells l^{-1} and 6.2×10^4 cells l^{-1}, respectively, while few *Pseudo-nitzschia* cells were counted at Stn 21 (Fig. 4B). Furthermore, the extent of morphological diversity (measured by aspect ratio) indicated that ≥3 species were present across the cruise track. *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 Stns 18, 19, and 21 (Fig. 4B).

DA in the northern Benguela Upwelling Zone

PDA was detected across a large expanse of coastal African waters, including both inside and outside the northern Benguela Upwelling Zone. We detected PDA in the surface waters of 4 regimes: the North Coastal regime (Stns 18 & 19), the Mid-Coastal regime (Stn 21), the South Coastal regime (Stns 22 to 24), and the Transition regime (Stns 25 to 27) (Fig. 5). We found a strong linear correlation ($r^2 = 0.93$) between DA values determined for 19 PDA samples analyzed by both LC-MS (0.24 to 103 ng l^{-1}) and ELISA (0.88 to 184 ng l^{-1}), therefore verifying the ELISA results. According to ELISA, PDA levels in the surface waters ranged between 1.05 and 184 ng l^{-1}, with the highest DA levels recorded at Stn 18 (94 ng l^{-1}) and Stn 19 (184 ng l^{-1}). In addition to PDA detected in surface waters (Fig. 5), moderately high PDA (85 ng l^{-1}) was detected at 50 m depth at Stn 24 (Fig. 5). Detectable dissolved DA levels were found only in 4 samples at near-surface depths at Stns 18 and 19, measuring between 0.68 and 0.78 ng l^{-1}. When normalized for total number of *Pseudo-nitzschia* cells enumerated in Niskin samples (Fig. 4B), PDA measured 1.5 pg $cell^{-1}$ at Stn 18, 3.0 pg $cell^{-1}$ at Stn 19, and 4.6 pg $cell^{-1}$ at Stn 21.

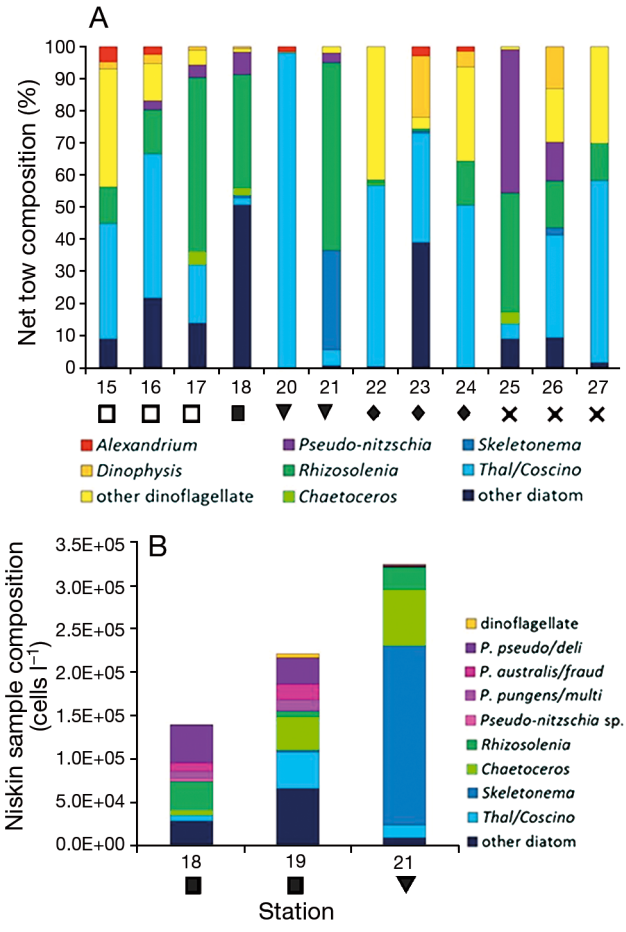

Fig. 4. Phytoplankton community composition in South Atlantic surface waters, as determined by light microscopy. (A) Net tow data for 12 stations, showing proportions of total dinoflagellate and diatom cells identified to genus level. (B) Niskin data for 3 stations, showing cells per liter of seawater identified as dinoflagellates, distinct genera of diatoms, and 3 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*. Sites are categorized according to environmental regimes, including North Open Ocean (□), North Coastal (■), Mid-Coastal (▼), South Coastal (◆), and Transition (✖)

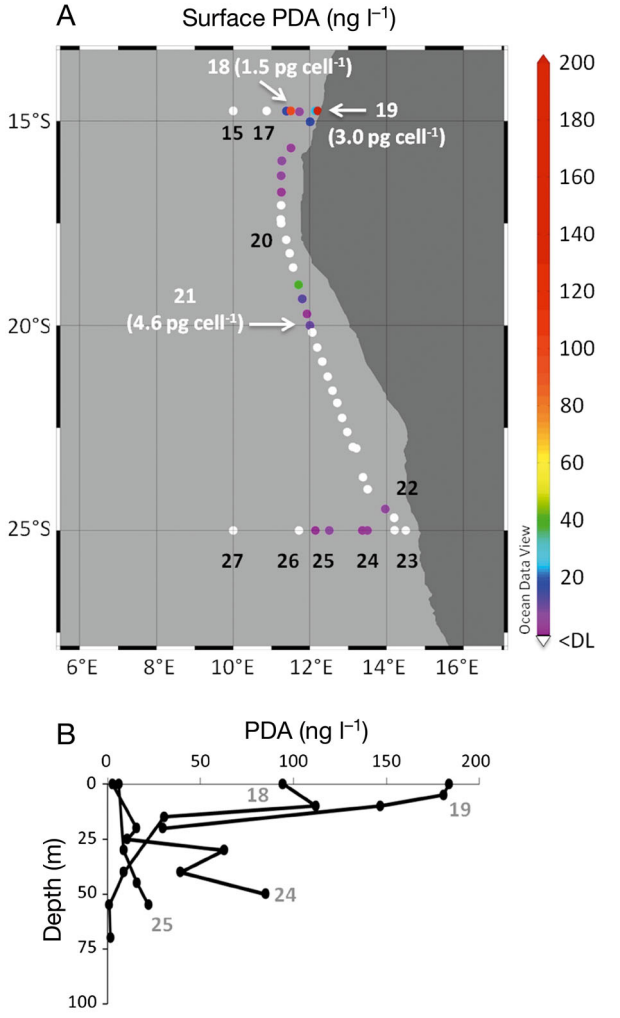

Fig. 5. (A) Particulate domoic acid (PDA) levels detected in surface waters at numbered cruise stations and underway points, as analyzed by enzyme-linked immunosorbent assay (ELISA). At stations where Niskin samples were collected (Stns 18, 19, and 21), DA expressed as pg cell^{-1} is also noted. The detection limit (DL) for ELISA is ~10 pg ml^{-1} in PDA extracts and DDA filtrates; white circles denote measurements <DL. (B) Depth profiles of PDA for Stns 18, 19, 24, and 25

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

We constructed partial ITS1 clone libraries for 4 communities representing 4 different environmental regimes: Stn 13 surface (North Open Surface regime), Stn 13 70 m (North Open Deep regime), Stn 19 surface (North Coastal regime), and Stn 25 surface (Transition regime). First, our clone library data validated the specificity of the PnAll primers for *Pseudo-nitzschia* as the majority of clones were identified as *Pseudo-nitzschia* and determined to be valid ARISA fragments (100 to 300 bp in length). The 227 validated clones were represented by 72 unique sequences. ARISA OTU type was predicted for these 72 sequences based on sequence length. By comparison with reference sequences from isolates with EM-verified species assignments, 10 previously described species and 1 unidentified species were represented among the South Atlantic sequences. Sequences were clustered based on a nucleotide divergence of <18%, which corresponded roughly to previously

Table 1. Identification of putative *Pseudo-nitzschia* species in 4 clone libraries from Stn 13 surface waters (S13 surface), Stn 13 deep chlorophyll maximum (S13 70 m), Stn 19 surface waters (S19), and Stn 25 surface waters (S25), along with predicted ARISA fragment lengths. Clones were clustered into species-groups defined by <18% nucleotide divergence and identified based on closest BLAST hit. 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. *denotes a novel ARISA fragment not reported previously

Species-group	No. of clones	ARISA fragment lengths (bp)	Community (clones/ genotypes)	Species and accession number	Closest reference sequence		
					Origin	Predicted ARISA length (bp)	Range of nucleotide divergence (%)
P. inflatula/ *P. micropora*	84	154*, 157*	S13 surface (26/4)	*P. inflatula*, DQ329204	Thailand	156	0–1.7
		163*, 164*	S13 surface (44/9), S13 70 m (2/1)	*P. inflatula*, DQ329204	Thailand	156	0–1.8
		170*, 171*	S13 surface (6/2)	*P. inflatula*, DQ329204	Thailand	156	0.80
		188*	S19 surface (3/1)	*P. micropora*, AY257847	Vietnam	146	6.80
		197*	S13 70 m (3/2)	*P. micropora*, AY257847	Vietnam	146	12
P. galaxiae	63	138*	S19 surface (22/4)	*P. galaxiae*, DQ336158	Australia	143	9.7–11.9
		149, 150*, 151, 152*	S19 surface (40/8)	*P. galaxiae*, EU327368	Spain	149	0–13.6
		156*	S19 surface (1/1)	*P. galaxiae*, EU327368	Spain	149	8.90
P. cf. *subpacifica* / *P. heimii* / *P.* sp. environmental samples	35	195	S19 surface (8/2)	*P. cf. subpacifica*, AY257858	Portugal	195	0–0.6
		196*	S25 surface (19/5)	*P.* sp. clone, EU068693 or	NE Pacific	195	5.6–7.5
				P. heimii, EU051655	NE Pacific	195	6.2–8.1
		217*, 218*, 219*, 221*	S25 surface (8/5)	*P.* sp. clone, EU068693 or	NE Pacific	195	5.6–9.3
				P. heimii, EU051655	NE Pacific	195	6.2–9.9
P. turgiduloides / *P. turgidula*	20	155*	S25 surface (12/5)	*P.* sp. clone, EU068695 or	NE Pacific	176	5.1–6.7
				P. turgiduloides, AY257839	Antarctica	175	8.2–9.1
		176, 177*, 178*, 179*	S25 surface (6/6), S13 70 m (2/1)	*P. cf. turgidula*, EU051653 or	NE Pacific	151	12–17
				P. sp. clone, EU068696	NE Pacific	176	0.7–19.4
P. cuspidata	18	216, 220*	S19 surface (10/5)	*P. cuspidata*, AY257852	Mexico	216	0.6–2.8
		226*	S19 surface (8/4)	*P. cuspidata*, AY257862	Australia	230	1.6–2.7
P. caciantha	3	225*	S19 surface (1/1)	*P. caciantha*, AY257861	Mexico	226	0
		229*, 230*	S13 surface (1/1), S19 surface (1/1)	*P. caciantha*, DQ813834	Italy	227	4.70
P. pungens	1	144	S19 surface (1/1)	*P. pungens*, EU327366	Spain	142	5.65
P. sp.	5	222, 223	S13 70 m (5/3)	No sequence <18% different			

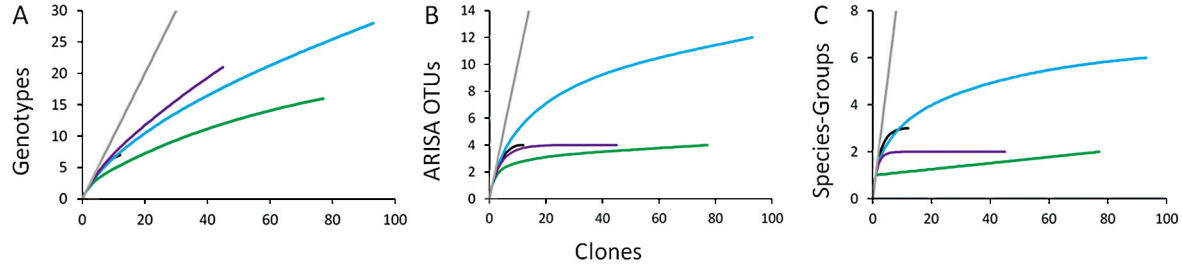

Fig. 6. Rarefaction curves constructed for 4 partial internal transcribed spacer 1 (ITS1) clone libraries, plotted according to increasing level of genotypic difference, including number of (A) distinct genotypes, (B) automated ribosomal intergenic spacer analysis (ARISA) operational taxonomic units (OTUs), or distinct ARISA peaks, and (C) species-groups, as defined by <18% nucleotide divergence. Lines denote Stn 13 surface community (green; North Open Surface regime), Stn 13 70 m community (black; North Open Deep regime), Stn 19 surface community (blue; North Coastal regime), Stn 25 surface community (purple; Transition regime), and 1:1 line (gray)

described species (Table 1). The 8 clusters or 'species-groups' (in order of abundance in the clone libraries) were (1) *P. inflatula/P. micropora*, (2) *P. galaxiae*, (3) *P. subpacifica/P. heimii*, (4) *P. turgiduloides/P. turgidula*, (5) *P. cuspidata*, (6) *Pseudo-nitzschia* sp., (7) *P. caciantha*, and (8) *P. pungens*.

Pseudo-nitzschia community composition was generally well sampled by clone libraries, and richness varied considerably across the South Atlantic (Fig. 6). Genotypic diversity, as defined by unique nucleotide sequences, did not plateau for any community (Fig. 6A), suggesting we sampled only a subset of ITS1 genotypes in all communities. However, in terms of predicted ARISA OTUs (Fig. 6B) and species-groups (<18% nucleotide divergence; Fig. 6C) the 4 communities appear more thoroughly sampled. Using these 2 metrics, the community at Stn 19 was the richest. ARISA OTU richness, which reflects intraspecific diversity (Hubbard et al. 2008), was approximately twice the magnitude of species-group richness. In clone libraries constructed from Stn 13 70 m and Stn 25, fewer *Pseudo-nitzschia* clones were found (e.g. only 12 clones at Stn 13 70 m). *Pseudo-nitzschia* cells may have been in lower relative abundance among these communities compared to the surface water communities at Stns 13 and 19.

The 4 South Atlantic *Pseudo-nitzschia* communities were markedly distinct based on putative species composition (Fig. 7). The *Pseudo-nitzschia* community in Stn 19 surface waters was the most diverse, comprising 6 of the 8 species-groups. Strikingly, only 2 species-groups were detected in >1 clone library: *P. inflatula/P. micropora* (Stn 13 surface, Stn 13 70 m, and Stn 19 surface) and *P. subpacifica/P. heimii* (Stns 19 and 25 surface). Overall, the most numerous sequences were from *P. inflatula* (78 clones), *P. galaxiae* (63 clones), and *P. heimii* (27 clones) (Table 1).

We detected a substantial amount of previously unreported diversity within the *Pseudo-nitzschia* genus, based upon ARISA fragment length. The majority of the predicted ARISA OTU fragment lengths differed from predicted lengths of the closest reference sequences (Table 1). Here, we report new ARISA types for 7 putative species. These include a *P. heimii*

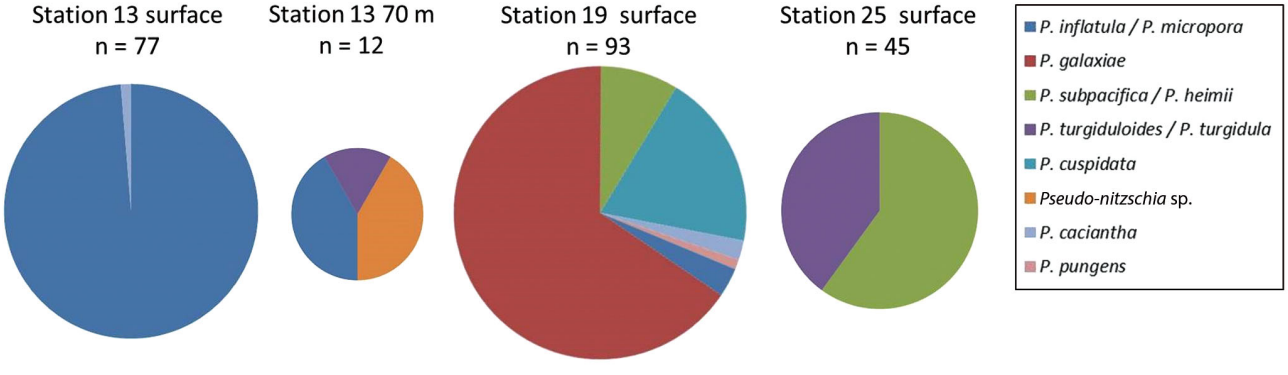

Fig. 7. Composition of 4 partial internal transcribed spacer 1 (ITS1) clone libraries based on 8 different *Pseudo-nitzschia* species-groups defined by <18% nucleotide divergence. Total number of clones per library (n) is also shown

type (ARISA = 217 to 221 bp), 2 new *P. galaxiae* types (ARISA = 138 and 156 bp), *P. inflatula* (163 to 164 bp, 170 to 171 bp), *P. micropora* (188 bp, 197 bp), *P. turgidula* (155 bp, 179 bp), *P. caciantha* (230 bp), and *P. cuspidata* (220 bp, 226 bp). Some of these cases may 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, with nucleotide divergence of 6.8% and 12%, respectively (not accounting for in/dels). Furthermore, genotypic richness is apparent among *Pseudo-nitzschia* types of the same species-group and ARISA OTU length. Many commonly detected ARISA types included different genotypes within the same community, such as *P. heimii* 196 bp (5 genotypes among 19 clones from Stn 25 surface), *P. galaxiae* 138 bp (4 genotypes among 22 clones from Stn 19 surface), *P. galaxiae* 149 to 152 bp (8 genotypes among 40 clones from Stn 19 surface), and *P. inflatula* 163 to 164 bp (9 genotypes among 44 clones from Stn 13 surface). However, despite the diversity of genotypes present for many ARISA types, a single genotype tended to dominate the clone sequences for each fragment type (data not shown).

In addition to the discovery of novel ARISA types, we detected several South Atlantic clones that were similar to reference sequences from geographically distant regions. For example, genotypes of *P. subpacifica* OTU 195 (detected at Stn 19 surface) were identical in ARISA fragment length to, and displayed only 0 to 0.6% nucleotide divergence from, *P. cf. subpacifica* isolated from Costa Nova, Portugal (Lundholm et al. 2003) (Table 1). *P. inflatula* OTUs 154/157 (Stn 13 surface) displayed 0 to 1.7% nucleotide divergence from the 156 bp long sequence of *P. inflatula* from Phuket, Thailand (Priisholm et al. 2002) (Table 1). Finally, some *P. turgidula* OTU 176-179 sequences (Stn 25) displayed as little as 0.7% nucleotide divergence with a 176 bp long *P.* sp. environmental clone detected at Ocean Station Papa in the Northeast Pacific (Marchetti et al. 2008).

Pseudo-nitzschia community composition and geographic distribution: ARISA

Members of the *Pseudo-nitzschia* genus were distributed widely throughout the South Atlantic Ocean and comprised diverse communities, as assessed by ARISA (Fig. 8). Compared to light microscope analyses, which detected *Pseudo-nitzschia* at only 7 stations, *Pseudo-nitzschia* were detected by ARISA at 17 of 20 stations, including sites within all 6 environmental regimes. In total, 37 distinct *Pseudo-nitzschia* ARISA OTUs were detected. Notably, we detected *Pseudo-nitzschia* in the surface waters and deep chlorophyll maxima of several stations in the open South Atlantic Gyre (Stns 7, 9, 11, and 13). Nine *Pseudo-nitzschia* OTUs (135, 149, 151, 153, 175, 179, 195, 219, and 225) were detected at ≥25% of the sites. Of the 37 OTUs detected, 10 OTUs contributed >30% of the total peak height within any single community (135, 139, 149, 153, 161, 163, 169, 195, 223, and 225 bp; Fig. 8). Only 3 of these 10 'dominant' OTUs were detected among ≥50% sites within an individual regime (Fig. 8). OTU 135 was dominant at North Open Deep sites, OTU 153 was dominant at North Open Surface sites, and OTU 225 was dominant at North Coastal sites and at Mid-Coastal sites. We were unable to assign a species to OTU 135 but putatively identified OTU 153 as either *P. inflatula* or *P. turgidula* and OTU 225 as either *P. cuspidata* or *P. caciantha*. Thus, overall, few *Pseudo-nitzschia* types were distributed broadly across the study region.

Pseudo-nitzschia community composition was significantly different across the different environments, including broadly between Open Ocean/Transition regimes and Coastal regimes, between surface and deep communities in the open ocean, and between inside and outside of the northern Benguela Upwelling Zone in the coastal ocean. First, ANOSIM tests determined that all of the pairwise community comparisons differed significantly, using either presence/absence or relative peak height data, for communities categorized *a priori* according to the 6 environmental regimes (Table 2). Second, cluster analysis of ARISA data resulted in 7 significantly different clusters, which (numbered from top to bottom, Fig. 8) represent mainly North Open and Transition DCM (Group 1), North Open and Transition surface (Group 2), South Coastal and Transition (Group 3), Stn 24 (Group 4), Stn 19 (Group 5), Stn 21 (Group 6), and Coastal/Transition regime communities (Group 7). Notably, in both analyses, *Pseudo-nitzschia* communities differed by depth in the open ocean, but not in coastal regions (Fig. 8, Table 2).

Biogeographic ranges were apparent for several OTUs (Fig. 8). Open ocean communities included OTUs 135 and 195, whereas OTUs 151, 221, and 287 were more predominant among DCM waters. Along the coast, OTUs 149 and 225 tended to co-occur (Fig. 8). The more broadly distributed OTU 225, putatively identified as *P. cuspidata* or *P. caciantha*,

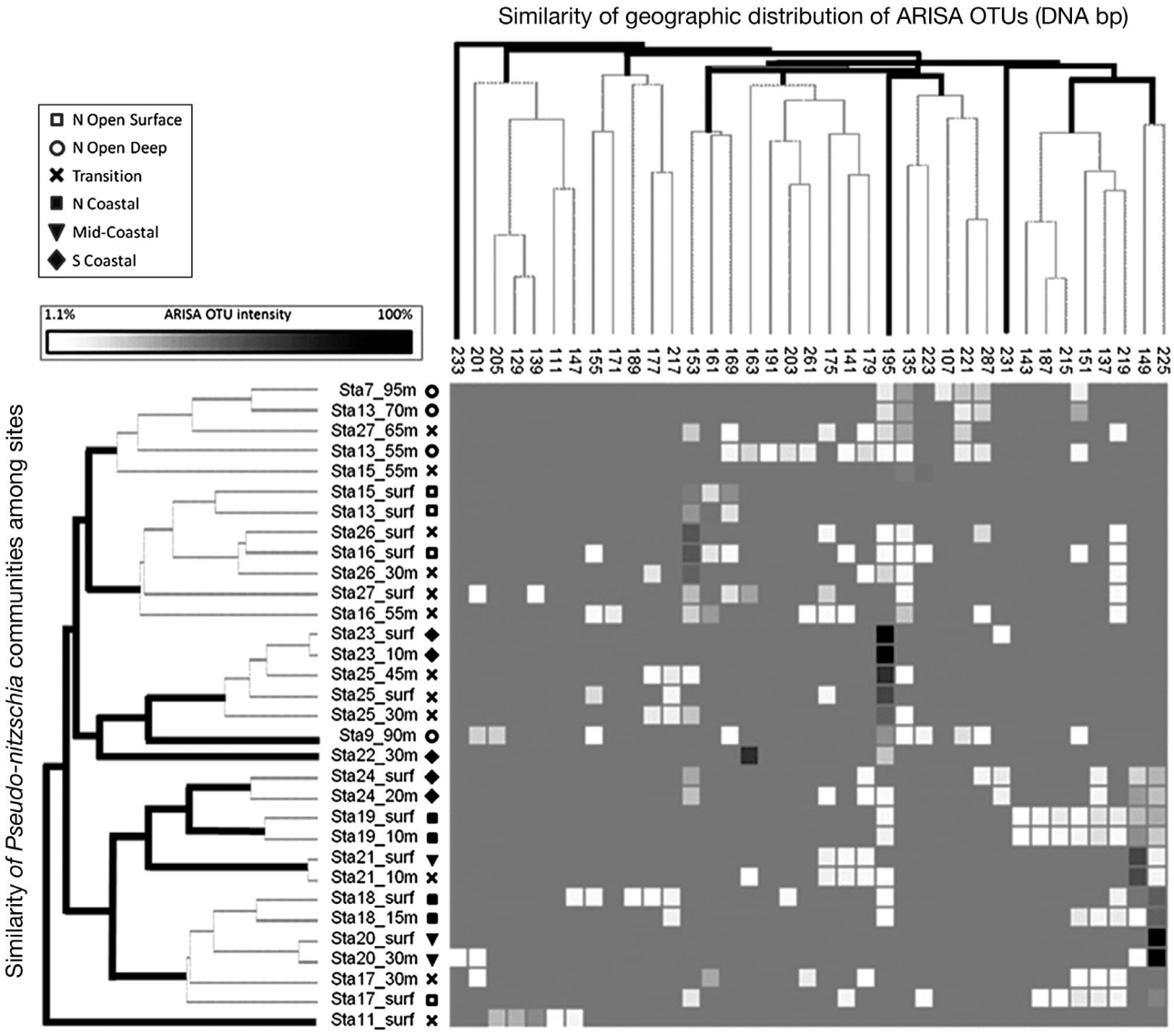

Fig. 8. *Pseudo-nitzschia* community composition in the South Atlantic Ocean based upon ARISA. Heat map shows relative contribution of each ARISA fragment (DNA bp), such that darker coloring indicates greater relative intensity of ARISA OTU within each individual community. Quantitative clustering dendrograms display Bray-Curtis similarity among whole profiles (left dendrogram) and similarity among individual OTU geographic distribution (upper dendrogram). Abbreviations in sample names indicate station and depth (deep chlorophyll maximum or surface), e.g. 'Sta7' = Stn 7; '95m' = deep chlorophyll maximum at 95 m depth; '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 ($p \leq 0.05$). Communities are coded according to 1 of 6 environmental regimes: North (N) Open Surface, North (N) Open Deep, Transition, North (N) Coastal, Mid-Coastal, and South (S) Coastal

was detected in 12 near-coastal communities (Fig. 8) but was not dominant in other communities. In contrast, *P. galaxiae* OTU 149 was found mainly in communities where DA was also detected (e.g. Stns 18, 19, 21, and 24; Fig. 5). A single OTU, 195, was detected in 20 of 32 communities examined (Fig. 8); however, based upon comparison of predicted *Pseudo-nitzschia* ARISA fragments and clone library sequence data, this OTU was likely represented by 2 different species-groups: *P. subpacifica*/*P. heimii* at Coastal and Transition stations and *P. micropora* in the open ocean.

We used local similarity analysis to generate hypotheses about the dominance of certain *Pseudo-nitzschia* ARISA types under specific environmental conditions. Significant correlations with environmen-

Table 2. ANOSIM statistics testing the hypothesis that *Pseudo-nitzschia* community composition (based on ARISA) differed significantly according to each of 6 significantly different environmental regimes, where 'N' signifies 'North' and 'S' signifies 'South.' Gray-shaded panels highlight significant differences ($p < 0.15$ and $R > 0.2$)

Groups	Semi-quantitative (Bray-Curtis similarity) R	p	Presence–absence (Sorensen similarity) R	p	Both analyses No. perm.
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

tal variables (those with both Pearson correlations [$p < 0.05$] and a low probability of false positives due to random chance [$q < 0.05$]; Xia et al. 2011, 2013) were observed for 10 OTUs. Of these, 5 were OTUs detected at ≥4 sites (Fig. 8): 135, 169, 195, 221, and 225 bp (Table 3). Unidentified OTUs 135 bp and 169 bp were distributed in both surface and DCM waters of Open Ocean and Transition regimes (Fig. 8) but were correlated with different nutrient ratios (Table 3), and their geographic distributions did not overlap completely (Fig. 8). *P. cuspidata/ P. heimii/ Pseudo-nitzschia* sp. OTU 221 displayed a different pattern: it was detected only in DCM waters of Open Ocean and Transition

Table 3. Significant Pearson correlations ($p < 0.05$, $q < 0.05$) determined by extended local similarity analysis between environmental parameters and *Pseudo-nitzschia* types (identified by ARISA fragment length in DNA bp and putative species identification [Table 1, clone library analysis]). Pearson correlation coefficent values are listed for significant correlations; '–' indicates insignificant correlations. In addition to nutrients and metals represented by standard symbols, 'DCM depth' denotes the depth of the deep chlorophyll maximum, 'Fluor' denotes chl *a* fluorescence, and 'PDA' denotes particulate domoic acid. OTUs detected at ≥4 sites (Fig. 8) are underlined

Pseudo-nitzschia type	DCM depth	Salinity	Fluor	NH_4	Si	Fe	Co	N:P	N:Fe	Si:Fe	PDA
107 bp unidentified OTU	0.551	0.585	–	–	–	–	–	–	–	–	–
135 bp unidentified OTU	–	–	–	–	–	–	–	–	0.639	0.729	–
143 bp *P. pungens*	–	–	0.529	–	–	–	–	–	–	–	0.667
169 bp unidentified OTU	–	–	–	–	–	–	–	0.504	–	–	–
195 bp *P. subpacifica, P. heimii,* or *P. micropora*	–	–	–	–	0.546	–	–	–	–	–	–
215 bp *P. cuspidata* or *P. heimii*	–	0.507	–	–	–	–	–	–	–	–	–
221 bp *P. cuspidata, P. heimii,* or *P.* sp.	0.516	–	–	–	–	–	–	–	–	–	–
223 bp *P. caciantha*, *P. heimii*, or *P.* sp.	–	–	–	–	–	–	–	–	0.577	0.595	–
225 bp *P. caciantha, P. cuspidata* or *P.* sp.	–	–	–	–	–	0.733	0.546	–	–	–	–
231 bp *P. caciantha*	–	–	–	0.617	–	–	–	–	–	–	–

Table 4. Compilation of culture studies, including a representative reference for lowest detected domoic acid (DA) in culture ('min'; often 'ND' or nondetectable by methods employed) and single highest reported DA level ('max') for species most similar to sequences detected in the South Atlantic Ocean via partial ITS1 clone libraries (Table 1). Two review papers cited herein present information on presence/absence of DA production (Table 1 in Lelong et al. 2012) and maximum DA per study (Table 3 in Trainer et al. 2012)

Species	No. of clones in library	Culture toxigenicity (min to max pg DA cell^{-1}l)	Reference (min DA; max DA)
Species detected at Stn 19 (184 ng l^{-1}; 3.0 pg cell^{-1})			
P. galaxiae	63	ND to 3.6 × 10^{-4}	Quijano-Scheggia et al. (2010); Cerino et al. (2005)
P. cuspidata	18	ND to 3.1 × 10^{-2}	Lelong et al. (2012); Lundholm et al. (2012)
P. subpacifica	8	ND	Lelong et al. (2012)
P. micropora	3	Not tested	Lelong et al. (2012)
P. caciantha	2	ND	Lelong et al. (2012)
P. pungens	1	ND to 0.47	Guannel et al. (2011); Rhodes et al. (1996)
Species detected at Stn 25 (5.98 ng l^{-1})			
P. heimii	27	ND	Marchetti et al. (2008)
P. turgiduloides	12	ND	Lelong et al. (2012)
P. turgidula	6	ND to 0.09	Marchetti et al. (2008); Bill (2011)

regimes (Fig. 8) and exhibited a significant correlation with DCM depth (Table 3). Significant correlations were also found for 2 OTUs that dominated Coastal and Transition regimes (Fig. 8): *P. caciantha*/*P. cuspidata*/*Pseudo-nitzschia* sp. OTU 225 was positively correlated with iron and cobalt, whereas *P. subpacifica*/*P. heimii*/*P. micropora* OTU 195 was positively correlated with silicic acid (Table 2).

We also compared *Pseudo-nitzschia* species composition at Stns 19 and 25 (Fig. 7) with DA production by these species determined in previously published culture studies (Table 4). At Stn 19 (PDA = 184 ng l^{-1}; 3.0 pg cell^{-1}), *P. galaxiae* sequences dominated the clone library (n = 63 of 93), suggesting that *P. galaxiae* cells were a major component of the *in situ Pseudo-nitzschia* community. However, in culture studies, DA production by *P. galaxiae* has been either not detected or several orders of magnitude lower (Table 4) than the per-cell toxigenicity found at Stns 18, 19, and 21 (1.5 to 4.6 pg DA cell^{-1}; Fig. 5). Similarly, only 2 of the other species detected at Stn 19 (*P. cuspidata, P. pungens*) have been reported to produce DA in culture (Table 4). Furthermore, the species composition at Stn 25 (PDA = 5.98 ng l^{-1}) included species that have not produced detectable DA in culture (*P. heimii*, *P. turgiduloides*) and 1 species (*P. turgidula*) that produced DA in culture (0.09 pg DA cell^{-1}) at orders of magnitude lower than per-cell DA values for Stns 18, 19, and 21 (1.5 to 4.6 pg DA cell^{-1}). Therefore, conflicting results of *Pseudo-nitzschia* species toxigenicity exist between laboratory culture findings and measured field toxigenicity in the present study.

DISCUSSION

Our survey of South Atlantic *Pseudo-nitzschia* communities revealed new diversity within the genus, at 2 major levels: intraspecific diversity (novel ARISA types) and potentially new species (based on nucleotide divergence). New ARISA types were detected for 7 putative species. For example, we detected at least 3 distinct ARISA types of *P. galaxiae* and 3 ARISA types most closely related to the single *P. inflatula* isolate reported in the literature (Priisholm et al. 2002). The 3 putative *P. inflatula* types coexisted with one another in at least 1 water parcel (Stn 13 surface waters), indicating either intraspecific niche differentiation or nonfunctional microdiversity. Second, some of the previously unreported *Pseudo-nitzschia* types may represent novel species. For example, 2 *Pseudo-nitzschia* types most closely related to *P. micropora* possessed large insertions in the ARISA fragment relative to the reference isolate. South Atlantic types measured 188 and 197 bp across this region compared to a predicted length of 146 bp for the reference isolate.

Our detection of *Pseudo-nitzschia* in the open South Atlantic Ocean builds upon an evolving view of *Pseudo-nitzschia* as common members of open-ocean environments; furthermore, we show for the first time that distinct depth-dependent populations exist. Initially, the presence of *Pseudo-nitzschia* in the open ocean was indicated by its dominance in open-ocean incubation and fertilization experiments (e.g. De Baar et al. 2005). Subsequently, *Pseudo-nitzschia* has been detected in open-ocean waters, using both molecular methods (Ribalet et al. 2010,

present study) and microscopy (Silver et al. 2010, Durkin et al. 2012). Here, we found *Pseudo-nitzschia* at many sites in the open South Atlantic Ocean. In addition, *Pseudo-nitzschia* community composition differed significantly between surface and DCM waters in the open ocean, a novel finding for *Pseudo-nitzschia* biogeography. *Pseudo-nitzschia* communities could be driven by parameters that vary with depth, such as light and nutrients, as has been observed in ecotypes of the diatom *Skeletonema* (Gallagher & Alberte 1985) and the cyanobacteria *Prochlorococcus* (Moore et al. 1998) and *Synechococcus* (Ahlgren & Rocap 2006). Similarly, photosynthetic and heterotrophic bacterial communities co-existing in these waters (sampled during the same cruise) differed between surface and DCM waters, with a more pronounced difference at open ocean sites (Morris et al. 2010, 2012).

Some South Atlantic *Pseudo-nitzschia* sequences displayed high similarity to geographically distant *Pseudo-nitzschia*, implying 'open-ocean' types may be able to survive a spectrum of conditions. Specifically, we found that *Pseudo-nitzschia* communities in the Transition regime included a mixture of types that dominated the North Open Surface and North Open Deep regimes, indicating the ability of 'open-ocean' *Pseudo-nitzschia* types to persist in waters closer to shore. In contrast, the coastal *Pseudo-nitzschia* communities differed markedly from the Open and Transition regimes. A similar scenario has been observed for *Pseudo-nitzschia* communities in the Northeast Pacific (Ribalet et al. 2010). In this case, the community at an ecotone (mixing of coastal and open-ocean waters) was also more similar to open-ocean communities than to coastal ones. The Northeast Pacific community included ARISA types identified as belonging to the *P. heimii*/*P. subpacifica* group as well as an unidentified ARISA type with length 176 bp. Our counterpart 'Transition' regime community at Stn 25 was most similar in nucleotide sequence to sequences from the NE Pacific (Table 1) and was dominated by *P. heimii*/*P. subpacifica* and *P. turgiduloides*/*P. turgidula*, including a 176 bp ARISA fragment we were able to assign to *P. turgidula.* These studies suggest that coastal *Pseudo-nitzschia* types may in fact be more geographically limited in comparison to open-ocean types, allowing open-ocean types to dominate waters at the boundary of oligotrophic and eutrophic regimes. Lelong et al. (2012) similarly reported that *P. turgidula* and *P. heimii* have been found in both open-ocean and coastal waters. These species may possess flexible physiologies, enabling them to persist despite differences between open-ocean and coastal waters in parameters such as temperature, salinity, and nutrients.

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 include common coastal *Pseudo-nitzschia* species such as *P. australis*, *P. multiseries*, and *P. pungens* (Hasle 2002). Strikingly, we did not detect either *P. australis* or *P. multiseries*, and we detected only a single clone of *P. pungens* (*P. pungens* var. *pungens*). In addition, we did not detect other common cosmopolitan species such as *P. delicatissima* and *P. fraudulenta* (Hasle 2002). Our detection of *P. galaxiae* was novel for African waters; this species has previously been detected near Mexico, Australia, and the Mediterranean and Sargasso Seas (Lundholm & Moestrup 2002, Hasle 2002, Cerino et al. 2005, McDonald et al. 2007, Quijano-Scheggia et al. 2010). Although we cannot make definitive conclusions about this disparity by comparing isolated, short-term studies, it is possible that seasonal patterns in species dominance exist throughout the Angola and Benguela systems, as shown for other ocean regions (Fryxell et al. 1997, Fehling et al. 2006, Klein et al. 2010). For example, *P. australis* was detected within this region during blooms in March and April (Marangoni et al. 2001, Seeyave et al. 2009), but not in the November to December sampling conducted in the present study.

Here, we present evidence of potentially ecologically harmful levels of DA (Scholin et al. 2000, Trainer et al. 2000) both inside and outside the northern Benguela Upwelling Zone, a region that includes organisms documented to suffer severe impacts of DA (e.g. sea lions) or to act as vectors of the toxin to humans (e.g. oysters). 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 timeframe of our study, surface currents indicated upwelling conditions throughout the sampling region. Therefore, it is likely that the DA production could 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 toward coastal shellfish beds (Adams et al. 2000, 2006, MacFadyen et al. 2005). As offshore organisms commonly act as vectors of DA (anchovies and sardines) or are sensitive to DA (sea lions and sea

birds), the occurrence of DA in the northern Benguela Upwelling Zone is a concern for impacts on marine ecosystems. Notably, however, DA levels of 5 pg cell^{-1} were measured in phytoplankton of Luanda Bay (northern Angola) in November of 2007, the same time frame we report here, and DA was also detected in bivalve samples, although at low levels (Blanco et al. 2010). 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. Simple correlations were not able to identify obvious environmental triggers of DA, perhaps because DA production was triggered by an unmeasured variable or by different variables in different samples or because of a time delay between environmental triggers and DA production. With the exception of a handful of stations (18, 19, and 25), the *Pseudo-nitzschia* communities we sampled, while consistently present, could not be considered to be in the midst of a bloom, making it more difficult to tease out environmental drivers of DA across the entire dataset.

The moderately high PDA levels we detected occurred at stations that hosted *Pseudo-nitzschia* species commonly believed to be weakly toxigenic or non-toxigenic — a finding that should caution stakeholders against making assumptions about public health risks based upon the presence of commonly known 'toxigenic' types. Indeed, the per-cell DA levels detected in the northern Benguela Upwelling Zone (1.5 to 4.6 pg DA cell^{-1}) were near the same level of magnitude (>1 pg DA cell^{-1}) of highly toxigenic species such as *P. multiseries*, *P. australis*, and *P. cuspidat*a (e.g. Trainer et al. 2012 for review). These DA levels exceed the previously reported highest per-cell DA levels off Lambert's Bay (Hubbart et al. 2012). Potential DA producers include *P. galaxiae* types, which dominated the clone library at the high-DA Stn 19 and were detected across other DA sites, although they were not correlated with PDA. *P. galaxiae* has been described elsewhere as a weakly toxigenic species, based upon laboratory studies (Table 4). At Stn 25, low levels of DA were detected in association with 2 other species groups, *P. heimii* and *P. turgiduloides*/*P. turgidula,* which have also previously been reported to produce low/undetectable DA in culture (Table 4). Similarly, in NW Subarctic Pacific communities (Buesseler et al. 2008), relatively high field levels of DA were reported by Silver et al. (2010) for species previously believed to be nontoxigenic or weakly toxigenic; specifically, up to 1.9 pg DA cell^{-1} was detected among *P.* cf *turgidula* and up to 0.7 pg DA cell^{-1} was detected among *P. lineola*. We attribute this disparity to the likelihood that cells in the field experience different environmental conditions compared to culturing conditions (i.e. different environmental triggers of DA production); therefore, the presence of any *Pseudo-nitzschia* species should be regarded as a warning for public health.

By documenting *Pseudo-nitzschia* and DA throughout coastal African waters inside and outside the northern Benguela Upwelling Zone, we provide further evidence for the possibility of ASP occurring in this region. Our findings highlight the likelihood that the toxigenicity of known *Pseudo-nitzschia* species has not been fully described. We also identified *Pseudo-nitzschia* sequences significantly divergent from those of known species, demonstrating that diversity within this genus remains to be explored. Consequently, researchers, resource managers, policymakers, and other stakeholders should avoid overreliance upon current understanding of *Pseudo-nitzschia* species toxigenicity when forecasting DA impacts. In time, increasingly greater numbers of *Pseudo-nitzschia* species may well be shown to have serious consequences for humans and other organisms.

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