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Connectivity of toxigenic *Pseudo-nitzschia* species assemblages between the Northeast U.S. continental shelf and an adjacent estuary

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A B S T R A C T

Pseudo-nitzschia harmful algal blooms have recently caused elevated domoic acid in coastal environments of the Northeast United States. In 2017, the toxigenic species *P. australis* was observed in Narragansett Bay, Rhode Island, a temperate estuarine ecosystem, for the first time since 2009 when DNA monitoring for *Pseudo-nitzschia* species began. This highly toxic species likely contributed to toxin-related shellfish harvest closures and is hypothesized to have been introduced by an offshore source. Little is known about offshore *Pseudo-nitzschia* spp. populations in the Northeast Continental Shelf marine ecosystem or how often toxigenic species enter Narragansett Bay through physical processes. Here, we collected filtered biomass samples from multiple time series sites within Narragansett Bay and along the Northeast U.S. Shelf Long-Term Ecological Research transect in winter and summer to investigate the frequency and seasonality of potential *Pseudo-nitzschia* spp. inflow from the continental shelf to the estuary. Species were taxonomically identified using DNA sequencing of the ITS1 region and domoic acid concentrations were quantified by liquid chromatography with tandem mass spectrometry and multiple reaction monitoring. During six years of sampling, *Pseudo-nitzschia* species assemblages were more similar between Narragansett Bay and the Northeast shelf in winter than summer, suggesting greater ecosystem connectivity in winter. These winter assemblages were often accompanied by higher domoic acid. Several *Pseudo-nitzschia* species co-occurred most often with domoic acid and were likely responsible for toxin production in this region, including *P. pungens* var. *pungens, P. multiseries, P. calliantha, P. plurisecta, P. australis*, and *P. fraudulenta*. Domoic acid was detected during periods of relatively low macronutrient concentrations in both seasons, warmer sea surface temperatures in winter, and colder temperatures in summer within this dataset. This study represents some of the first domoic acid measurements on the offshore Northeast U.S. Continental Shelf, a region that supplies water to other coastal environments and could seed future harmful algal blooms. The elevated domoic acid and frequency of hypothesized inflow of toxigenic *Pseudo-nitzschia* spp. from the Northeast continental shelf to Narragansett Bay in winter indicate the need to monitor coastal and offshore environments for toxins and harmful algal bloom taxa during colder months.

1. Introduction

Pseudo-nitzschia is a diatom genus found across a variety of marine environments, from the open ocean to coastal ecosystems where it can cause toxic harmful algal blooms (HABs). There are more than 60 species within the *Pseudo-nitzschia* genus, and about half have been confirmed in culture to produce domoic acid (DA), a neurotoxin that bioaccumulates in marine organisms (reviewed in Bates et al., 2018; Li et al., 2018; Dong et al., 2020; Chen et al., 2021; Percopo et al., 2022; Niu et al., 2023; von Dassow et al., 2023). Toxigenic strains of

Abbreviations: DA, domoic acid; NB, Narragansett Bay; NES, Northeast U.S. Shelf; NES-LTER, Northeast U.S. Shelf Long-Term Ecological Research program.

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Pseudo-nitzschia facultatively produce DA, and a variety of biotic and abiotic conditions may influence the frequency and toxicity of environmental DA events (reviewed in Bates et al., 2018). Therefore, it is critical to understand which species are present in an ecosystem and the conditions in which toxin production increases to better predict HAB events.

Narragansett Bay (NB), Rhode Island, an estuary in the Northeast U.S., experienced a shellfish harvest closure due to elevated DA in 2017 (Sterling et al., 2022). During this closure, *P. australis* was observed for the first time in this ecosystem since 2009 when DNA monitoring for *Pseudo-nitzschia* species began at the Narragansett Bay Long Term Plankton Time Series (NBPTS) site (Fig. 1; Roche et al., 2022). This high-toxin producing species, which was involved in the largest documented *Pseudo-nitzschia* HAB on the U.S. west coast (McCabe et al., 2016; Zhu et al., 2017), appeared occasionally at the NBPTS site for several years following the 2017 closures (Roche et al., 2022). Whether it persisted or was re-introduced several times to NB and other Northeast U.S. coastal ecosystems remains unclear (Clark et al., 2021; Roche et al., 2022). Furthermore, a separate shellfish harvest closure due to elevated DA in the Northeast U.S. region preceded this 2017 event by several months but with the absence of *P. australis*, meaning other toxigenic species were responsible for the initial 2016 HAB in NB (Roche et al., 2022; Sterling et al., 2022).

Pseudo-nitzschia HABs in estuarine environments may result from either increased growth and toxicity of endemic species, or they may be seeded by the introduction of toxigenic species from offshore sources. Water masses that enter semi-enclosed ecosystems from offshore through currents, tides, and storm events not only influence the physical and chemical properties of the estuarine water column (Banas and Hickey, 2005; Cloern and Jassby, 2012), but they can also transport or introduce new planktonic organisms (Winder et al., 2011) or in some cases HAB taxa (Velo-Suarez et al., 2014; Clark et al., 2021). Since *P. australis* was never observed prior to the 2017 toxic event in NB, its occurrence is hypothesized to be linked to transport from an offshore population. To examine whether *P. australis* and other toxigenic species may periodically or episodically enter NB from an external source and stimulate toxin production, it is necessary to characterize species composition in offshore water masses.

NB is located within the Northeast U.S. Continental Shelf marine ecosystem, which spans the Gulf of Maine to the Mid-Atlantic Bight of the U.S. east coast. This highly productive region, characterized by strong seasonality (Li et al., 2015; Friedland et al., 2020), exchanges water with NB, primarily through tidal processes and wind-driven intrusions into the East Passage of NB with outflow through the West Passage (Kincaid et al., 2003; Pfeiffer-Herbert et al., 2015). There is some evidence that wind-driven physical exchange between NB and the continental shelf region varies between winter and summer (Kincaid et al., 2003; Kincaid et al., 2008), which may influence the seasonal frequency at which *Pseudo-nitzschia* species enter NB from the shelf region. Prior modeling work examining the physical transport of toxigenic *Pseudo-nitzschia* species on the Scotian shelf northeast of NB found that *P. australis* was likely introduced to the Gulf of Maine in 2016 via the subpolar gyre and Labrador current (Clark et al., 2021). The introduction of *P. australis* to the Gulf of Maine occurred about six months prior to its appearance in NB, and source populations in the continental shelf region could seed both NB and the Gulf of Maine due to their proximity. However, there may be differences in local currents and geography that drive the physical connectivity of the shelf region to the Gulf of Maine's Bay of Fundy differently than to NB, warranting investigation of local species occurrence patterns.

Despite the prevalence of toxic *Pseudo-nitzschia* blooms on the Northeast U.S. shelf and globally, particularly in the past decade, causative drivers of DA production in environmental *Pseudo-nitzschia* assemblages remain somewhat elusive. Extensive work has been done to investigate biological, chemical, and physical drivers of DA production in various cultured *Pseudo-nitzschia* species. Some biological factors that have been shown to stimulate DA in culture include grazing (Zhang et al., 2024), competition with other phytoplankton (Prince et al., 2013), and the presence of certain bacterial taxa (Bates et al., 1995; Lelong et al., 2014; Sison-Mangus et al., 2014). Individual abiotic factors like temperature (e.g. Thorel et al., 2014), salinity (e.g. Pednekar et al., 2018), irradiance (e.g. Auro and Cochlan, 2013), macronutrients (e.g. Pan et al., 1996; Hagström et al., 2011), micronutrients (e.g. Wells et al., 2005), and pCO_2 (e.g. Sun et al., 2011) have been evaluated with varying influences on toxicity and growth rate, partially due to inter- and intraspecies variability. Nutrient limitation, in particular, has long been thought to stimulate DA production through cellular stress (as reviewed in Trainer et al., 2012; Bates et al., 2018), however, recent work examining DA production rates and partitioning of DA phases between particulate and dissolved fractions suggests a more complex relationship between DA and nutrient limitation (Cochlan et al., 2023). One study found that total DA production rates were highest for one strain of *P. australis* in the nutrient-replete exponential growth, and higher DA was found in the dissolved phase during phosphate- and silicate-depleted stationary growth, suggesting that pDA concentration varies with growth stage (Cochlan et al., 2023). Furthermore, multi-stressor and mesocosm experiments that more closely approximate *in situ* conditions have shown that some physical and chemical drivers of toxin production, including temperature, nutrients, and pCO_2, can have interacting effects on toxicity (Kelly et al., 2023; Xu et al., 2023). These culture-based and mesocosm approaches provide insight into potential drivers of environmental DA in single species, but further investigation is required to better understand the complexity of *in situ* dynamics.

Here, we examined *Pseudo-nitzschia* species composition and particulate DA concentrations both inside NB and in adjacent continental shelf waters to investigate the hypothesized connectivity of the two ecosystems. We collected samples from five sites in NB and six stations along the Northeast Shelf (NES) Long-Term Ecological Research (LTER) program transect, which originates 50 km southeast from the mouth of NB and terminates at the continental shelf break (Fig. 1). Using DNA metabarcoding and targeted DA metabolite detection, we compared seasonal patterns in *Pseudo-nitzschia* species composition, DA, and potential environmental drivers of toxin production over a six-year time series. These findings will inform HAB monitoring practices in the Northeast U.S. and enable better understanding of offshore sources of toxigenic *Pseudo-nitzschia* species and environmental DA production.

2. Methods

2.1. Study area and sample collection

Samples were selected from the NES-LTER transect and various time series sites in NB during winter and summer periods from January 2018 through February 2023 to compare seasonal and regional patterns of *Pseudo-nitzschia* species composition and DA, as well as environmental drivers. NB and NES will henceforth be referred to as subregions of the larger Northeast U.S. Continental Shelf region, with NES specifically referring to the area spanned by the NES-LTER transect (Fig. 1A). Samples were collected on NES-LTER cruises (*R/V* Endeavor, *R/V* Atlantis) from 11 stations along a 150 km transect (Fig. 1; $n = 77$) each winter (January-February) and summer (July-August). Samples from three to four stations per cruise were used in this dataset spanning innershelf (L1), midshelf (L3, L4) and outershelf (L7, L8, L10) sections of the transect. The northernmost station, L1, is about 50 km from the mouth of NB. To collect plankton biomass for nucleic acid isolation, CTD rosette seawater from the surface and subsurface chlorophyll maximum (SCM) were passed via peristaltic pump over 25 mm 5 μm pore size filters (Sterlitech, Kent, WA, USA). Biomass filters were either flash frozen in liquid nitrogen (2018–2022) or placed in DNA/RNA shield (2023; Zymo Research, Irvine, CA, USA) and stored in a 80 °C freezer. These sample storage methods yielded no statistical differences in ASV diversity in other microbial studies (i.e. Pratte and Kellogg, 2021). The

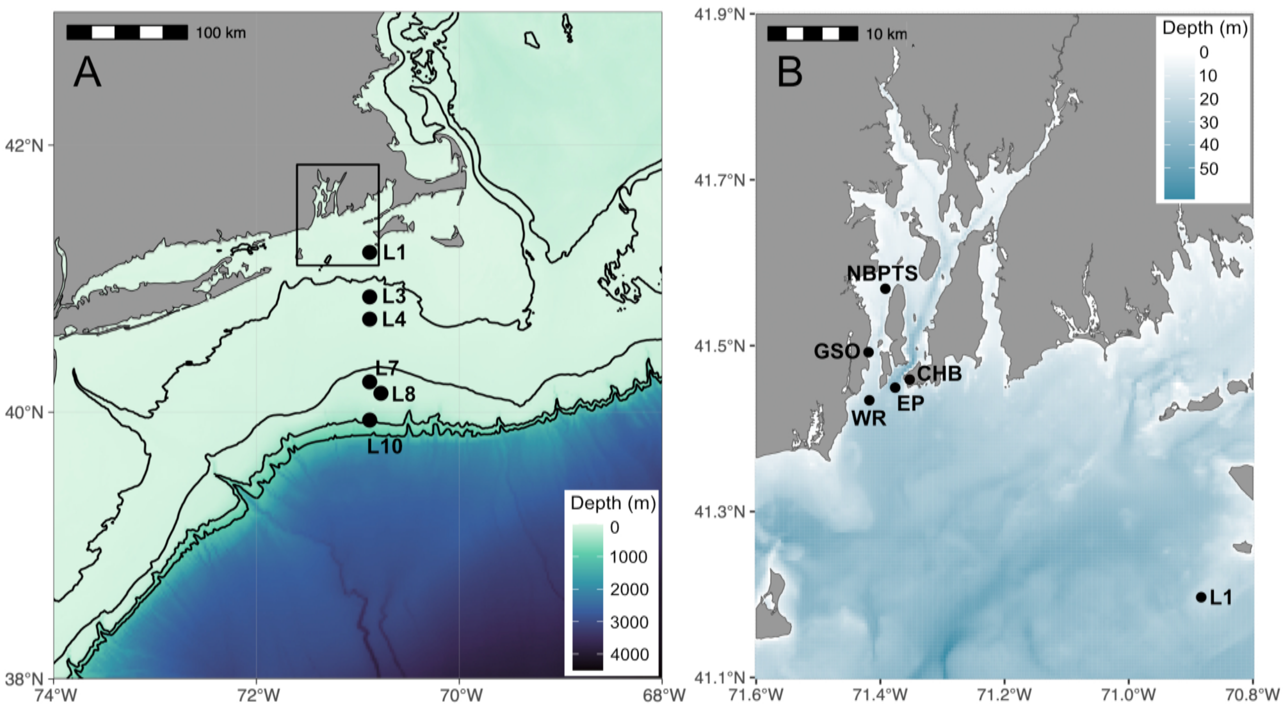

Fig. 1. Map of study sites in Narragansett Bay (NB), Rhode Island, USA (A) and the Northeast Continental Shelf (NES) in the northwest Atlantic Ocean (B) with bathymetry. The box in (A) indicates the latitudinal and longitudinal range shown in (B). The NES-LTER program samples 11 stations, 6 of which were used in this study, along a transect that originates southwest of Martha's Vineyard, MA and terminates at the continental shelf break. Two time series programs sample the Narragansett Bay Plankton Time Series (NBPTS) and Whale Rock (WR) sites weekly, and secondary sites including the University of Rhode Island Graduate School of Oceanography dock (GSO), East Passage (EP), and Castle Hill Beach (CHB), which are added during periods of high *Pseudo-nitzschia* cell abundance.

SCM depth varied as observed by *in situ* chlorophyll fluorescence, with a median depth of 28 m for summer and 19 m for winter samples. In cases where the SCM was not well defined due to water column mixing that typically took place in winter and at nearshore stations, a sampling depth between 20 and 30 m was targeted.

In NB, surface seawater samples were collected from various sites in the East and West Passages including the Narragansett Bay Long-Term Plankton Time Series (NBPTS) site, Whale Rock (WR), Castle Hill Beach (CHB), East Passage (EP), and University of Rhode Island Graduate School of Oceanography (GSO) dock (Fig. 1B). Seawater was transported back to the laboratory and passed over 25 mm 5 μm pore size filters (Sterlitech, Kent, WA, USA) using a peristaltic pump before flash freezing in liquid nitrogen and storage at −80 °C. To fill in several missing dates from this time series, six samples collected separately in the NBPTS (ht tps://web.uri.edu/gso/research/plankton/) sampling program were used. These samples differed in collection methodology only by the filter pore size used (0.22 μm, Express Plus, Millipore Sigma) and vacuum as opposed to peristaltic filtration. A prior comparison of *Pseudo-nitzschia* species composition on inline 5 μm and 0.2 μm filters showed that all species diversity was captured on the 5 μm filters, making them comparable to whole seawater filtered onto 0.2 μm pore size filters (Roche et al., 2022). All NB samples were collected as part of a weekly time series, however, only samples within 28 days of the start of NES-LTER cruises and 28 days following the end of the cruises were included in this study in order to investigate potential inflow of species from the NES to NB. This timing was selected based on the average residence times of NB, which are 26 days in summer and 19 days in winter, and which range from 10 to 40 days throughout the year (Kincaid et al., 2003). Within these timeframes, available samples from all sites were used. NBPTS samples were always available ($n = 86$), and this site was supplemented with additional samples from WR ($n = 52$), CHB ($n = 10$), EP ($n = 7$), and GSO ($n = 5$) to enhance spatial resolution. Each season and year timeframe included a full set of weekly NB samples during 28 days before and after NES-LTER cruise transects, with the exception of summer 2022 when samples were only available 21 days before and after. Some samples used in this dataset were previously sequenced ($n = 49$; Sterling et al., 2022). Raw sequencing reads for these samples were obtained from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject PRJNA690940, and domoic acid, environmental metadata, and ASVs were accessed through the National Science Foundation Biological and Chemical Oceanography and Data Management Office (BCO-DMO; Jenkins and Bertin 2021a, 2021b).

2.2. DNA extraction, sequencing, and bioinformatic processing

DNA was extracted from most NB and NES samples ($n = 219$) using a modified version of the DNeasy Plant Kit (Qiagen, Germantown, MD, USA) that included a 1-minute bead beating step (0.1 mm and 0.5 mm Zirconia/Silica beads, BioSpec Products, Bartlesville, OK, USA) and two-part elution into a total of 45 μL Buffer AE. Similarly, the six NBPTS samples were extracted using a modified version of the DNeasy Blood & Tissue kit (Qiagen, Germantown, MD, USA) with a 1-minute bead beating step and final elution into 50 μL Buffer AE. Some NES samples ($n = 18$) were extracted using the Quick-DNA/RNA Miniprep Plus Kit (Zymo Research, Irvine, CA, USA) with a 1-minute bead beating step (0.4 mm Zirconium Beads, OPS Diagnostics, Lebanon, NJ, USA) and final elution into 50 μL nuclease-free water. DNA from each sample was amplified with a primer set that targets the eukaryotic internal transcribed spacer region 1 (ITS1) and effectively distinguishes *Pseudo-nitzschia* species (White et al., 1990; Sterling et al., 2022). Briefly, DNA was diluted to 1–4 ng/μL and 2 μL of template was added to 25 μL PCR reactions with Phusion Hot Start High-Fidelity Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) and HPLC-purified forward and reverse primers at 0.5 μM concentration with Illumina MiSeq adapters (Integrated DNA Technologies, Coralville, IA, USA). A stepwise thermocycle was used as described in Sterling et al. (2022).

DNA amplicons were sequenced at the Rhode Island-INBRE

Molecular Informatics Core on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA). There, libraries were prepared by cleaning ITS1 PCR products with KAPA pure beads (KAPA Biosystems, Woburn, MA, USA) and attaching sequencing indices and adapters using PCR. This amplification was performed with the Illumina Nextera XT Index Kit (Illumina, San Diego, CA, USA) and Phusion High Fidelity Master Mix, followed by a second round of cleaning with KAPA pure beads and visualization with gel electrophoresis. The quality of select samples was assessed on a Bioanalyzer DNA1000 chip (Agilent Technologies, Santa Clara, CA, USA) and all samples were quantified on a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The final library was pooled, quantified with qPCR on a LightCycler480 (Roche, Pleasanton, CA, USA) using a KAPA Biosystems Illumina Kit (KAPA Biosystems, Woburn, MA, USA), and sequenced on the Illumina MiSeq using v3 chemistry and 2×250 paired-end reads. Samples were sequenced across five separate MiSeq runs using identical methods and blank negative controls.

Raw sequencing read quality was assessed using FastQC and MultiQC (v.0.11.9, v1.11; Andrews, 2010; Ewels et al., 2016) before and after primer and adapter trimming in Cutadapt (v3.2; Martin, 2011). The Divisive Amplicon Denoising Algorithm (DADA2) was used in R to estimate sequencing error and identify distinct amplicon sequence variants (ASVs) (v1.20.0; Callahan et al., 2016). DADA2 was run separately for each sequencing run because it is designed to account for run-specific error. Taxonomy was assigned to ASVs from all sequencing runs using a dual approach to maximize the number of ASVs identified to the species level and enhance confidence. First, the scikit-learn naïve Bayes machine learning classifier in QIIME2 (v2022.11; Bolyen et al., 2019) and a curated reference database were used to assign taxonomy with a confidence threshold of 0.8. This curated database from Roche et al. (2022) included 302 unique *Pseudo-nitzschia* spp. ITS1 sequences from NCBI GenBank with 51 different species represented (retrieved June 1, 2021). Next, to ensure that relevant *Pseudo-nitzschia* spp. ITS1 taxonomy was not omitted from the curated database, a megablast search was performed using the entire BLAST nt database. Additional ASVs classified by megablast were retained if there was >99% identity and >99% query cover to NCBI *Pseudo-nitzschia* species. If QIIME2 and megablast taxonomic assignment did not match for a particular ASV, no species-level taxonomy was assigned. Samples containing no reads identified to the *Pseudo-nitzschia* species level were removed from the analysis ($n = 15$). To avoid potentially falsely detected taxa, ASVs classified as a species that appeared in only one sample across the dataset were discarded ($n = 6$).

2.3. Environmental measurements and domoic acid quantification

Various biological, chemical, and physical data were collected during each sampling event. In NB, surface temperature and salinity were measured using multiparameter sondes (6920 V2 for samples collected at NBPTS and WR; ProDSS for samples collected at CHB and GSO dock; YSI, Yellow Springs, Ohio, USA). During NES sampling, temperature and salinity were measured using two SBE911 CTD sensors (Sea Bird Electronics, Bellevue, WA, USA) and the mean of the two measurements was used in the final analysis. Dissolved macronutrient samples were collected by freezing 0.2 μm seawater filtrate at 20 °C. NB site nutrients were analyzed at the University of Rhode Island Marine Science Research Facility (URI MSRF, Narragansett, RI, USA) on a QuickChem 8500 (Lachat, Milwaukee, WI, USA) while NES samples were measured at the Woods Hole Oceanographic Institution Nutrient Analytical Facility (Woods Hole, MA, USA) on a four-channel segmented flow AA3 HR Autoanalyzer (SEAL Analytical, Mequon, WI, USA). Both instruments measured nitrite + nitrate, ammonium, silicate, and phosphate. Nitrite + nitrate and ammonium values were summed and used in the analysis as dissolved inorganic nitrogen (DIN). Any measurements below each instrument's limit of detection for each nutrient type were replaced with zero.

Biomass for particle-associated DA analysis (>5 μm) was collected, extracted, and analyzed via liquid chromatography with tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring. All samples were chromatographically separated in an identical fashion to Sterling et al. (2022), and the majority of samples ($n = 167$) were analyzed using a 4500 QTRAP mass spectrometer (SCIEX, Framingham, MA, USA). A subset of samples ($n = 42$) were measured using a 1290 Infinity II UHPLC system coupled to a 6470 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The peak of DA eluted at 10.41 min. Analysis was carried out in positive mode, and three transitions from the protonated DA molecule were used and optimized for quantification: m/z 312 → 266, m/z 312 → 161, and m/z 312 → 105 determined by MassHunter Optimizer (Agilent, Santa Clara, CA, USA) including the optimized fragmentor (112), collision energy (20, 28, 48), and cell accelerator voltage (4) settings. The m/z 312 → 266 transition was used for quantification following acquisition from both mass spectrometry instruments. Particle-associated DA was quantified to ng particulate DA L^{-1} of filtered seawater using an external calibration curve created with pure DA standards of increasing concentrations included in each analysis (DA Certified Reference Material, National Research Council Canada, Halifax, Nova Scotia). A comparison of the standard curves from the two instruments can be found in Figure S1, and the instrument used to quantify each sample is described in Table S1. The limit of detection for each instrument was quantified by determining the minimum detectable concentration (MDC) of DA. Initial LC-MS/MS analysis with MRM indicated that a 0.1 ng/mL injection concentration of DA gave a reproducible signal that could be differentiated from the baseline. Next, 0.5 ng/mL DA was repeatedly injected ($n = 3$) and the standard deviation (s) of the peak area for the DA signal was calculated. Three DA calibration curves (Fig. S1) for each instrument were generated to calculate the mean slope (m) of the calibration curves. The MDC was calculated as $3s/m$ (3 times the standard deviation of the 0.5 ng/mL solutions divided by the slope of the calibration curve). The MDC was below 0.1 ng/mL for both instruments, and the quantification limit at 0.1 ng/mL was retained for consistency in the complete data set.

Pseudo-nitzschia spp. abundance was quantified using light microscopy cell counts of live (NBPTS site) and preserved (all other sites) samples. For preserved samples, acidic Lugol's solution was added to whole seawater for a final concentration of 1% Lugol's and stored at 4 °C until enumeration. A Sedgewick-Rafter counting chamber (Science First/Wildco, Yulee, FL, USA) and a BX40 light microscope (Olympus, Tokyo, Japan) were used to identify and enumerate cells at the genus level, since many *Pseudo-nitzschia* species are morphologically cryptic under light microscopy (e.g. Quijano-Scheggia et al., 2009; Lundholm et al. 2012; Percopo et al., 2016).

2.4. Statistical analysis and visualization

Amplicon sequencing data are semi-quantitative due to biases in extraction efficiency and PCR amplification (Koid et al., 2012), sequencing depth variation and saturation of sequencing platforms (Santoferrara, 2019), and variation in copy number of the target amplicon sequence between taxa (Mäki et al., 2017). For these reasons, all statistical analyses and visualizations of metabarcoding in this study used presence-absence, or binary occurrence, metrics instead of absolute or relative sequencing read abundances. Furthermore, due to the importance of species-focused patterns, all ASVs of the same *Pseudo-nitzschia* species were agglomerated in all analyses. For several visualizations, we calculated frequency of occurrence from these presence-absence species patterns, defined here as the proportion of a sample set in which a particular species occurred. These sample sets included NES compared to NB samples and DA-detected samples compared to the entire dataset.

This study's dataset was designed to test hypothesized patterns of species inflow into NB from the NES by determining the presence or absence of each species during three time periods and locations: 1) in NB 28 days prior to the NES-LTER research cruise, 2) on the NES during the

NES-LTER cruise, and 3) in NB 28 days following the NES-LTER research cruise. Patterns of presence-absence across those three time periods were assessed for each *Pseudo-nitzschia* species in the dataset. Of all possible permutations of presence (P) and absence (A) across those three time periods, we only evaluated four that were relevant to the aims of this study: APA, APP, PAP, PPP (Table 1). The other four permutations not used here either provided little information (AAA) or assumed patterns that were untestable based on the sampling design (PAA, PPA, AAP). To test differences in these species patterns by season, Mann Whitney U tests were performed on the number of species exhibiting each pattern in winters (2018–2023) and summers (2018–2021, 2023), which were not normally distributed (Shapiro Wilk test; p 0.05). Summer 2022 was excluded from this analysis because NB samples were only available during 21 days before and after NES-LTER transect cruises.

Several statistical tests were employed to compare species composition, toxin presence, and environmental conditions. To assess the similarity of *Pseudo-nitzschia* species assemblages across time and space, we used Analysis of Similarities (ANOSIM) and Permutational Multivariate Analysis of Variance (PERMANOVA) tests on Jaccard distances of presence-absence transformed sequencing reads. ANOSIM performs better with unbalanced sampling designs when variance within sample sets is low, while PERMANOVA is more robust to balanced sample numbers regardless of within-set variance (Anderson and Walsh, 2013). Because many of our sample sets were unbalanced, we used ANOSIM when possible (beta dispersion not significant, 999 permutations; $p >$ 0.05), while PERMANOVA was used for several comparisons only when beta dispersion was significant (p 0.05) and sample numbers were relatively equivalent between groups (Table S2). A Fisher's exact test was used to compare frequencies of species occurrence when toxin was detected and not detected (p 0.05). To evaluate environmental drivers of DA that may vary over spatiotemporal scales, samples were subset by location (estuary (NB), offshore (NES)), season (winter, summer), and whether or not DA was detected. Environmental variables included sea surface temperature, salinity, DIN, silicate, and phosphate, which were compared between DA-detected and DA-not-detected samples for each season and subregion. Independent sample *t*-tests were used when environmental sample sets were normally distributed (Shapiro Wilk test; $p >$ 0.05), and Mann Whitney U tests were used when data were not normally distributed (Shapiro Wilk test; p 0.05).

All data analysis and visualization took place in Rstudio (v1.3.1056) using various packages including phyloseq (v1.46.0; McMurdie and Holmes, 2013) for metabarcoding data transformation and subsetting; vegan (v2.6.4; Dixon, 2003) for ordination, ANOSIM, and PERMANOVA; dplyr (v1.1.4; Wickham et al., 2019) and tidyr (v1.3.0; Wickham and Wickham, 2017) for data manipulation; viridis (v0.6.2; Garnier et al., 2021) for colorblind-accessible color palettes; and ggplot2 (v3.4.4; Wickham, 2011) for visualization. The maps were generated using ggOceanMaps (v2.2.0; Vihtakari. M., 2024) and marmap (v1.0.10; Pante and Simon-Bouhet, 2013) with shapefiles and bathymetry from the General Bathymetric Chart of the Oceans (Fig. 1A; GEBCO Compilation Group, 2023) and the National Ocean Service Coastline of the Northeastern United States (Fig. 1B; NOS80 K, NOAA, 1994).

Table 1
Acronyms for presence-absence patterns of *Pseudo-nitzschia* species in three timeframes to examine hypothesized inflow of species from the Northeast U.S. Shelf (NES) to Narragansett Bay (NB).

Pattern acronym	NB within 28 days prior to NES-LTER cruise	NES-LTER cruise transect	NB within 28 days after NES-LTER cruise
APA	absent	present	absent
APP	absent	present	present
PAP	present	absent	present
PPP	present	present	present

3. Results

3.1. Regional and seasonal patterns of Pseudo-nitzschia species composition

Sequencing of 237 samples yielded a total of 189 ASVs that were identified to the *Pseudo-nitzschia* species level, representing 21 unique species. These species exhibited varying temporal patterns in the estuary and offshore sites. There were greater differences in *Pseudo-nitzschia* species composition between NB and NES in summer compared to winter (Fig. 2). ANOSIM of the Jaccard dissimilarity matrices of all samples containing *Pseudo-nitzschia* spp. reads (n = 222) revealed significantly different winter and summer assemblages (p 0.05, Fig. 2). To investigate finer scale similarities by subregion, we also used ANOSIM and PERMANOVA to assess differences in species composition between NB and the NES for each season in each year (Table S2). In each summer that met the assumptions for a multivariate test (2018, 2020, 2022), there was a significant difference in species composition between the two subregions (Table S2). All winter Jaccard dissimilarity matrices met the assumptions for ANOSIM, and of the six winters, three displayed a significant difference in *Pseudo-nitzschia* species composition by subregion (2021–2023). Each of the other winters (2018–2020) did not demonstrate significant differences in species composition between NB and the NES.

We tested the hypothesis that individual species were transported from offshore sites into the estuary by comparing their occurrence in NB for 28 days prior to research cruises, on the NES during LTER transects, and in NB for 28 days following research cruises (Table 1). The number of species that were observed only on the NES during this timeframe (represented by "APA") was significantly higher in the summers than winters (Mann Whitney U test; p 0.01) (Fig. 3). Furthermore, in winter there was a significantly higher number of species that were not observed in NB prior to the research cruise, were then observed on the NES transect, and were also observed in NB following the research cruise (APP) (Mann Whitney U test; p 0.05) than species exhibiting this pattern in summer. The APP pattern only occurred with one species on one summer NES-LTER transect, while the pattern occurred in five out of six winter transects with an average of three species per cruise. Neither the number of species that were observed only in NB during the timeframe (PAP) nor the number of species present in both subregions (PPP) differed significantly between seasons (Fig. 3).

There were nine *Pseudo-nitzschia* species that exhibited the APP pattern across six different season/year timeframes, all of which are considered toxin-producing species (Fig. 4; reviewed in Bates et al., 2018). Three of these species displayed the APP pattern twice: *P. australis* (winters 2018 and 2022), *P. fraudulenta* (winters 2018 and 2021), and *P. multiseries* (winters 2018 and 2021). The other six species that exhibited the pattern in one cruise were *P. calliantha* (winter 2018), *P. delicatissima* (winter 2018), *P. pungens* var. *pungens* (winter 2018), *P. turgidula* (winter 2019), *P. hasleana* (winter 2023), and *P. galaxiae* (summer 2018). Each of the species displaying the APP pattern was observed at the NES-LTER transect station L1, the closest station to the mouth of NB, with the exception of *P. calliantha* in winter 2018 that was observed only at station L4, a mid-shelf location (Fig. 4, Table S4). For the majority of these events (58%), the species observed in NB were present at 3 to 4 of the NES-LTER transect stations sampled and often at both surface and SCM depths (Table S4).

Several *Pseudo-nitzschia* species were observed for the first time in the Northeast U.S., and some species were only observed in NB or on the NES. Three species were identified in this region for the first time: *P. sabit* (2 occurrences in NB, 10 occurrences on the NES), *P. qiana* (1 occurrence in NB, 6 occurrences on the NES), and *P. mannii* (7 occurrences on the NES). The first dates each of these species were observed were 2/4/19 (WR), 8/23/19 (L10 SCM), and 7/22/18 (L10 surface) respectively. Three species were observed on the NES but never in NB during the study timeframes: *P. mannii, P. caciantha* (3 occurrences), and

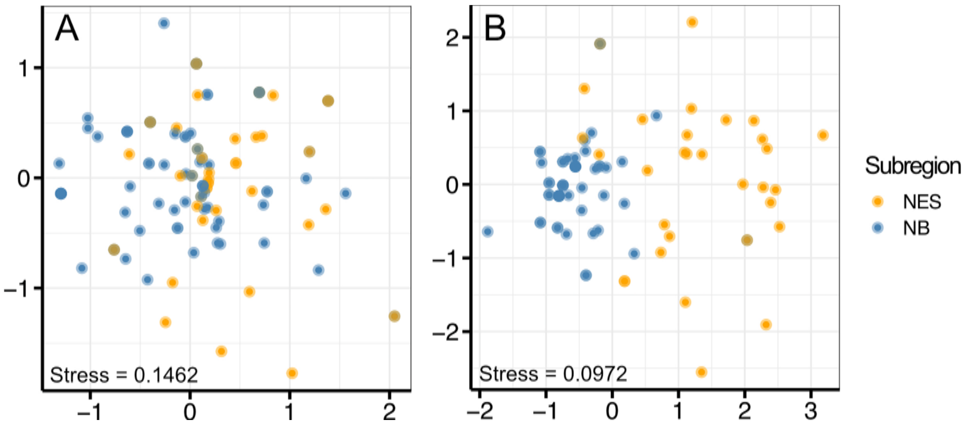

Fig. 2. Non-metric multidimensional scaling (NMDS) of the Jaccard distance matrix of *Pseudo-nitzschia* spp. presence-absence in winter (A) and summer (B). Each point represents the species composition of samples in Narragansett Bay (NB; blue) and the Northeast U.S. Shelf (NES; gold), and proximity of points indicates assemblage similarity.

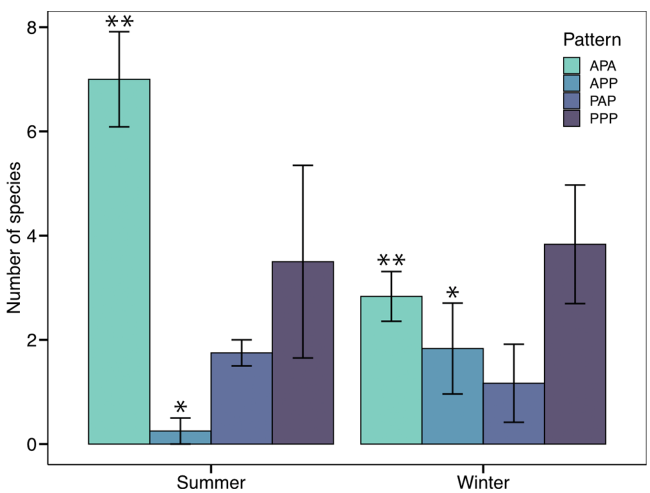

Fig. 3. The average number of *Pseudo-nitzschia* spp. exhibiting each spatial pattern for four summer timeframes (2018–2021) and six winter timeframes (2018–2023). The pattern names correspond to presence (P) or absence (A) in three time periods and locations: Narragansett Bay (NB) during 28 days prior to the cruise, Northeast U.S. Shelf (NES) during the cruise transect, and NB for 28 days following the cruise. For example, APP indicates that a species was absent in NB before the cruise, present on the NES transect, and present in NB following the cruise (Table 1). Error bars represent the standard error. Two asterisks indicate p 0.01 and one asterisk indicates p 0.05 for significance testing of the average number of species for each pattern between seasons.

P. inflatula (15 occurrences). However, *P. caciantha* and *P. inflatula* were previously observed in NB during other times of the year (Sterling et al., 2022).

While many *Pseudo-nitzschia* species were observed across wide temperature ranges, there were some species that occurred more frequently at certain temperatures, and these patterns differed by subregion (Fig. 5). The temperature ranges in each subregion within this dataset were more similar in winter than in summer. Winter NES (2.3 – 13.7 °C) and winter NB (1.1 – 8.0 °C) temperatures spanned similar ranges, with averages of 7.1 °C and 3.7 °C respectively. Summer NES temperatures spanned a wider range (10.0 – 27.4 °C) than summer NB (17.9 – 25.5 °C) and averaged 19.4 °C and 21.8 °C in this sample set (Fig. 5). It should be noted that some of the colder summer NES temperatures represent samples collected deeper in the stratified water column. Four toxin-producing species – *P. pungens* var. *pungens, P. multiseries, P. calliantha*, and *P. plurisecta* – were the most frequently observed species during NB summers (39%, 35%, 32%, and 11% of all NB samples respectively), though these species also occurred in a high proportion of NB winter samples (29%, 23%, 12%, 17% respectively). *P. americana*, a non-toxigenic species, most frequently occurred in NB winter, usually between 2 and 6 °C. A high toxin-producing species, *P. australis*, was observed in both NES and NB winter temperature ranges, also typically between 2 and 6 °C. This species did not occur in any NB summer samples from 2018 to 2022. Several other species also occurred more often in winter, including *P. fraudulenta* (NES) and *P. delicatissima* (NES and NB). At the highest temperatures observed on the NES during this study (24–28 °C), many of the least common species of *Pseudo-nitzschia* were present, including *P. hasleana, P. galaxiae, P. mannii, P. inflatula, P. sabit, P. qiana*, and *P. caciantha.*

3.2. Particulate domoic acid and co-occurrence with Pseudo-nitzschia species

During this study, DA varied seasonally, by subregion, and along the NES-LTER transect (Fig. 6). DA above the limit of detection (0.1 ng L^{-1} seawater filtered) was measured in 83 out of the 209 available samples and did not have a linear relationship with *Pseudo-nitzschia* spp. cell abundance (Fig. S2). On the NES, DA was detected most frequently in winter, occurring in 55% of winter samples compared to 23% of summer ones (Fig. 6). DA above the detection limit was measured during each winter transect cruise, typically at two or three of the stations and within surface and SCM samples (Fig. 6). The highest DA observed was in winter 2021 at the station L1 SCM (27 ng L^{-1} seawater), which was nearly three times greater than the highest NB DA measurement in this study (10 ng L^{-1} seawater, summer 2019, WR). In summer on the NES, however, DA almost never surpassed the limit of detection with the exception of one sample in summer 2019, one sample in summer 2022, and four samples in summer 2021, all of which measured 0.3 ng L^{-1} or lower. In both seasons, DA was most frequently detected at the innershelf (L1) and midshelf (L3, L4) stations, and infrequently detected at offshore stations (L7, L8, L10) (Fig. 6). DA was not always coupled between surface and SCM samples, and several times was measured at one depth and not detected at another. DA in NB was also detected more frequently in winter than summer. Each season in which DA was observed in NB, there was at least one DA detection along the NES transect (Fig. 6).

Out of the 21 *Pseudo-nitzschia* species observed in this study, 17 co-occurred with DA in at least one sample (Fig. 7). Based on a Fisher's exact test, there was a significant difference in the frequency of occurrence of species in samples where DA was detected compared to those where no DA was detected (p 0.05), indicating an association between toxin production and species occurrence. Some species co-occurred with DA more than half the time it was detected, including *P. pungens* var. *pungens* (84%), *P. multiseries* (69%), and *P. calliantha* (57%) (Fig. 7). Six

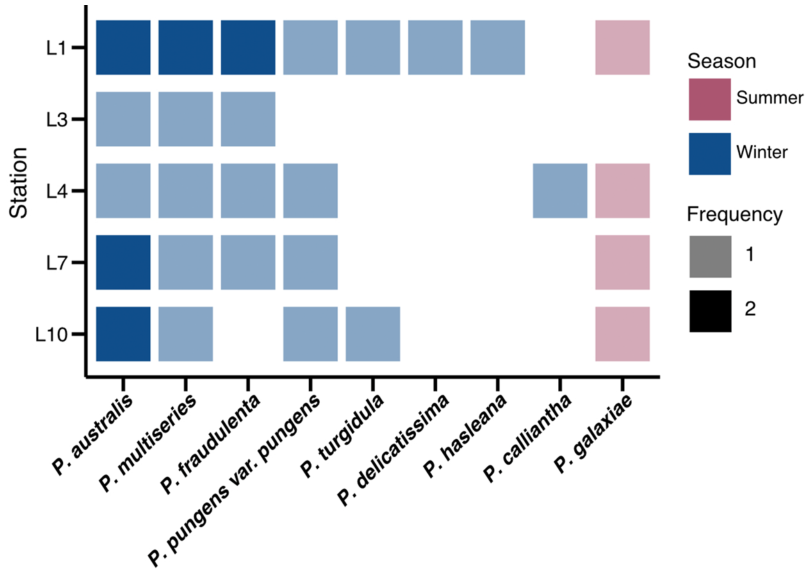

Fig. 4. Location on the Northeast U.S. Shelf (NES) transect and seasonality of *Pseudo-nitzschia* spp. exhibiting the APP (absent in Narragansett Bay (NB) prior to cruise, present on NES-LTER transect, present in NB following cruise) spatiotemporal pattern for four summer timeframes (2018–2021) and six winter timeframes (2018–2023). Color indicates season and shading intensity represents the number of cruises in which a species exhibiting the APP pattern was observed at an NES-LTER station. The specific years and depths at which these species occurred is included in Table S4. Species exhibiting the APP spatial pattern are ordered by season and frequency of observation from left to right for each season. Toxigenic species as designated by Bates et al. (2018) are shown in bold.

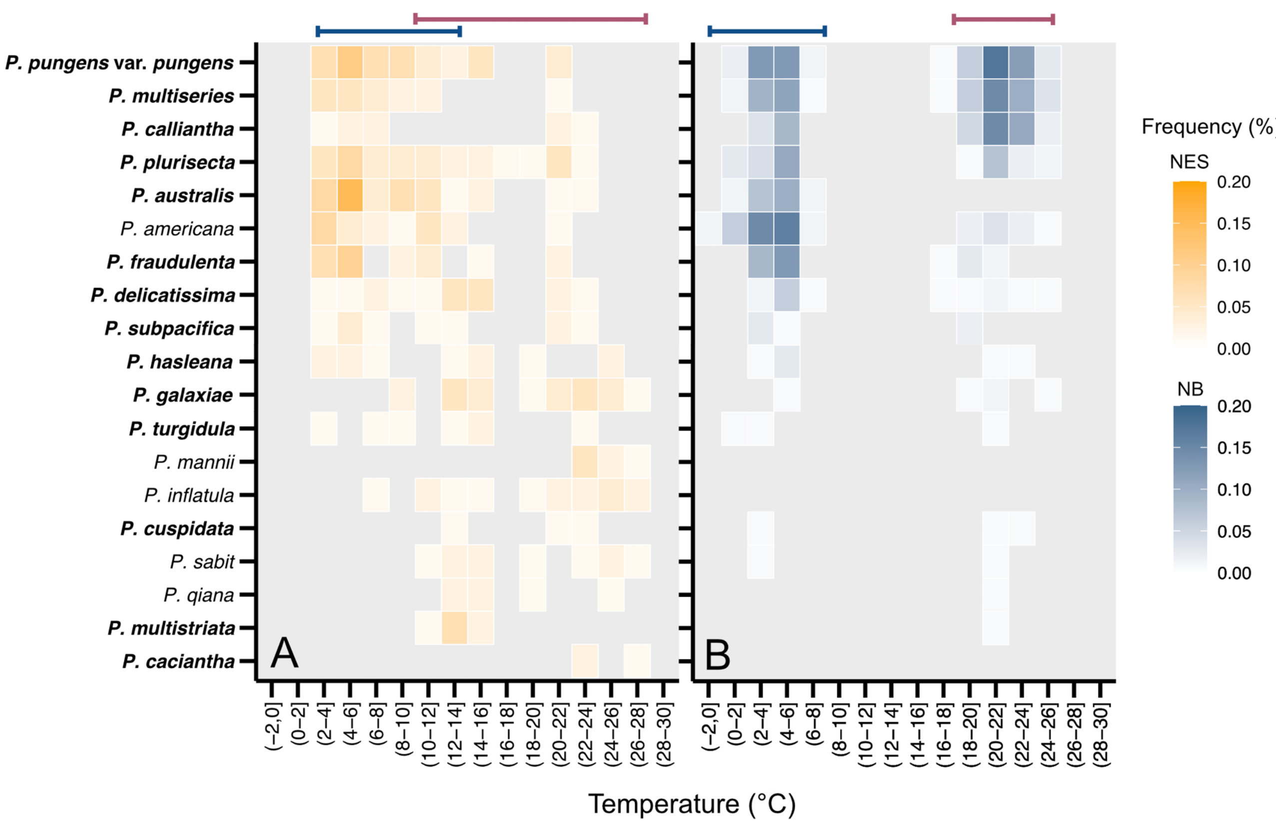

Fig. 5. Temperature niches of *Pseudo-nitzschia* spp. on the Northeast U.S. Shelf (NES; A) and in Narragansett Bay (NB; B) during summers (2018–2022) and winters (2018–2023). The color range indicates the frequency of occurrence within respective sample sets (NES and NB) of a particular species in a 2 °C temperature bin. Toxigenic species as designated by Bates et al. (2018) are shown in bold. The overall temperature minima and maxima for NES and NB winters (blue) and summers (pink) in study samples are indicated by bars at the top of each heatmap. NES temperatures include surface and SCM, while NB includes only sea surface temperatures.

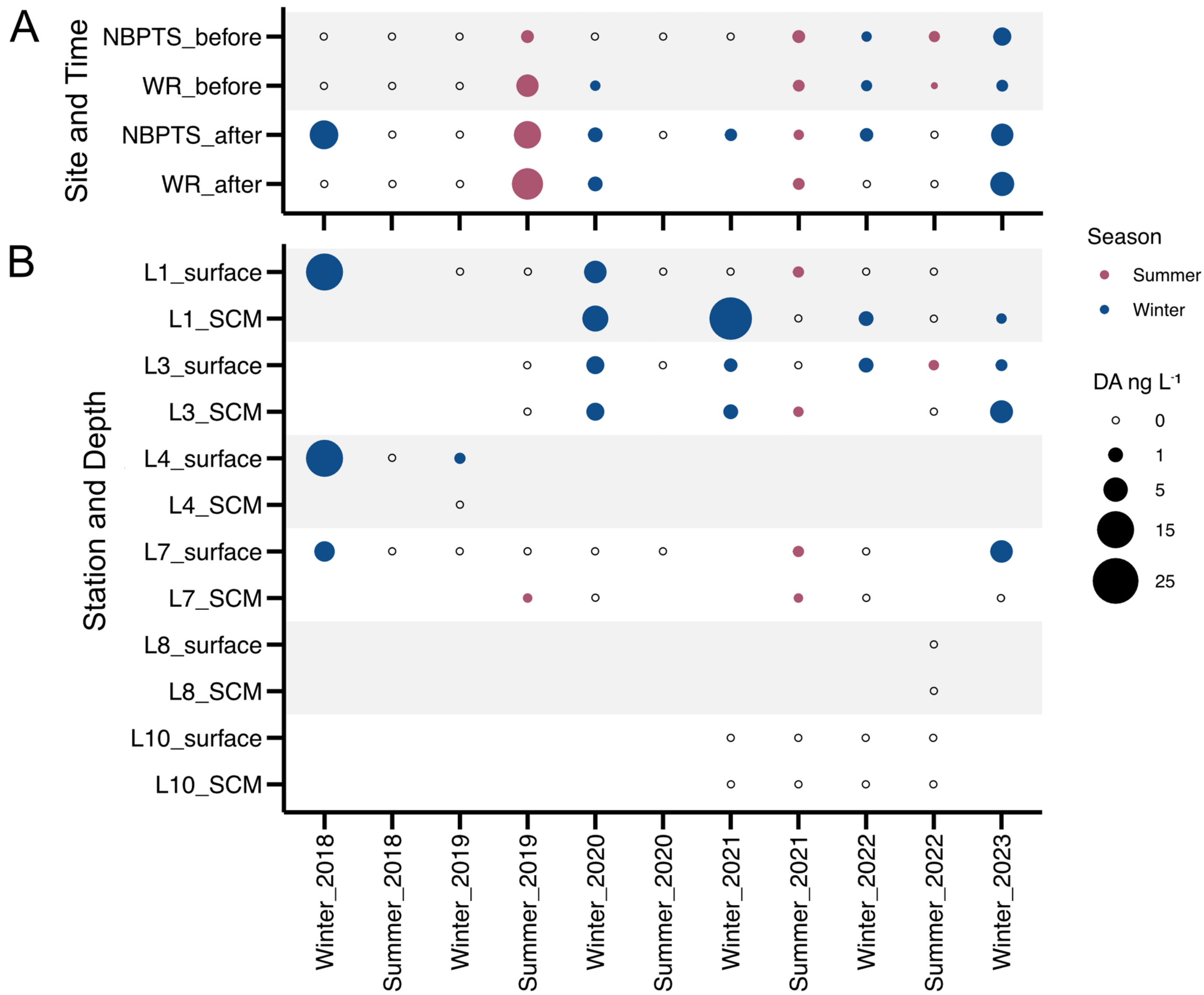

Fig. 6. Domoic acid measurements at 2 sites in Narragansett Bay (NB; A) and 6 stations on the Northeast U.S. Shelf (NES) transect (B) from 2018 to 2023. NB measurements represent the maximum DA recorded at each site during the 28 days prior to (before) and following (after) NES-LTER cruises at sites near the mouth of the bay (WR) and mid-bay in the West Passage (NBPTS). NES measurements represent one sample at the surface and one sample at the subsurface chlorophyll max (SCM) depth at each station when available. Color represents season and quantity of DA in ng L^{-1} seawater filtered is indicated by the size of each circle, with a limit of detection of 0.1 ng L^{-1}. Open circles indicate samples where DA was measured but not detected.

species co-occurred with DA more often than their frequency of occurrence across the entire dataset, including *P. pungens* var. *pungens, P. multiseries, P.* calliantha, *P. plurisecta, P. australis*, and *P. fraudulenta* (DA frequency >4% higher than total frequency). Furthermore, several species occurred with DA detection more often in the summer (*P. pungens* var. *pungens, P. multiseries*, and *P. calliantha*) while others commonly coincided with DA in the winter (*P. pungens* var. *pungens, P. plurisecta, P. australis*, and *P. fraudulenta*). *P. americana*, a non-toxigenic species, also co-occurred with DA in a large proportion of winter samples (Fig. 7).

A subset of environmental parameters were significantly different when DA was detected compared to samples with DA below the limit of detection for each subregion and season. Significant results of *t*-tests and Mann Whitney U tests (p 0.05) are included in Table S3. In NB in the winter, DA was detected at significantly higher sea surface temperature, higher salinity, lower DIN, and lower silicate than in samples without DA (Fig. 8A-D). During the summer in NB, DA was associated with significantly lower temperature, lower silicate, and lower phosphate (Fig. 8E-G). Similar to NB winter, on the NES in winter, DA coincided with significantly lower DIN. However, unlike NB, NES DA was associated with lower salinity than samples with no DA detected (Fig. 8H-I). There were no significant drivers of DA on the NES during the summer.

4. Discussion

Pseudo-nitzschia HABs are an emerging issue in coastal ecosystems of the Northeast U.S. Therefore, understanding potential offshore sources of these blooms is critical for HAB prediction and management. In this study, we leveraged six years of sampling from time series programs in NB and the NES to investigate the potential inflow of species from a continental shelf to an estuarine system, as well as compare *Pseudo-nitzschia* species composition, DA, and environmental conditions between the two subregions. The ITS1 metabarcoding approach used here delineates nearly all known species of *Pseudo-nitzschia*, which is essential for understanding potential for toxicity, since not all species produce DA. Furthermore, this approach is more effective for *Pseudo-nitzschia* species delineation than imagery-based methods like light microscopy and imaging flow cytometry because *Pseudo-nitzschia* species are morphologically cryptic without the use of resource-intensive electron microscopy (Amato and Montresor, 2008; Lundholm et al., 2012; reviewed in Bates et al., 2018). Across this dataset, we observed 21 *Pseudo-nitzschia* species in NB and the NES with varying seasonal and

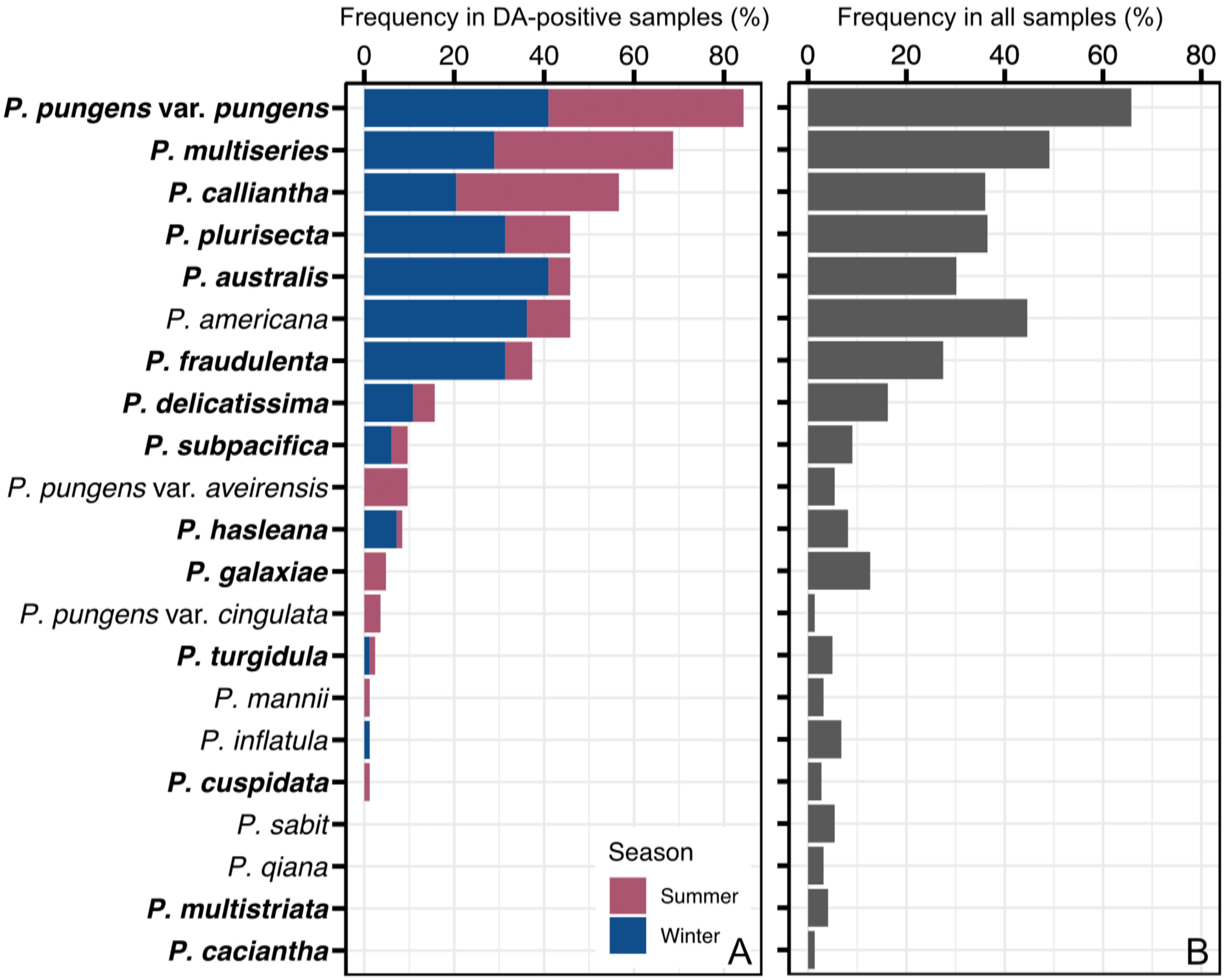

Fig. 7. Frequency of occurrence of *Pseudo-nitzschia* spp. in samples with DA detected (A) and across the entire dataset (B). Toxigenic species as designated by Bates et al. (2018) are shown in bold. Color indicates occurrence by season.

regional patterns of occurrence, 14 of which have identified toxigenic strains (Fig. 7; reviewed in Bates et al., 2018). The highly sensitive LC-MS/MS method used to quantify DA alongside species delineation allowed us to examine periods of toxicity below levels of concern to fisheries, with a range of 0.1 ng DA L^{-1} seawater filtered (limit of detection) to 27 ng L^{-1} (Fig. 6). These DA measurements along the NES transect represent the most comprehensive DA quantification at offshore sites in this region and expand upon the first observations of DA on the NES in 2018–2019 (Sterling et al., 2022). Since the mean circulation in this subregion is in the southwestward direction (Chapman and Beardsley, 1989), toxigenic *Pseudo-nitzschia* spp. and DA on the NES may seed HAB events in other coastal ecosystems along the eastern U.S.

4.1. Enhanced ecosystem connectivity between the continental shelf and estuary in winter

The comparison of *Pseudo-nitzschia* species composition between NB and the NES revealed greater similarity of assemblages in the winter than the summer (Fig. 2, Table S2), suggesting stronger ecological connectivity between the two subregions during winter months. The number of species that occurred on the NES but did not appear in NB during the specified timeframe was significantly higher in the summer (Fig. 3, APA). This could mean that species from the NES did enter NB but were met with unfavorable conditions for survival, for example higher temperatures, or it could indicate that there is less physical exchange from the NES into NB in summer. In contrast, there was a significantly higher number of species in winter that occurred on the NES transect and in NB following the cruise that did not appear in NB prior to the cruise (Fig. 3, APP), which may represent an inflow of species to NB from the NES subregion. While it is possible that the indicated species were residing elsewhere in NB prior to the NES-LTER cruises and coincidentally occurred in the surface water of our sampling sites following the cruises, this is unlikely due to the shallow and well-mixed winter water column (Nixon et al., 2009) and homogeneity of species composition at the varied sampling locations in the East and West Passages of NB (Fig. 1, Fig. S3, Fig. S4).

There are several physical mechanisms that could support enhanced inflow from the NES to NB in winter, and thus explain the observations of species in NB following their occurrence on the NES. Circulation and exchange between the NES and NB are highly complex because of NB's multiple openings to the shelf region and the dynamic variation in wind-driven currents in the Rhode Island Sound (Kincaid et al., 2008). Wind forcing is important for circulation in this region, with coastline-parallel winds and wind relaxation events driving the most estuary-shelf exchange (Kincaid et al., 2008). These wind relaxation events, or changes in wind direction, have been reported to be common in winter in this region, though they can also occur in summer (Kincaid et al., 2008). A second mechanism that could increase inflow to NB is strengthening of the counterclockwise flow that brings water in through the East Passage and out the West Passage. This flow is enhanced by high runoff events,

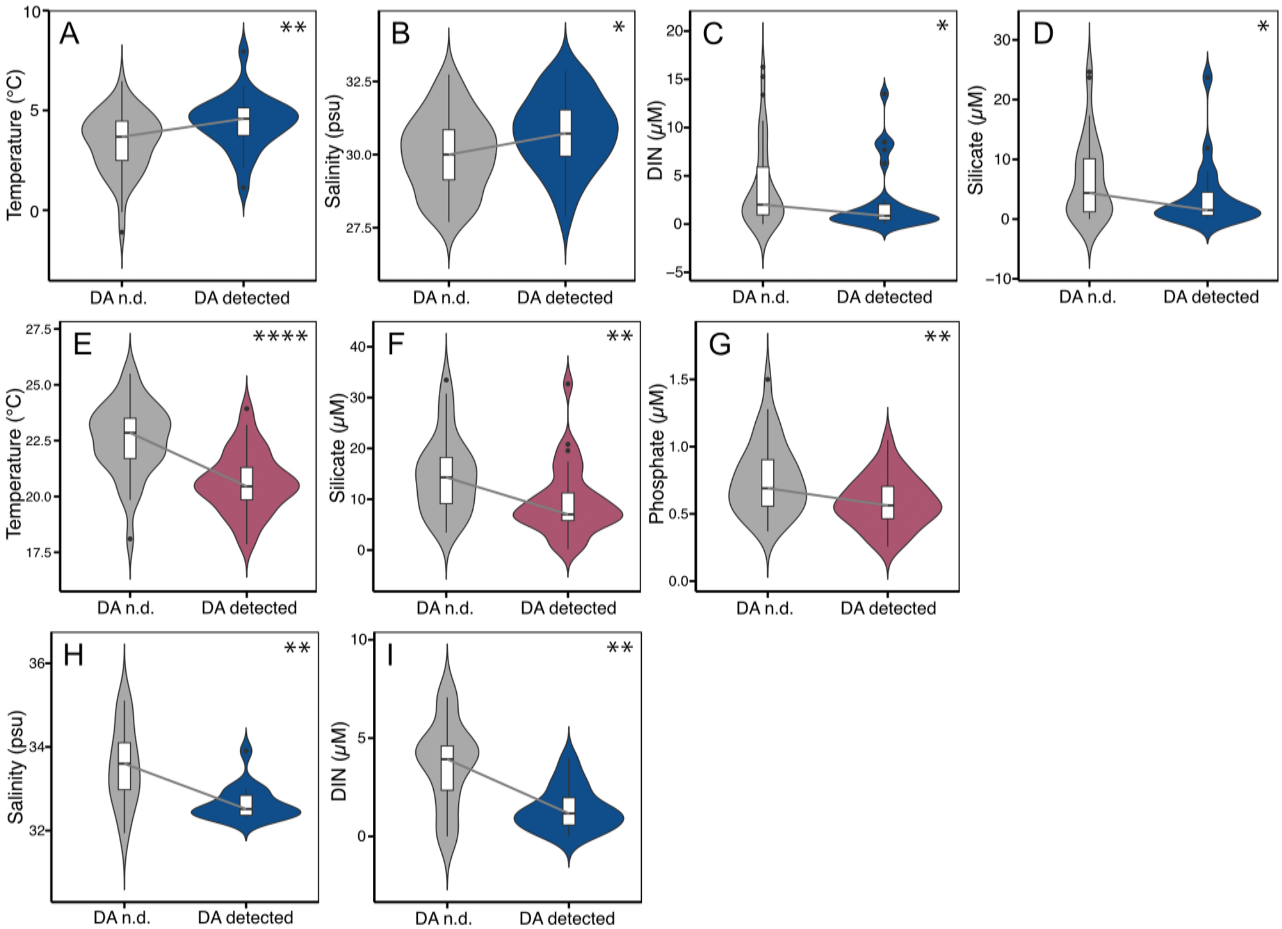

Fig. 8. Environmental correlates of DA detection in Narragansett Bay (NB) during winter (A-D), in NB during summer (E-G), and on the Northeast U.S. Shelf (NES) during winter (H-I). Color indicates the season of samples in which DA was detected, with winter shown in blue and summer in pink. Gray violins are shown for samples in which DA was not detected (n.d.). Only environmental parameters with significantly different means between periods of DA detection and non-detection are shown (p 0.05 = *, p 0.01 = **, p 0.001 = ***, p 0.0001 = ****), with statistical test results in Table S3. Median values of environmental parameters for DA-detected and DA-not-detected samples are connected with gray lines.

which occur more often in winter and could increase the amount of NES water entering the East Passage (Kincaid et al., 2008). Lastly, NB water outflow has a lower advection efficiency in winter compared to summer (Kincaid et al., 2003). In the summer, a strong cyclonic coastal current outside the mouth of NB enhances the advection of outflow to the southwest, preventing it from re-entering NB in a flood tide (Kincaid et al., 2003). In contrast, winter weakening of this current increases the proportion of an ebb tide that re-enters NB during flood tide (Kincaid et al., 2003). It is possible that this sequential re-entering of shelf water to NB allows multiple opportunities for exchange of the plankton communities that move with the water masses, supporting the ecosystem connectivity observed in the *Pseudo-nitzschia* species composition in winter. Because this region has highly complex physical dynamics, further work is necessary to understand the connectivity of offshore *Pseudo-nitzschia* populations with those of NB, and particle-tracking modeling efforts like those used in the Gulf of Maine to elucidate pathways of toxigenic *Pseudo-nitzschia* spp. introduction could be used here (Clark et al., 2021).

Each of the nine *Pseudo-nitzschia* species that exhibited the pattern of hypothesized inflow to NB from offshore have documented toxigenic strains (reviewed in Bates et al., 2018), indicating the potential for toxic events in NB during winter months. *P. australis*, a toxigenic species new to the Northeast U.S. that contributed to the 2016 and 2017 DA-related shellfish harvest closures (Clark et al., 2019; Roche et al., 2022; Sterling et al., 2022), was one of the most frequent species to exhibit the APP pattern during the study period (Fig. 4). Each time *P. australis* exhibited this spatiotemporal pattern, it was accompanied by DA detected both on the NES and in NB in the locations it was observed (winter 2018, winter 2022; Fig. 6, Table S4). While the *Pseudo-nitzschia* species hypothesized to have entered NB were often observed across 3 to 4 stations within the same NES-LTER transect, the most common station at which they occurred was Station L1, located closest to the mouth of NB (Fig. 4, Table S4). Therefore, it may be more important to monitor toxigenic species and DA at the nearshore NES-LTER stations during winter months to aid in predicting toxic events in NB.

The winter 2018 period serves as a case study for how time series observations could be used to capture instances of toxic HAB species entering an estuary from offshore and leading to events of elevated toxin. *Pseudo-nitzschia* species inflow to NB from the NES is likely a continuous process, and we may not have captured the full extent during the intervals in which we sampled. However, in winter 2018, we were able to observe the potential inflow of six toxigenic *Pseudo-nitzschia* species to NB accompanied by elevated DA. For a month prior to the winter 2018 NES-LTER cruise, *P. americana*, a non-toxigenic species, was the only *Pseudo-nitzschia* species observed in NB (Fig. S5; reviewed in Bates et al., 2018) and DA was not detected (Fig. 6). On the NES-LTER cruise transect, elevated DA occurred in the surface of all three stations sampled and six toxigenic species that were observed during this cruise also appeared at NB sites during the four weeks after (Fig. 3, Table S4). It should be noted that there were three additional species observed on the NES that did not occur in NB during the following four weeks (Fig. S5), so it is possible that these species also entered NB and did not survive or were not captured by the timing and volume of sampling efforts. Concurrent with the hypothesized inflow of species, DA at the NBPTS site was elevated following the research cruise (Fig. 6), indicating the potential for the seeding of species from offshore to initiate toxic events. It is essential that time series monitoring continues in this region to ensure events such as this one are captured with real-time sampling efforts.

4.2. *Pseudo-nitzschia* species vary in seasonality and toxin production

Temperature is a key factor that influences phytoplankton growth, distribution, and physiology (Anderson et al., 2021; Toseland et al., 2013), and some *Pseudo-nitzschia* species have unique *in situ* thermal ranges (Roche et al., 2022). In this study, NB *Pseudo-nitzschia* species assemblages displayed two main modes: a summer assemblage comprising predominantly *P. pungens* var. *pungens, P. multiseries, P. calliantha,* and *P. plurisecta* and a winter assemblage containing those four species in addition to *P. australis, P. americana, P. fraudulenta,* and *P. delicatissima* (Fig. 5B). Overall greater diversity was observed in NB within winter temperature ranges. Notably, *P. australis* was never observed in the NB summer samples in this study, while it was present during winter. During two winters, the occurrence of *P. australis* was linked to the hypothesized inflow from the NES (Fig. 4). The NES displayed less distinctive seasonal assemblages based on temperature, which could be due in part to the large cross-shelf temperature ranges and depth variation from surface and SCM samples, leading to overlap in temperature ranges in winter and summer. The NES subregion exhibits strong within-season variations in sea surface temperature, and these seasonal temperature ranges are expanding through time (Friedland and Hare, 2007). As the summer and winter temperature ranges expand and further overlap, we may observe even more similarity in winter and summer *Pseudo-nitzschia* species assemblages on the NES.

Pseudo-nitzschia spp. vary in DA production at the species and strain level (reviewed in Bates et al., 2018). Moreover, environmental species assemblages are dynamic and complex, making it challenging to determine contributions of individual species. Here, we used two sensitive 'omics approaches – DNA metabarcoding and targeted metabolite detection – to estimate which species may be responsible for toxin production based on species co-occurrence with DA. It is important to note that co-occurrence alone does not indicate that a species is actively producing toxin, however, these patterns provide insight into which species are likely to produce DA in these ecosystems. In this study, the six species that co-occurred with DA in a higher proportion of samples than their frequency across the entire dataset are considered to be probable toxin producers in this region, including *P. pungens* var. *pungens, P. multiseries, P.* calliantha, *P. plurisecta, P. australis*, and *P. fraudulenta* (Fig. 7). Strains of each of these species have been shown to produce DA in culture (reviewed in Bates et al., 2018), including a *P. australis* isolate from the Gulf of Maine (Clark et al., 2021) as well as *P. pungens* var. *pungens* and *P. multiseries* isolates from NB (unpublished data). Knowing that these six species are likely to produce DA in NB and the NES aids in targeted monitoring efforts. Furthermore, several of these species are more likely to co-occur with DA in winter or summer specifically, which can further inform seasonal monitoring. *P. pungens* var. *pungens, P. multiseries*, and *P. calliantha* are the most common species that co-occur with DA in summer, while other toxigenic species like *P. plurisecta, P. australis*, and *P. fraudulenta* may be more likely to produce DA in the winter (Fig. 7). Methods for monitoring DA biosynthesis gene expression (e.g. *dabA*), including metatranscriptomics and qPCR, have been developed to elucidate species-specific toxin production in other ecosystems (Brunson et al., 2018; Coyne et al., 2022; Brunson et al., 2024). Similar methods could be deployed on the NES in the future to further validate species-specific toxicity.

4.3. Elevated domoic acid in winter and under various environmental conditions

DA varied seasonally, annually, and spatially throughout this six-year dataset. In both NB and the NES, DA was more frequently observed in winter, and the highest DA measurement of the dataset was recorded at station L1 during the winter 2021 NES-LTER transect cruise (Fig. 6). Previously, winter DA events in the Northeast U.S., including the March 2017 toxic event in NB that led to shellfish harvest closures, were somewhat anomalous, with blooms more consistently occurring in summer and fall (Clark et al., 2021; Sterling et al., 2022). In NB, winter is typically dominated by the non-toxigenic *P. americana* (Sterling et al., 2022). However, elevated DA in the winter, particularly on the NES, indicates that toxigenic species are present and have the potential to cause toxic HAB events in winter conditions. Furthermore, DA varied across the shelf and was more frequently observed at the nearshore stations (Fig. 6). Since toxigenic species that entered NB from the NES were also observed most often at the nearshore stations (Fig. 4), these are critical sites to monitor with time series observations.

The relationship between *Pseudo-nitzschia* spp. cell abundance and DA concentration in environmental settings in not always direct or consistent. In this study, DA concentration did not directly correlate with *Pseudo-nitzschia* spp. cell abundance as enumerated by light microscopy (Fig. S2), which has been shown previously in NB and on the U. S. west coast (Marchetti et al., 2004; Sterling et al., 2022). However, some environmental studies have shown the contrary, with DA increasing as a function of *Pseudo-nitzschia* spp. cell abundance (McCabe et al., 2016; Smith et al., 2018). Here, DA was detected at least once in each subregion and season when cell abundance was below detection (1000 cells L^{-1}) in samples enumerated from 1 mL of whole seawater. However, DNA metabarcoding recovered *Pseudo-nitzschia* sequences from those same samples, underscoring the utility and sensitivity of DNA-based approaches for determining *Pseudo-nitzschia* spp. occurrence. It should be noted that the sensitivity of this approach may be partially due to the integration over a larger volume of seawater, as an average of 407 mL was filtered for DNA biomass in this study.

DA concentration is dependent on both the presence of toxigenic species and certain environmental conditions, although these conditions are not well understood and may vary by geographic location (reviewed in Bates et al., 2018). As with several prior studies of environmental DA, here there were no simple correlations of single environmental parameters with absolute DA concentration (Smith et al., 2018; Clark et al., 2021; Sterling et al., 2022). However, we identified potential environmental drivers of DA production in each subregion and season based on differences in samples where DA was detected and not detected (Fig. 8). During NB winters, DA was detected at significantly higher temperatures and salinities, and significantly lower DIN and silicate. In contrast, lower temperature, silicate, and phosphate coincided with DA in NB during the summer (Fig. 8). These opposite temperature trends between seasons indicate that DA is more common in NB when temperatures are not approaching either extreme. These patterns could represent intrusions of NES water to NB since shelf water tends to be warmer and saltier than NB in winter and colder than NB in summer (Kincaid et al., 2008). If these shelf water intrusions also bring toxigenic populations, the observed DA could be driven by both the inflow of toxigenic species and toxin-promoting environmental conditions. Consistent with culture-based macronutrient limitation studies, DA in NB was detected at significantly lower macronutrient concentrations in both summer and winter (Fuentes and Wikfors, 2013; Lema et al., 2017; Radan and Cochlan, 2018). In environmental assemblages, expression of the *dabA* DA biosynthesis gene has been shown to correlate with silicate limitation (Brunson et al., 2024). On the NES, there were no significant patterns of DA with environmental parameters in the summer, however, DA was observed at significantly lower salinity and DIN in winter (Fig. 8). This low salinity could represent enhanced precipitation, local variation in river runoff, or mixing with less saline water masses (Mountain, 2003). Notably, the NES region is mainly influenced by two water sources, the cool and fresh Labrador Current and the warm and saline Gulf Stream (Gonçalves Neto et al., 2021), so the DA coinciding with low salinity could indicate input from the Labrador Current.

4.4. Pseudo-nitzschia composition and toxicity on a warming continental shelf

Over the past century, sea surface temperatures on the NES have risen by nearly 1 °C, two times faster than the rate of warming of the regional atmospheric temperature (Shearman and Lentz, 2010; Gonçalves Neto et al., 2021). This region has also experienced enhanced seasonal temperature variation in the past century, which plays a role in increased stratification (Friedland and Hare, 2007). Strong stratification prevents nutrient-rich deep water from reaching the surface layer (Van de Waal and Litchman, 2020). Since DA was detected at lower nitrogen levels on the NES, an increase in stratification and surface nutrient limitation could lead to increased toxicity of *Pseudo-nitzschia* spp. Moreover, climate change has been predicted to cause shifts in phytoplankton community composition (Barton et al., 2016) and may also contribute to shifts in *Pseudo-nitzschia* species composition based on thermal niches (Clark et al., 2022; Roche et al., 2022). *P. multiseries* is one of the most commonly observed species in NB and on the NES, and it was one of the most frequent species to exhibit hypothesized inflow to NB following NES-LTER cruises (winters 2018 and 2021, Table S4). This species has been predicted as a threat to ecosystems that are warming with climate change due to its ability to acclimate and augment DA production at higher temperatures (Roche et al., 2022; Xu et al., 2023). Lastly, elevated DA coincided with warmer winter sea surface temperatures in NB, so intrusions of warm water in winter months or sea surface warming may also increase toxicity. As the coastal Northeast U.S. warms, seasonal variation intensifies, and *Pseudo-nitzschia* species composition shifts, there is potential for greater frequency and intensity of toxic HAB events in this region.

5. Conclusion

In this study, we investigated offshore sources of toxigenic *Pseudo-nitzschia* spp. that may seed toxic events in an estuarine system. We used DNA metabarcoding of the ITS1 marker to delineate *Pseudo-nitzschia* species composition and identified 21 *Pseudo-nitzschia* species, including three species that had never been observed in this region previously. During the six years of sampling, there was greater ecosystem connectivity and exchange of toxigenic *Pseudo-nitzschia* species from the NES to NB during winter, which included the potential inflow of the highly toxic *P. australis* during two separate winters. Winter samples were accompanied with higher and more frequent DA than in summer, and in particular, elevated DA was observed more often at coastal NES stations. We identified six *Pseudo-nitzschia* species as likely toxin-producers in NB and on the NES due to their high co-occurrence with DA, and DA was linked to various environmental conditions that differed by season and subregion. These findings highlight the importance of time series sampling efforts and suggest that winter HAB monitoring in this region is critical for capturing future toxic events.

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CRediT authorship contribution statement

Katherine M. Roche: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Isabella N. Church:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Alexa R. Sterling:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Tatiana A. Rynearson:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Matthew J. Bertin:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Data curation. **Andrew M. Kim:** Writing – review & editing, Methodology, Data curation. **Riley D. Kirk:** Writing – review & editing, Methodology, Data curation. **Bethany D. Jenkins:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing financial or personal relationships that could have influenced the work presented in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2024.102738.

Data availability

For samples collected at the NBPTS, physical, nutrient, and cell count data are publicly available at: https://web.uri.edu/gso/research/plankton/data/. For samples collected at WR, temperature and salinity data are publicly available at: https://web.uri.edu/gso/research/fish-trawl/data/. NES-LTER physical and nutrient data are available at: portal.edirepository.org. Raw DNA sequencing data are accessible in the NCBI Sequence Read Archive under BioProject PRJNA1129077 (SAMN42123204–42,123,391) and BioProject PRJNA690940 (SAMN17268730-SAMN17268922). ASV sequences identified to the *Pseudo-nitzschia* species level can be accessed in NCBI GenBank under accession numbers PQ002243-PQ002350 and MW447658-MW447770. ASV sequences used in this study with annotated taxonomy, a presence-absence matrix of *Pseudo-nitzschia* species in each sample, and associated environmental and domoic acid data are available at BCO-DMO (Roche et al., 2024a; Roche et al., 2024b).

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