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A Decade of Time Series Sampling Reveals Thermal Variation and Shifts in *Pseudo-nitzschia* Species Composition That Contribute to Harmful Algal Blooms in an Eastern US Estuary

Katherine M. Roche[1], *Alexa R. Sterling*[2], *Tatiana A. Rynearson*[1], *Matthew J. Bertin*[3] *and Bethany D. Jenkins*[1,2]*

[1] Graduate School of Oceanography, University of Rhode Island, Narragansett, RI, United States, [2] Department of Cell and Molecular Biology, University of Rhode Island, Kingston, RI, United States, [3] Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI, United States

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***Correspondence:**
Bethany D. Jenkins
bdjenkins@uri.edu

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In 2016-17, shellfish harvesting closed for the first time in Narragansett Bay, Rhode Island, USA, from domoic acid (DA), a neurotoxin produced by diatoms of the *Pseudo-nitzschia* genus. *Pseudo-nitzschia* have occurred frequently for over 60 years in Narragansett Bay's Long-Term Plankton Time Series (NBPTS), therefore it is surprising that the first closure only recently occurred. *Pseudo-nitzschia* species are known to vary in their toxin production, thus species identification is critical for understanding the underlying ecological causes of these harmful algal blooms (HABs). DNA in plankton biomass can be preserved for many years, so molecular barcoding of archived samples is useful for delineation of taxa over time. This study used amplification of the *Pseudo-nitzschia*-specific 18S-5.8S rDNA internal transcribed spacer region 1 (ITS1) in plankton samples and high throughput sequencing to characterize *Pseudo-nitzschia* species composition over a decade in Narragansett Bay, including eight years before the 2016-17 closures and two years following. This metabarcoding method can discriminate nearly all known *Pseudo-nitzschia* species. Several species recur as year-round residents in Narragansett Bay (*P. pungens* var. *pungens, P. americana, P. multiseries*, and *P. calliantha*). Various other species increased in frequency after 2015, and some appeared for the first time during the closure period. Notably, *P. australis*, a species prevalent in US West Coast HABs and known for high DA production, was not observed in Narragansett Bay until the 2017 closure but has been present in several years after the closures. Annual differences in *Pseudo-nitzschia* composition were correlated with physical and chemical conditions, predominantly water temperature. The long-term composition trends of *Pseudo-nitzschia* in Narragansett Bay serve as a baseline for identifying the introduction of new species, understanding shifting assemblages that contributed to the 2016-17 closures, and monitoring species that may be cause for future concern.

Keywords: ***Pseudo-nitzschia***, **DNA metabarcoding, Narragansett Bay, harmful algal blooms (HAB), long-term trends**

INTRODUCTION

Pseudo-nitzschia, a cosmopolitan genus of diatom, causes harmful algal blooms (HABs) through the production of the neurotoxin domoic acid (DA), which bioaccumulates in primary and secondary consumers and causes the potentially fatal illness Amnesic Shellfish Poisoning in humans (Bates et al., 1989). *Pseudo-nitzschia* HABs are frequent on the US Gulf and Pacific coasts (Del Rio et al., 2010; McCabe et al., 2016), though the Northeast US had not experienced levels of DA high enough to prompt shellfish harvest closures until 2016, followed by additional closures in 2017 (Clark et al., 2019; Sterling et al., in press). This included Narragansett Bay, Rhode Island, where for the first time a closure in RI was triggered by DA in shellfish meat exceeding National Shellfish Sanitation Program limits (reviewed in Bates et al., 2018; NSSP, 2019; Sterling et al., in press). This recent emergence of blooms was unexpected, as the RI Department of Environmental Management (RI DEM) has monitored *Pseudo-nitzschia* HABs in Narragansett Bay since the 1990s without a closure incident (Pers. comm. David Borkman, RI DEM), and *Pseudo-nitzschia* have been recorded for over 60 years at the site of the Narragansett Bay Long-Term Plankton Time Series (NBPTS) (Smayda, 1959-1997; https://web.uri.edu/gso/research/plankton/, 1999-2022).

Only half of the known *Pseudo-nitzschia* species are confirmed toxin producers, which makes identification of species important for monitoring toxic events (reviewed in Bates et al., 2018). Additionally, many *Pseudo-nitzschia* species are morphologically cryptic under light microscopy (Amato & Montresor, 2008; Lundholm et al., 2012). High throughput sequencing techniques and genus-specific amplicon metabarcoding have made it possible to accurately and cost-effectively identify species at high taxonomic resolutions (Canesi & Rynearson, 2016; Lopes dos Santos et al., 2022). Furthermore, this method can be applied to previously archived biomass samples, including those of the NBPTS. Thus, amplicon sequencing is an effective way to analyze the role of species composition in the development of HABs over long periods of time (Lopes dos Santos et al., 2022). For example, a previous study that used *Pseudo-nitzschia*-specific metabarcoding to distinguish species in Narragansett Bay found that the high toxin-producing species *P. australis* likely contributed to the 2017 shellfish harvest closure and several *Pseudo-nitzschia* species more commonly observed at the NBPTS contributed to the precautionary closure in 2016 (Sterling et al., in press). From 2017 – 2019 in Narragansett Bay, low levels of plankton-associated DA were observed with fall and summer maxima, indicating that toxic species of *Pseudo-nitzschia* remained present in seasonally distinct species assemblages (Sterling et al., in press).

Similar to *Pseudo-nitzschia* HABs recently appearing in new locations like Narragansett Bay, they are also increasing in frequency and intensity in many regions of the ocean as climate change increases sea surface temperatures and impacts the phenology of biogeochemical cycling (reviewed in Wells et al., 2015; Bates et al., 2018; Testa et al., 2018). One example of this was in 2015 on the US West Coast, when a large bloom of *P. australis* led to record levels of DA, and an anomalously warm water mass was implicated in bloom formation (McCabe et al., 2016). Narragansett Bay has also been impacted by climate change, with surface water temperatures that increased by 0.23± 0.1°C per decade from 1984 – 2020 and more pronounced winter warming than other seasons (Fulweiler et al., 2015; Benoit and Fox-Kemper, 2021). Additionally, this location has experienced climate-driven nutrient cycle changes as well as a reduction in nutrient inputs due to recent management changes of sewage treatment (Oviatt et al., 2017). Examining whether the long-term patterns in *Pseudo-nitzschia* species composition correlate with these shifting environmental conditions is necessary for understanding the emergent DA events in Narragansett Bay and predicting future HABs.

In this study, we investigated the following questions: (1) Have particular species of *Pseudo-nitzschia* increased in prevalence during the closures and subsequent years? (2) Is *P. australis* a new species in Narragansett Bay? and (3) How have changing environmental conditions influenced *Pseudo-nitzschia* species composition? To address these questions, we examined more than a decade of archived phytoplankton biomass samples collected weekly since 2008 by the NBPTS for DNA analysis, with corresponding chemical and physical measurements. We used metabarcoding of the ITS1 region to identify nearly all known *Pseudo-nitzschia* species during the timeframe prior to closures (2008 - 2015), the years in which closures occurred (2016 and 2017), and the subsequent years (2018 - 2019). Characterizing these long-term patterns in species assemblages and environmental conditions provides a baseline for understanding the changes in closure years and following, informing how future *Pseudo-nitzschia* HABs will be monitored in Narragansett Bay.

METHODS

Phytoplankton Biomass and Field Sampling

Narragansett Bay, a temperate estuary on the northeast continental shelf of the United States, receives riverine freshwater inputs from the north and saline tidal flow from the Atlantic Ocean in the south (Raposa, 2009). Weekly surface water samples from December 2008 to November 2019 were collected at the NBPTS (**Figure 1**), a mid-bay site located in the West Passage (41° 34.2' N, 71° 23.4' W). In this study, two sample sets were combined to analyze over a decade of data: (1) December 2008 - August 2017 samples collected by the NBPTS and (2) September 2017 - November 2019 samples collected at the same location and processed by Sterling et al., in press. The data from Sterling et al., in press are publicly available online through the National Science Foundation Biological and Chemical Oceanography and Data Management Office (BCO-DMO; Jenkins & Bertin, 2021a; Jenkins & Bertin, 2021b). For a comparison of sample set metadata and methods, refer to **Table S2**.

Biomass from the seawater samples collected by the NBPTS was filtered onto 25 mm 0.22 µm pore size ExpressPlus filters (MilliporeSigma, Burlington, MA, USA) and stored at -80°C until DNA extraction. The volume filtered for biomass capture varied and was based on the observed Secchi depth to normalize

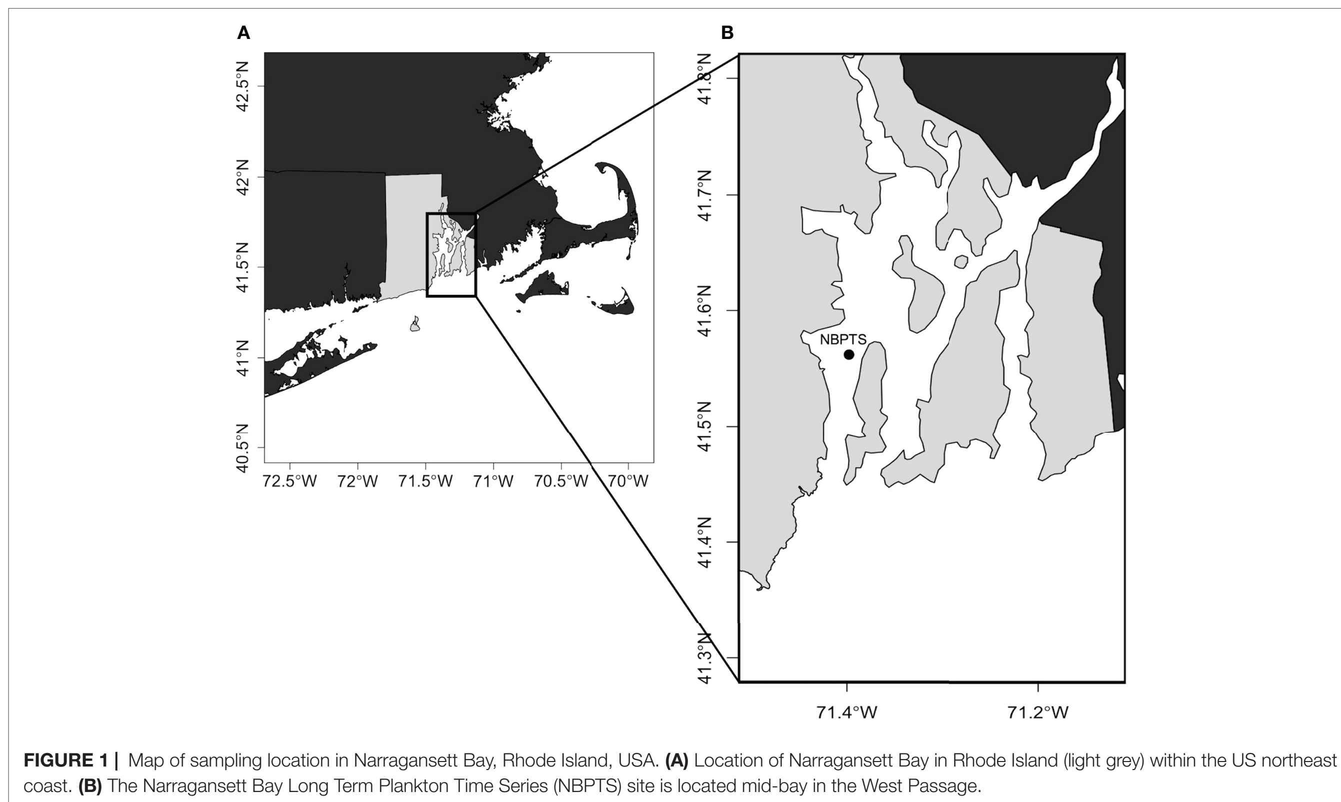

FIGURE 1 | Map of sampling location in Narragansett Bay, Rhode Island, USA. **(A)** Location of Narragansett Bay in Rhode Island (light grey) within the US northeast coast. **(B)** The Narragansett Bay Long Term Plankton Time Series (NBPTS) site is located mid-bay in the West Passage.

biomass collected, with 100 mL of seawater filtered per 1 m of Secchi depth. The volume filtered for NBPTS samples averaged 300 mL and ranged 100 – 600 mL. The Sterling et al., in press biomass samples were passed over 25 mm 5.0 µm pore size polyester membrane filters (Sterlitech, Kent, WA, USA), with an average of 240 mL and range of 75 – 430 mL filtered. Filters were then flash frozen in liquid nitrogen and stored at -80°C until DNA extraction. To ensure the comparability of sample sets using different pore sizes, we performed a comparison of species richness captured on each pore size that is outlined in the supplemental materials **Figure S1**. Sea surface temperature, sea surface salinity, chlorophyll *a* concentrations, *Pseudo-nitzschia* spp. cell counts, and nutrient measurements (nitrate, nitrite, phosphate) were obtained from the NBPTS prior to August 2017 and from the BCO-DMO dataset after September 2017 (NBPTS; Jenkins and Bertin, 2021a; Sterling et al., in press).

Sample Selection, DNA Extraction and Sequencing

DNA from 65 previously extracted NBPTS samples from December 2008 to April 2017 was used in this study (Canesi & Rynearson, 2016; Rynearson et al., 2020; Sterling et al., in press). An additional 76 NBPTS biomass samples from March 2009 to August 2017 were extracted. NBPTS samples with the highest corresponding *Pseudo-nitzschia* spp. cell counts under light microscopy for each month were selected. If all cell counts during a month were zero, samples were selected at random. The following frequency of samples was chosen when available: one sample per month from winter (December - February) and spring (March-May), and two samples per month from summer (June-August) and fall (September-November) due to the more frequent occurrence of high *Pseudo-nitzschia* abundance during these months (Sterling et al., in press). Additionally, 70 publicly available sequenced samples collected at the NBPTS site from September 2017 - November 2019 were used (Jenkins and Bertin, 2021b; Sterling et al., in press). Because only a partial time series exists for 2012, samples from that year were not analyzed as part of this study. In total, the dataset contained 211 samples from the NBPTS site during December 2008 - November 2019.

For all NBPTS samples, including those extracted prior to this study, DNA was extracted from biomass filters using a modified version of the DNeasy Blood & Tissue DNA extraction kit (Qiagen, Germantown, MD, USA) with the addition of 4 µL RNase, a 1 min bead beating step (0.1 mm and 0.5 mm Zirconia/Silica beads, BioSpec Products, Bartlesville, OK, USA), and elution into a total volume of 100 µL Buffer AE. DNA was amplified using a eukaryotic ITS1 forward primer 5' TCCGTAGGTGAACCTGCGG 3' (White et al., 1990) and 5.8S reverse primer 5' CATCCACCGCTGAAAGTTGTAA 3' (Sterling et al., in press), with MiSeq adapters added to the 5' ends of each primer for high throughput sequencing: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG - forward primer; 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG - reverse primer. This reverse primer was designed by Sterling et al., in press using a curated database of *Pseudo-nitzschia* sequences representing 41 species, thus the primer

set can discriminate nearly all known *Pseudo-nitzschia* species with high specificity, though it is also expected to amplify some other diatom and dinoflagellate genera. The ITS region (both ITS1 and ITS2) have been used to distinguish *Pseudo-nitzschia* species in several studies (Lundholm et al., 2003; Amato et al., 2007; Casteleyn et al., 2008; Kaczmarska et al., 2008). The ITS1 region alone has been demonstrated as an effective marker to distinguish intra- and interspecific variation of *Pseudo-nitzschia* species (Hubbard et al., 2008; Hubbard et al., 2014). In *Pseudo-nitzschia*, the ITS1 locus is naturally variable in length, so the expected PCR product length ranged from 235 to 370 base pairs (White et al., 1990; Sterling et al., in press).

For PCR amplification, the following reagents were used in 25 µL reactions: Phusion Hot Start High-Fidelity Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA), HPLC-purified forward and reverse primers at 0.5 µM concentration (Integrated DNA Technologies, Coralville, IA, USA), and 2 µL of DNA template. A stepwise thermocycle protocol was used to amplify samples: 30 s denaturation at 98°C, 15 cycles of 98°C (10 s), 64.1°C (30 s), 72°C (30 s), 15 cycles of 98°C (10 s), 72°C (30 s), 72°C (30 s), and 10 min final extension at 72°C (Sterling et al., in press). Positive and negative sequencing controls were used as reported in Sterling et al., in press. PCR products were submitted to the RI Genomics and Sequencing Center (Kingston, RI, USA) for sequencing library preparation and high throughput sequencing. There, ITS1 PCR products were cleaned with KAPA pure beads (KAPA Biosystems, Woburn, MA, USA), sequencing indices and adapters were attached using PCR (50 ng template DNA, 8 cycles) and the Illumina Nextera XT Index Kit (Illumina, San Diego, CA, USA) with Phusion High Fidelity Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA), and PCR products were cleaned again with KAPA pure beads before being visualized by agarose gel electrophoresis. Selected samples were run on a Bioanalyzer DNA1000 chip (Agilent, Santa Clara, CA, USA). All samples were quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) prior to pooling, and the final pooled library was quantified with qPCR in a LightCycler480 (Roche, Pleasanton, CA, USA) with the KAPA Biosystems Illumina Kit (KAPA Biosystems, Woburn, MA, USA). Samples were analyzed using v3 chemistry, 600 cycles, and 2x250 bp paired-end sequencing on an Illumina MiSeq (Illumina, Inc., San Diego, CA, USA). These sequencing methods were exactly the same as those in Sterling et al., in press.

Sequence Processing and Taxonomic Assignment

Illumina Miseq adapters and primers were trimmed using Cutadapt (v3.2; Martin, 2011) and sequences were quality checked before and after trimming using MultiQC (v1.9; Ewels et al., 2016). Amplicon sequence variants (ASVs) were delineated in DADA2 in R (v1.18.0; Callahan et al., 2016). One sample contained no reads following analysis with DADA2 and was removed. All ASVs, including those sequenced for this study and those from Sterling et al., in press, were assigned taxonomy using the scikit-learn naïve Bayes machine learning classifier in QIIME2 with the default confidence threshold of 0.7 (v2021.4.0; Bolyen et al., 2019). For taxonomic assignment, the curated reference database used in Sterling et al., in press was updated with an additional 170 unique *Pseudo-nitzschia* National Center for Biotechnology Information (NCBI) GenBank sequences, for a total of 302 sequences representing 51 species (retrieved June 1, 2021) (**Table S1**). To maximize the number of ASVs classified to the species level, additional ASVs were assigned by manual inspection of a megablast search that reported the top hit of each ASV from the BLAST nt database (retrieved June 24, 2021). Additional ASVs classified using the megablast search required >98% identity and >98% query cover to an NCBI *Pseudo-nitzschia* species, otherwise classifications were discarded. From this additional classification, we recovered only species already represented in our custom database from the pool of ASVs. All ASVs of the same species were agglomerated in R for downstream analyses.

Analysis of Species Composition and Environmental Conditions

Data were analyzed and visualized in R (v4.0.2; R Core Team, 2017) within RStudio (v1.3.1056; RStudio Team, 2020) using the following packages: phyloseq (v1.34.0; McMurdie & Holmes, 2013) for ASV dataset manipulation and transformations; ggplot2 (v3.3.3; Wickham, 2016) for scatterplot, heatmap, bar plot, and sample frequency plots; vegan (v2.5.7; Dixon, 2003) for dispersion tests and ANOSIM; indicspecies (v1.7.9; De Cáceres & Legendre, 2009) for indicator species analysis; raster (v3.5.15; Hijmans et al., 2015) for the sampling site map; and viridis (v0.6.2; Garnier et al., 2021) for colorblind-friendly figure color palettes. Relative abundances were calculated as the proportion of a species out of the total *Pseudo-nitzschia* sequencing reads for each sample, and these were only used in one visualization (**Figure 3**). Presence-absence Jaccard distances of composition data generated in phyloseq were used in all statistical analyses to avoid distorted relative abundance metrics that may arise from eukaryotic gene copy number variation (reviewed in Canesi & Rynearson, 2016; Gloor et al., 2017). This is a better approach for metabarcoding data than using relative or absolute abundance of sequencing reads (Zaiko et al., 2015; Canesi & Rynearson, 2016; Rynearson et al., 2020).

Similarities between temporal groupings of species composition were characterized using an analysis of similarity (ANOSIM) on a Jaccard distance matrix. An assumption of ANOSIM is relatively equal variances, or lack of dispersion, between groups being compared. Prior to ANOSIM, dispersion of groups was determined using betadisper() and permutest() (R: vegan) with 999 permutations and a significance level of 0.05. Only groups that did not have significant dispersion were used in ANOSIM, which included year and timeframe, the latter defined as two time periods: before closures (2009 - 2015) and during/after closure years (2016 - 2019). The groupings of samples by both month and season exhibited significant dispersion of groups and thus did not meet the assumptions to test for similarity in ANOSIM ($p=0.023$; $p=0.014$). ANOSIM was performed using anosim() (R: vegan) with 999 permutations and a significance level of 0.05. To determine which species preferentially

occurred before and during/after closures, an indicator species analysis (ISA) was performed on a Jaccard distance matrix using multipatt() (R: indicspecies), 9999 permutations, and a significance level of 0.05 on p-values adjusted for multiple testing.

A Best Subset of Environmental Variables (BIOENV) multivariate analysis was performed using bioenv() (R: vegan) to correlate environmental conditions with species composition. Environmental variables, including sea surface temperature, sea surface salinity, dissolved inorganic nitrogen (DIN), dissolved inorganic phosphorus (DIP), and chlorophyll *a* concentration were standardized using log-transformation prior to comparison with a Jaccard distance matrix. The BIOENV analysis was performed for all samples with complete physical and chemical data (n=178), as well as for each timeframe (before: n=81, during/after: n=97).

RESULTS

Sequencing and Taxonomic Assignment

Within the 141 samples from December 2008 – August 2017 that were sequenced for this study, there were a total of 12.3 x 10^6 read pairs. Initial reads per sample ranged from 4,821 to 115,087, with an average of 70,104 reads per sample. After DADA2 sequencing error inference, there was an average of 39,673 reads per sample, with a range of 1,335 to 66,903 reads. This is comparable to the average of 35,550 reads per sample reported in the Sterling et al., in press dataset. In total, 5,117 ASVs were recovered in the newly sequenced samples. Taxonomic assignment yielded 57 ASVs at the *Pseudo-nitzschia* species level (46 QIIME2, 11 megablast). When re-classifying the ASVs from Sterling et al., in press with the updated database, 27 ASVs were assigned to the species level (20 QIIME2, 7 megablast). The number of ASVs per sample ranged from 1 to 15 and an average of 5,773 sequencing reads per sample were assigned to *Pseudo-nitzschia* spp. Each species was represented by a minimum of 2 and maximum of 17 ASVs, and after aggregating ASVs by species, 17 *Pseudo-nitzschia* species were characterized in the samples.

Pseudo-nitzschia Species Composition During Periods of High Cell Abundance

During the study period, December 2008 – November 2019, *Pseudo-nitzschia* species were observed by light microscopy each year, excluding the gap in 2012 (**Figure 2**). Cell counts at the genus level surpassed the RI DEM HAB abundance threshold of 20,000 cells per liter each year for which there was abundance data except 2015 (**Figure 2**) (RI DEM, 2021). Specifically, weekly samples surpassed the cell abundance threshold 69 times across all seasons, though more frequently during spring (n=20) and summer (n=33) months (**Figure 2**). The closure years in 2016 and 2017 alone comprised 38% of these high cell abundance samples: 2016 (n=11) and 2017 (n=17). A subset of our total sequenced samples (n=55) have corresponding cells counts that surpassed the RI DEM HAB threshold and were used to analyze *Pseudo-nitzschia* species composition during high abundance periods. *P. pungens* var. *pungens* occurred most frequently on dates of high cell counts, followed by *P. multiseries* and *P. americana* (**Figure 3**). Only one sample contained just one species (*P. pungens* var. *pungens*; Aug 07, 2017), while all other samples contained between 2 and 7 species (**Figure 3**).

Long-Term Patterns of *Pseudo-nitzschia* Species Composition

The 17 species of *Pseudo-nitzschia* identified in the total dataset exhibited varying annual patterns of occurrence (**Figure 4**). Four species were observed most frequently in over 40% of samples - P. *pungens* var. *pungens* (66%), *P. americana* (66%), *P. multiseries* (55%), and *P. calliantha* (44%) - and were found in samples from each year of the dataset (**Figure 4A**). Several species appeared for the first time or increased in prevalence during closure and subsequent years. Most notably, *P. australis*, a well characterized toxin producer, was not present in any of the sequenced samples prior to 2017 and appeared for the first time on February 6, 2017, several weeks before the closure (**Figure 4B**). But from 2017 – 2019, *P. australis* continued to be observed, appearing in 23% of sequenced samples. Similarly, the toxin-capable species

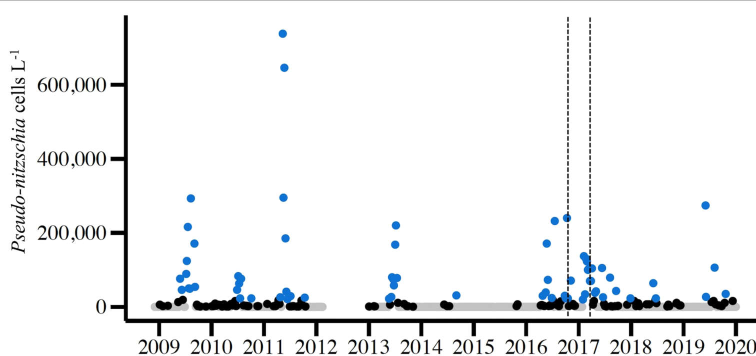

FIGURE 2 | Abundance of live *Pseudo-nitzschia* spp. cells from light microscopy counts of weekly NBPTS surface seawater samples spanning Dec 2008 – Dec 2019 (n=523). The NBPTS paused during Mar 2012 – Dec 2012, so no cell counts were collected. Blue points represent *Pseudo-nitzschia* abundance that surpassed the RI Department of Environmental Management (DEM) HAB abundance threshold of 20,000 cells per liter, while black points are non-zero abundances below the threshold and gray points are zero counts. Dotted lines denote the 2016 precautionary shellfish closure (Oct 7–30) and 2017 closure (Mar 1–24).

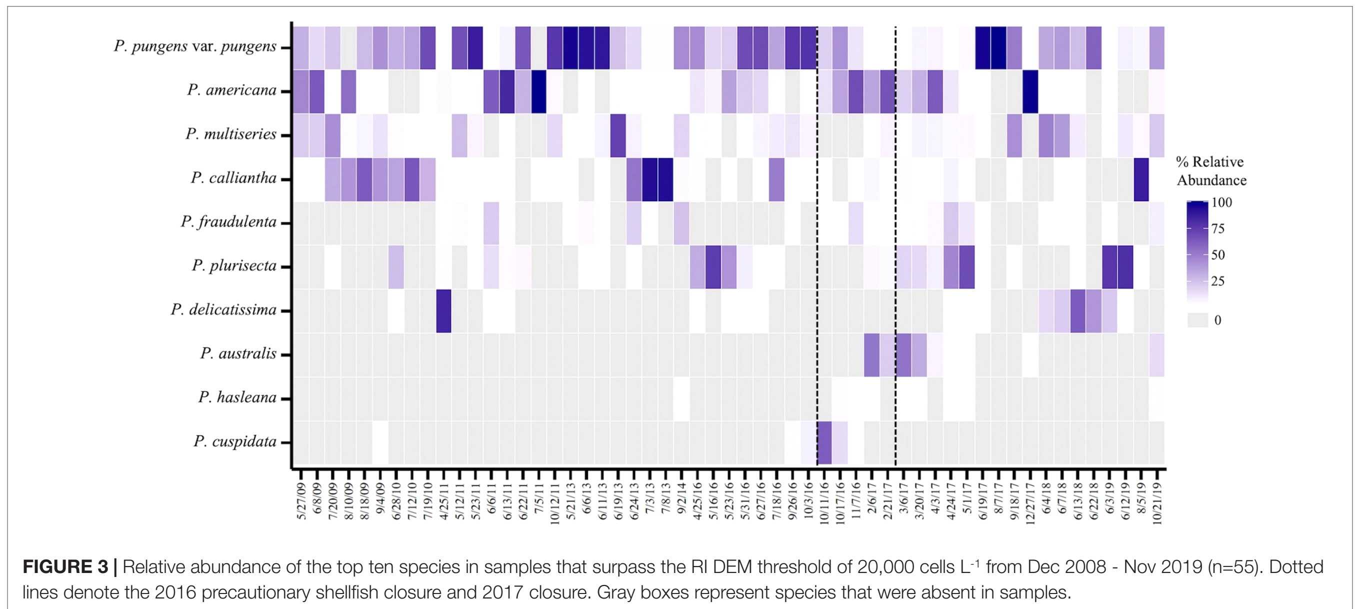

FIGURE 3 | Relative abundance of the top ten species in samples that surpass the RI DEM threshold of 20,000 cells L^{-1} from Dec 2008 - Nov 2019 (n=55). Dotted lines denote the 2016 precautionary shellfish closure and 2017 closure. Gray boxes represent species that were absent in samples.

P. hasleana and *P. subpacifica* appeared relatively infrequently in the years prior to the shellfish closures, however, each became more prevalent in frequency and were found in nearly each year from 2015-2019 (**Figure 4B**). Two additional toxin-capable species *P. fraudulenta* and *P. plurisecta* were present in low proportions of samples (0 - 32%) in the years prior to closures, but both were observed in higher proportions of samples (25 - 74%) from 2016-2019 (**Figure 4B**). Several rare *Pseudo-nitzschia* species that occurred in less than 10% of samples from the entire dataset were more prevalent during the closure years than any other timeframe, including *P. hasleana* in 2016 and 2017 and *P. cuspidata* in 2016 (**Figure 4B**).

To determine if species assemblages differed temporally, ANOSIM was used on the Jaccard distance matrix of *Pseudo-nitzschia* species composition. Two groupings were determined to be viable for ANOSIM based on dispersion of group tests (n=199): year (p=0.286), and timeframe (p=0.658), the latter of which was defined as before closures (2009 - 2015) and during/after closures

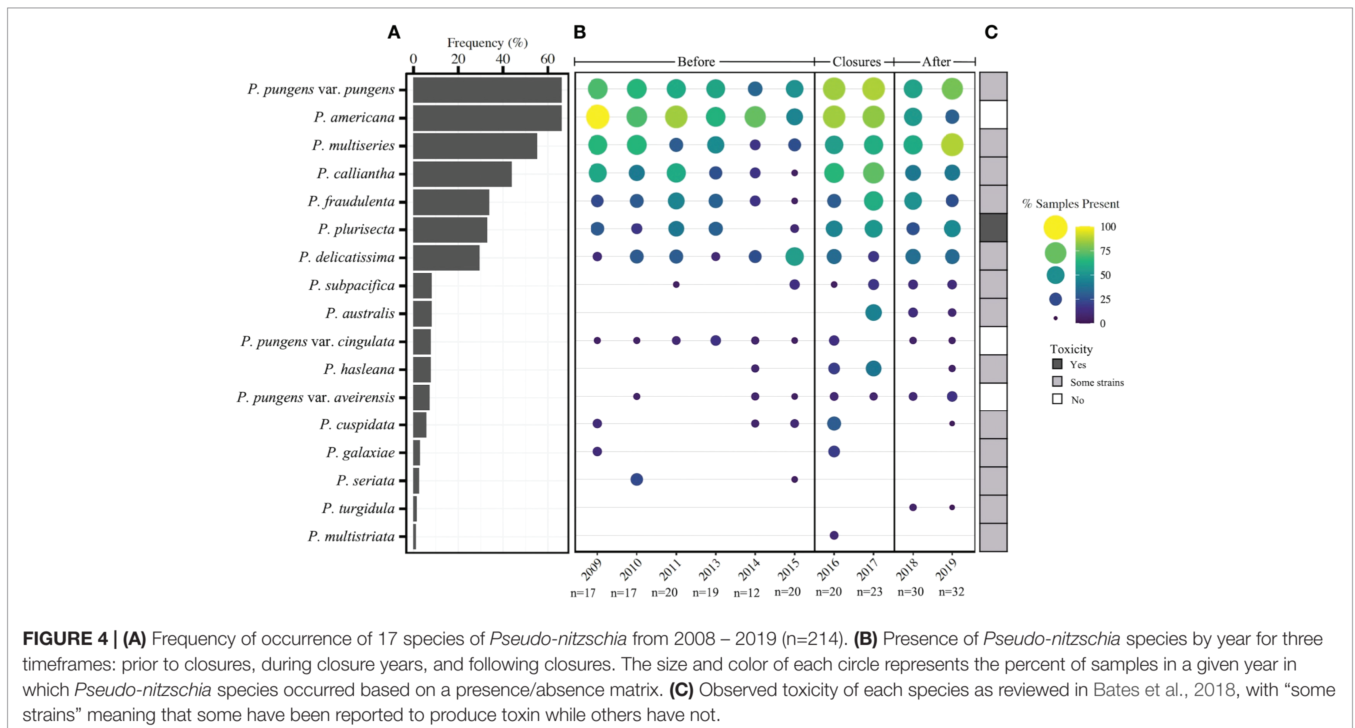

FIGURE 4 | (A) Frequency of occurrence of 17 species of *Pseudo-nitzschia* from 2008 – 2019 (n=214). **(B)** Presence of *Pseudo-nitzschia* species by year for three timeframes: prior to closures, during closure years, and following closures. The size and color of each circle represents the percent of samples in a given year in which *Pseudo-nitzschia* species occurred based on a presence/absence matrix. **(C)** Observed toxicity of each species as reviewed in Bates et al., 2018, with "some strains" meaning that some have been reported to produce toxin while others have not.

(2016 - 2019). ANOSIM revealed significant differences between species assemblages in each temporal grouping (p=0.001, ANOSIM statistic 0.1082 and 0.0625 respectively) (**Figure S2**). On average, species richness by year increased over time (**Figure S3**). An ISA was also performed on a Jaccard matrix to determine which species preferentially occurred in the timeframes before and during/after closures, since the species assemblages between these two timeframes were significantly different (ANOSIM, **Figure S2**). This analysis revealed that the strongest indicator species of the timeframe prior to closures were *P. americana* (p=0.003) and *P. seriata* (p=0.025). Eight species were significant indicators of the timeframe during and after closures, including *P. australis* (p=0.0001), *P. hasleana* (p=0.0002), *P. multiseries* (p=0.0027), *P. plurisecta* (p=0.017), *P. pungens* var. *aveirensis* (p=0.031), *P. pungens* var. *pungens* (p=0.033), *P. subpacifica* (p=0.045), and *P. calliantha* (p=0.046). All other species included in the analysis did not preferentially occur in either of these two timeframes.

Environmental Correlates of Species Composition

A multivariate correlation analysis (BIOENV) showed that *Pseudo-nitzschia* species composition correlated with various environmental conditions. The parameters that best correlated with species composition (0.1545) were surface temperature and DIP (**Table 1**). When the same multivariate correlation analysis was performed on each timeframe (before and during/after closures), the highest correlates prior to closures were temperature and DIP (0.678), while the during/after closure timeframe was most correlated with temperature, DIP, and DIN (0.2725) (**Table 1**).

Pseudo-nitzschia species were found at a wide variety of temperatures in Narragansett Bay (**Figure 5**). During the study period, temperatures ranged from -1.31 – 26.47°C, and *Pseudo-nitzschia* were present in sequenced samples through nearly this entire range, from -1.31 – 25.51°C. The seven most frequently observed species- *P. pungens* var. *pungens, P. americana, P. multiseries, P. calliantha, P. fraudulenta, P. plurisecta*, and *P. delicatissima*- were present over wide temperature ranges that did not differ greatly in the before closure (2008 - 2015) timeframe and during/after closure (2016 - 2019) timeframe. Several less prevalent species had smaller and more distinct temperature ranges. *P. subpacifica* (7.6 – 23.3°C), *P. pungens* var. *cingulata* (4.48 – 22.18°C), *P. pungens* var. *aveirensis* (15.4 – 22.18°C), and *P. cuspidata* (9.97 – 22.4°C) were observed during relatively higher temperatures, while *P. australis* (1.6 – 15.4°C) and *P. hasleana* (2.24 – 22.18°C) tended to occur at relatively lower temperatures (**Figure 5**).

DISCUSSION

Multiple Species Responsible for High Cell Abundance Blooms

Multiple species were present during *Pseudo-nitzschia* blooms in Narragansett Bay, defined here as periods where *Pseudo-nitzschia* cells surpass the RI DEM cell abundance threshold of 20,000 cells per liter (**Figure 2**) (RI DEM, 2021). Exceeding this threshold prompts DA Scotia testing of phytoplankton tow net samples followed by DA screening of shellfish meat, which can trigger shellfish harvest closures (RI DEM, 2021). More weekly light microscopy samples surpassed the cell abundance threshold in 2016 and 2017 than any other year of the study period (**Figure 2**). From 2016 - 2017, *P. pungens* var. *pungens. P. fraudulenta*, and *P. americana* were present in each of the bloom samples, with the first appearance of *P. australis* occurring in 2017 (**Figure 3**). Though *P. calliantha* was the fourth-most prevalent species across the entire study period, it occurred in less than half of the bloom samples (**Figures 3**, **4A**). Most samples that surpassed the abundance threshold contained diverse multi-species assemblages containing as many as seven of the top ten most abundant *Pseudo-nitzschia* species, with the exception of one bloom sample in which just *P. pungens* var. *pungens* occurred (**Figure 3**).

Resident and Indicator Species of Narragansett Bay

From 2008 – 2019, 17 species of *Pseudo-nitzschia* were detected in Narragansett Bay (**Figure 4**). All species were previously observed except *P. seriata*, which was recorded annually from 2012 to 2016 in the nearby Gulf of Maine and has only ever been observed in the North Atlantic (Hasle, 2002; Clark et al., 2019; Sterling et al., in press). Some patterns in species composition differ in the years prior to shellfish harvest closures, while others remain consistent. The four resident species (*P. pungens* var. *pungens, P. americana, P. multiseries*, and *P. calliantha*) that occurred in each year of the dataset followed similar patterns of prevalence before, during, and after the closure periods (**Figure 4**). Furthermore, these resident species occurred across the widest ranges of temperatures and were present through nearly the entire temperature range of Narragansett Bay (**Figure 5**). This prevalence across temperature ranges indicates a plasticity of these species to a range of environmental conditions. Consistent with our observations, *P. pungens* var. *pungens* and *P. multiseries* have been previously characterized as cosmopolitan, occurring in both equatorial and cold-water regions (Hasle,

TABLE 1 | BIOENV results of the three models with the highest Spearman rank correlation coefficient (ρ) for three timeframes: 2008 – 2019; 2008 – 2015; 2016 – 2019.

Entire dataset (n=178) 2008 – 2019		Before closures (n=81) 2008 – 2015		During/after closures (n=97) 2016 – 2019	
Parameters	ρ	Parameters	ρ	Parameters	ρ
Temp + DIP	0.1545	Temp + DIP	0.0678	Temp + DIP + DIN	0.2725
Temp + DIP + DIN	0.1474	Temp + DIP + Chla	0.0410	Temp + DIP	0.2695
Temp + DIP + DIN + Chla	0.1337	Temp + DIP + DIN + Chla	0.0255	Temp	0.2397

The environmental variables that appear in the strongest models are sea surface temperature (Temp), dissolved inorganic phosphorus (DIP), dissolved inorganic nitrogen (DIN), and chlorophyll a of the phytoplankton community (Chla).

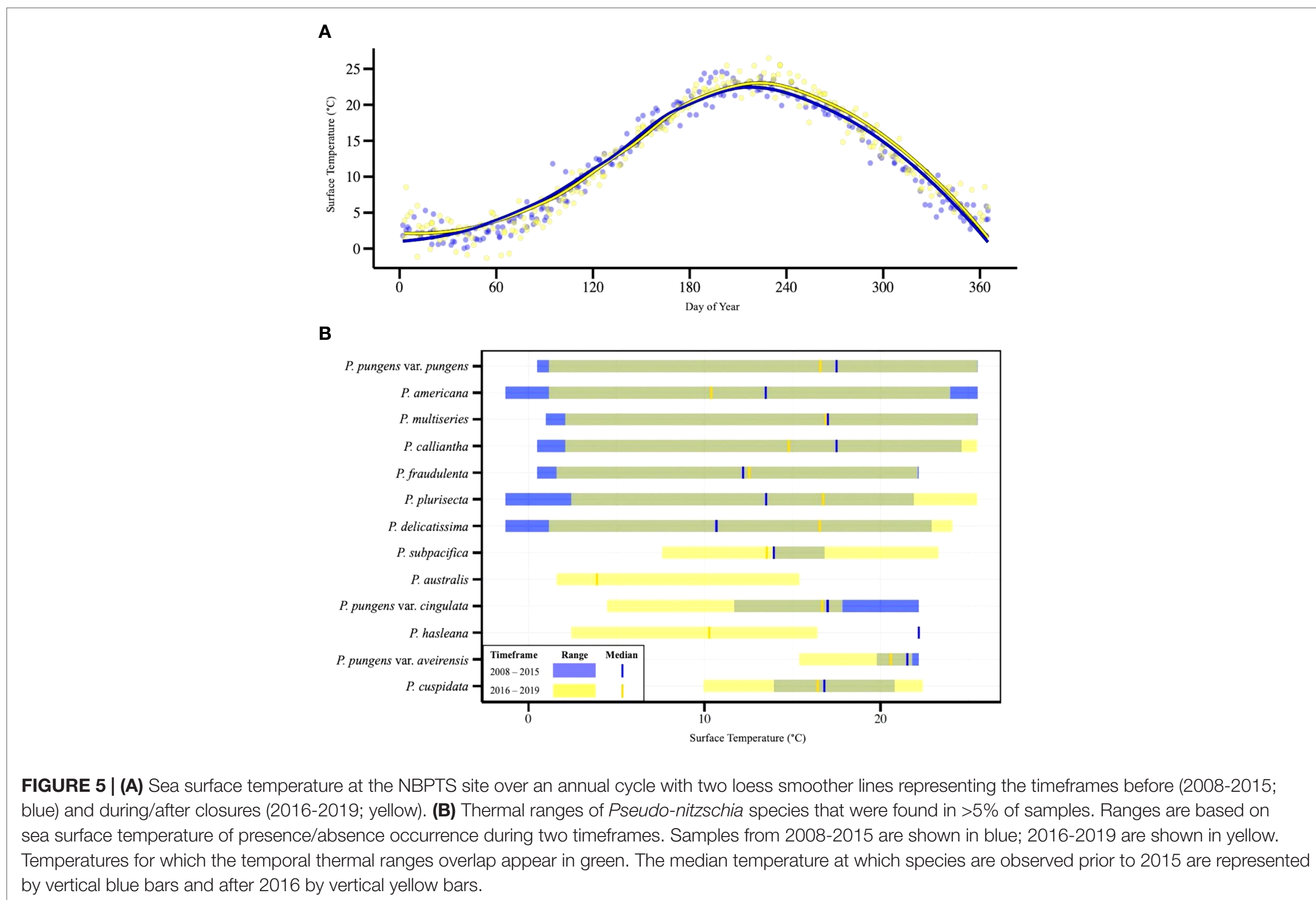

FIGURE 5 | (A) Sea surface temperature at the NBPTS site over an annual cycle with two loess smoother lines representing the timeframes before (2008-2015; blue) and during/after closures (2016-2019; yellow). **(B)** Thermal ranges of *Pseudo-nitzschia* species that were found in >5% of samples. Ranges are based on sea surface temperature of presence/absence occurrence during two timeframes. Samples from 2008-2015 are shown in blue; 2016-2019 are shown in yellow. Temperatures for which the temporal thermal ranges overlap appear in green. The median temperature at which species are observed prior to 2015 are represented by vertical blue bars and after 2016 by vertical yellow bars.

2002). Additionally, *P. americana* has been observed in a wide range of environments, including the Gulf of Maine, Malaysia, Mexico, and Namibia (reviewed in Bates et al., 2018). *P. calliantha*, is also a confirmed cosmopolite with occurrences in warm and cold environments (Stonik et al., 2011).

Species assemblages varied significantly between years and timeframes (before and during/after closures) (**Figure S2**). Though prior work in Narragansett Bay observed distinct species assemblages by season (Sterling et al., in press), the seasonal groupings in this dataset were unable to be tested for similarity because the monthly and seasonal groups displayed significant dispersion. Additionally, distinct species groupings by year and timeframe suggest that composition patterns have shifted over time in Narragansett Bay. The indicator species from each of these timeframes explains some of the temporal shifts. Before the closure periods, *P. americana*, which is a non-toxigenic year-round resident of Narragansett Bay, was the strongest indicator species. This timeframe was characterized by lower species richness and lower prevalence of toxigenic species (**Figure 4**; **Figure S3**). *P. multiseries* and *P. plurisecta* were two of the strongest indicator species for the during closure/after timeframe, and both toxigenic species increased in prevalence 2014 – 2019 (**Figure 4B**). Several other important indicator species of the during closure/after timeframe were *P. hasleana, P. subpacifica*, and *P. pungens* var. *aveirensis*, all of which were present in very few samples prior to closures but persisted in nearly each year of the closures and following (**Figure 4B**).

Temperature was the only consistent environmental driver that appeared in each of the top three multivariate models for the entire species composition dataset, the timeframe prior to closures, and the timeframe encompassing closures and subsequent years (**Table 1**). This is supported by previous work in Narragansett Bay that showed temperature also correlated with both *Thalassiosira* spp. and *Skeletonema* spp. assemblage composition from 2008 - 2014 (Canesi & Rynearson, 2016; Rynearson et al., 2020). The importance of sea surface temperature to phytoplankton community composition may be partially attributed to the wide range of temperatures that Narragansett Bay experiences throughout the year (**Figure 5**). As sea surface temperatures in Narragansett Bay continue to increase over time due to climate change (Fulweiler et al., 2015; Benoit & Fox-Kemper, 2021), this driver of *Pseudo-nitzschia* assemblage composition may lead to further shifts in the prevalence of certain species. This could include continued increases in the frequency of *P. australis, P. hasleana, P. galaxiae*, and *P. subpacifica*, all of which became more prevalent in Narragansett Bay in closure and subsequent years.

Additionally, availability of nutrients, including both DIP and DIN, impacted *Pseudo-nitzschia* species composition. DIP was a driver of species composition in all but one of the top multivariate models (**Table 1**). Phosphorus is a required

macronutrient for phytoplankton growth, though its relationship to *Pseudo-nitzschia* HAB formation is complex because high DIP increases *Pseudo-nitzschia* growth rate but low DIP correlates with increased DA toxin production (Pan et al., 1998; Sun et al., 2011; Brunson et al., 2018). Furthermore, DIN appears in the multivariate model with the highest correlation in the during closures/after timeframe (**Table 1**). The importance of DIN concentration to both *Pseudo-nitzschia* assemblage composition and toxin production in Narragansett Bay is supported by Sterling et al., in press, who found that nitrate and DIN : DIP correlated with species composition and low DIN correlated with elevated particulate DA. The recently reduced DIN in Narragansett Bay following wastewater treatment changes may play a role in impacting the composition and physiology of these *Pseudo-nitzschia* assemblages (Oviatt et al., 2017).

P. australis Introduction and Persistence in Narragansett Bay

P. australis was not observed in Narragansett Bay prior to 2017 (**Figure 4**) and was the strongest indicator species in the during closure/after timeframe as compared to samples prior to the 2016 – 2017 HABs. It appears to be recently introduced to the North Atlantic coasts of the US and Canada during the record HABs of 2016 (Clark et al., 2019, reviewed in Bates et al., 2018). Notably, it was absent during the RI precautionary closure of 2016 in which DA below the NSSP threshold was observed in shellfish meat (**Figure 3**; reviewed in Bates et al., 2018; Sterling et al., in press), meaning there were other species responsible for this event. Toxigenic species present during the 2016 closure included *P. pungen* var. *pungens* and *P. cuspidata*. *P. australis* continued to persist in Narragansett Bay in about one-fifth of the samples from 2017 – 2019, suggesting it remained in the region and may be a cause for concern in future toxic events. However, it was not the only toxic species in Narragansett Bay during the 2016 – 2017 closures, so continued monitoring of *Pseudo-nitzschia* at the species level is important for understanding which species contribute to producing closure levels of DA.

P. australis has been connected to a large 2015 HAB event on the US west coast where increases in both *Pseudo-nitzschia* abundance and DA were attributed to anomalously warm water temperatures and nutrient-rich upwelling (McCabe et al., 2016). Sea surface temperatures in the North Pacific were 2.5°C higher than long term means, ranging about 12 – 18°C during the closure period from May – November 2015 (McCabe et al., 2016). Increased DA production in West Coast *P. australis* strains was also associated with warmer temperatures (Zhu et al., 2017). In contrast to this, we found that *P. australis* occurred at a lower relative thermal range than any other species and was only observed in Narragansett Bay between 1.6 – 15.4°C, with a median temperature of 3.9°C. During the closure in February – March of 2017, the temperatures at which *P. australis* was observed ranged 2.44 – 3.26°C, so this particular toxic event was not initiated by a warm water mass. Increased toxin concentration in shellfish meat has been linked to higher growth potential of *P. australis* (McCabe et al., 2016). Clark et al. (2021) reports that the optimal growth temperature for a strain of *P. australis* isolated from the nearby Gulf of Maine was about 15°C, while studies with US West Coast isolates have found optimal growth temperatures of 17 – 18°C (Monterey Bay, California 2015 isolate; McCabe et al., 2016) and 23 – 26°C (Southern California isolate; Zhu et al., 2017). The presence of *P. australis* at much lower temperatures in Narragansett Bay suggests there may be other factors influencing its growth and toxin production, such as nutrient availability, bacterial associations, or zooplankton grazing (Maldonado et al., 2002; reviewed in Lelong et al, 2012; Lundholm et al., 2018).

CONCLUSION

In this study, we used a DNA metabarcoding approach on more than a decade of plankton samples from Narragansett Bay, RI to characterize *Pseudo-nitzschia* species diversity before, during, and after two shellfish harvest closures. We found that periods of high *Pseudo-nitzschia* cell abundances correspond to a wide diversity of species present, supporting the complexity of bloom-forming assemblages. We characterized several species as residents of Narragansett Bay, which occurred in samples in nearly each year of the study period. *P. australis*, a high toxin producing species, was not present in any of the samples until 2017, and thus is likely to be a newly introduced species. Additionally, species composition was most strongly influenced by water temperature. As water temperatures continue to rise in Narragansett Bay and toxigenic species become more prevalent, it is imperative to continue monitoring *Pseudo-nitzschia* species composition for increased frequency and potential introduction of toxigenic species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below:
https://www.ncbi.nlm.nih.gov/genbank/, OM672116 - OM672173.
https://www.ncbi.nlm.nih.gov/, SAMN25894732 - SAMN25894875.

AUTHOR CONTRIBUTIONS

Conception and design, KR, BJ. Sample collection, TR. Methodology, KR, AS, TR. Formal analysis, KR. Investigation, KR. Resources, BJ. Data curation, KR. Writing—original draft preparation, KR, BJ. Writing—review and editing, all authors. Visualization, KR. Supervision, BJ. Project administration, BJ, TR. Funding acquisition, BJ, MB, TR. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.889840/full#supplementary-material

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