Detecting the Diatom HAB Genus *Pseudo-nitzschia* in the Public Shellfish Harvesting Area of Huntington Beach State Park Using a Nanopore Sequencing Approach

Megan Cevasco[1], Aramis Lawson[2], Zachary Padgett[3], and Elena Renshaw[4]

AUTHORS: [1]Associate Professor, Department of Biology, Coastal Carolina University, Conway, SC, 29526, USA. [2]Student, Department of Biology, Coastal Carolina University, Conway, SC, 29526, USA. [3]Student, Department of Chemistry, Coastal Carolina University, Conway, SC, 29526, USA. [4]Student, Department of Biological Sciences, University of South Carolina, Columbia, SC, 29208, USA.

Abstract. Harmful algal blooms (HABs) cause detrimental economic, ecological, and human health impacts. A major group of HAB taxa of particular interest in coastal shellfish harvesting grounds consists of domoic-acid-producing species within the cosmopolitan marine diatom genus *Pseudo-nitzschia*. Domoic acid (DA) is a neurotoxic amino acid that bioaccumulates in the tissue of filter-feeding shellfish and is responsible for amnesiac shellfish poisoning (ASP) in humans and other marine life. To minimize human health risks and economically damaging shellfish harvesting closures, there is a need to develop proactive monitoring efforts that focus on determining the environmental conditions likely to support *Pseudo-nitzschia* HABs. This project uses Nanopore sequence data from PCR amplicons using eDNA collected from the public shellfish harvesting area in Huntington Beach State Park. The aim of this research is to explore the use of ribosomal primers in providing baseline taxonomic data supporting effective management of shellfish harvesting areas along the South Carolina coast.

INTRODUCTION

Harmful algal blooms (HABs) and the detrimental impacts they generate appear to be increasing on a global scale. The purpose of this study is to explore the use of multiple ribosomal primers in providing baseline taxonomic data indicating the diversity of the HAB-associated diatom genus, *Pseudo-nitzschia*, in South Carolina coastal waters. These data will provide the basis for developing cost-effective taxonomic assays that target locally relevant *Pseudo-nitzschia* taxa. The development of such an assay will enhance and support effective management of shellfish harvesting areas by providing rapid and specific information to decisionmakers determining the level of human health risk at a given location and potentially reducing the number and duration of shellfish harvesting closures.

BACKGROUND AND RELATED WORK

As both the frequency and severity of harmful algal blooms (HABs) continue to increase worldwide, so too do risks of detrimental economic, ecological, and human health impacts arising from these events (McCarthy et al. 2015; Kelchner et al. 2021). In coastal marine environments, HABs associated with domoic acid (DA) producers within diatom genus *Pseudo-nitzschia* are of particular concern (Dong et al. 2020, Moore et al. 2019). Currently, 26 species of *Pseudo-nitzschia* are identified producers of DA, a potent neurotoxin that bioaccumulates within the tissue of filter-feeding organisms (Bates et al. 2018). High concentrations of DA detected in tissue samples using enzyme-linked immunosorbent assays (ELISA) have been shown to cause adverse effects in multiple vertebrate taxa as a result of trophic transfer in the marine food web (Berdalet et al. 2015; Kelchner et al. 2021). Notably, human consumption of shellfish with elevated DA concentrations can cause Amnesic Shellfish Poisoning (ASP), a condition characterized by gastrointestinal distress, mental disorientation, short-term memory loss, seizures, and, in rare occurrences, death (Grant et al. 2010, Wright et al. 1989). To minimize human health risks and economically damaging shellfish harvesting closures, there is a need to develop proactive monitoring efforts that focus on determining the microbial community structure and environmental conditions associated with *Pseudo-nitzschia* HABs (Kelchner et al. 2021; Van Meerssche et al. 2018).

Monitoring populations of DA-producing *Pseudo-nitzschia* in coastal waters commonly employs a combination of approaches to gather microscopic and ELISA data. While light microscopy remains a standard methodology for observing *Pseudo-nitzschia*, multiple cryptic species within the genus severely limits the identifiability of DA-producing species (Tenorio et al. 2021). This limitation poses a challenge for conducting observationally-based estimates of *Pseudo-nitzschia* species diversity and for monitoring shifts in the relative abundance of HAB taxa (Liu et al. 2020). HAB monitoring and management efforts also often rely on DA-specific ELISA results to indicate the neurotoxin's concentration in tissue samples but not the identity of the taxa producing the DA. For example, in 2016, a HAB of *Pseudo-nitzschia* spp. resulted in a regional closure of shellfish harvesting grounds in the Gulf of Maine based on DA concentrations exceeding 20 μg g^{-1} in shellfish tissue, yet the relative contributions of individual *Pseudo-nitzschia* species during this event remains an area of active research (Clark et al. 2021).

The development of molecular screening implemented in quantitative polymerase chain reaction (qPCR) and DNA metabarcoding methodologies has served both to improve taxonomic resolution and to explore the relative abundances of *Pseudo-nitzschia* species within the context of HAB dynamics (Ajani et al. 2021; Liu et al. 2020). Genotyping via qPCR typically involves amplification of a short (75–200 base-pair) DNA fragment immediately followed by high-resolution melting (HRM) to denature the amplified product and create a melt profile reflecting a taxon-specific nucleotide composition (Penna and Galluzzi 2013). As genotyping using qPCR/HRM requires both that the primers used for sequence amplification exclusively target the taxa of interest within isolated DNA, and that the amplicons contain enough nucleotide variability to resolve species-level differences, establishing a baseline understanding of location specific *Pseudo-nitzschia* molecular diversity is essential for the success of this approach (Pugliese et al. 2017; Ajani et al. 2021). In contrast, molecular barcoding and metabarcoding approaches developed using next-generation sequencing technology are capable of recovering sequence data from amplicon libraries of isolated environmental DNA (eDNA) representing variable taxonomic ranges contingent on the specificity of primers. As such, high-throughput sequencing approaches offer advantages in avoiding taxonomic biases introduced during culturing and provide a means of capturing both diversity and relative abundance data inclusive of both invasive and rare taxa in samples (Liu et al. 2020). Additionally, improved sequencing depths (10^3 - 10^5 reads per amplicon) facilitated by modern sequencing platforms allows for simultaneous investigation of microbial taxa co-occurring with *Pseudo-nitzschia* HABs (Nishimura et al. 2021).

The efficacy of molecular approaches in capturing *Pseudo-nitzschia* species diversity from eDNA samples containing complex marine microbial biota requires optimization of methodological factors pertaining to primer design, sequencing chemistries, and bioinformatic considerations. As both qPCR and amplicon sequencing analyze amplified DNA, the use of nucleotide primers with high binding affinity for DNA templates in target taxa is critical to their success (Penna and Galluzzi 2013). Nuclear-encoded ribosomal genes provide templates favorable for designing primers that can be anchored in highly conserved sequences but also span variable inter-specific regions capable of distinguishing multiple *Pseudo-nitzschia* taxa (Pugliese et al. 2017). Specifically, the D1–D3 sequence region coding for the ribosomal large subunit (LSU) contains nucleotide variability appropriate for genotype screening of *Pseudo-nitzschia* cultures (Nishimura et al. 2021; Pugliese et al. 2017). Ribosomal sequence amplification targets also provide the advantage of aiding in bioinformatic identification by having strong representation in reference databases (Singer et al. 2019). Primers designed to amplify multiple taxa within marine microbial communities, however, often result in uneven taxonomic coverage (Taberlet et al. 2012). Moreover, amplicon length constraints dictated by next-generation sequencing platforms and chemistries can further skew diversity estimates (Nishimura et al. 2021). The specificity and sensitivity of primers to comprehensively genotype all the 58 known *Pseudo-nitzschia* species using eDNA templates remains untested.

PROJECT GOAL

The goal of this study is to explore the efficacy of multiple primer sets in detecting *Pseudo-nitzschia* species diversity present in water samples collected from the public shellfish harvesting area at Huntington Beach State Park (HBSP), SC, using the high-throughput sequencing approach offered by Oxford Nanopore Technology's (ONT) portable MinION system. In this exploratory investigation of Nanopore sequencing to identify *Pseudo-nitzschia* in eDNA samples, collection efforts were restricted to a single location in order to assess and to optimize the methodological approach for future application in multiple shellfish harvesting areas along the SC coast. The MinION system is a third-generation hand-held sequencing platform which uses nanopore biosensors inserted into a synthetic polymer membrane to read nucleotide bases from single-stranded DNA molecules being transported through the pores causing temporary detectable changes in voltage (Mason and Elemento 2012). Nanopore sequencing was selected for this primer exploration project based upon the system's relatively low cost (~$2K MinION device and library preparation kit) and flexibility to accommodate real-time sequencing of variably-sized amplicons ranging from 102 to over 105 nucleotide

bases (Wang et al. 2021). As such, the MinION has potential to serve as a portable, rapid, and relatively low-cost option for researchers and water management professionals in the identification of target taxa in water samples. To this end, the sequence data recovered in this study explores both *Pseudo-nitzschia* species diversity present at HBSP and primer efficacy in detecting this diversity using five different primer sets with specific rDNA nucleotide targets and designed to resolve variable taxonomic-levels.

MATERIALS AND METHOD

Water samples of 500 mL each were collected at a depth of 1 meter from the public shellfish harvesting area (Oyster Landing) at HBSP (33.52106°, -79.06093°). Samples were collected in 2022 (6/7, 6/14, 6/21, 6/28, 7/14, 7/19) in duplicate from three locations roughly 25 meters apart along a North/South transect across the harvesting area. To capture broad taxonomic representation within eDNA samples, collections took place under a range of environmental conditions occurring during June and July 2022, as recorded in Table 1.

After collection, water samples were immediately transported to Coastal Carolina University for processing. Each 500 mL sample was filtered with 4.5 µm membrane filters (MilliporeSigma) to select for eukaryotic microbiota. DNA extraction was done aseptically in a UV-sterilized laminar flow hood (AirClean Systems) using the DNeasy PowerWater Kit (Qiagen) and following the manufacturer's instructions for lysis by bead-beating including a 10 min. incubation of bead tubes at 65°C followed by 10 min. of vortexing. After extraction, the eDNA was quantified using a Qubit 2.0 fluorometer (Thermo Fisher) with a Qubit dsDNA HS Assay Kit (Thermo Fisher) prior to amplification by polymerase chain reaction (PCR).

The oligonucleotide primers selected for eDNA amplification consist of three broad spectrum and two *Pseudo-nitzschia*-specific primer pairs. Two of the broad-spectrum primer sets, Euk (Medlin et al.1988) and 28S (Rousseau et al. 1997) target the amplification of microeukaryotes from multiple phyla but amplify different rDNA regions (~1200 base pairs from the small subunit, 18S rDNA, and ~680 base pairs from the large subunit, 28S rDNA, respectively (see Figure 1)). The third broad spectrum primer set, 18S (Pillet et al. 2011), was designed to capture diversity within the diatoms (class Bacillariophyceae) by amplifying an ~830 base pair fragment of SSU rDNA from multiple diatom genera. In contrast to the broad-spectrum primers, the HRM primer set (Pugliese et al. 2017) specifically targets the genus *Pseudo-nitzschia* by amplyfing ~132 base pairs in the LSU ribosomal gene region and was designed to distinguish *P. calliantha*, *P. delicatissima/P. arenysensis* complex and *P. pungens* in Mediterranean Sea samples using a qPCR/HRM methodology. Partially overlapping both the HRM and 28S primer sets, the AL2 primer set was developed for this study as a *Pseudo-nitzschia*-specific primer amplifying an ~ 138 bp fragment within the DI and D2 regions of LSU rDNA. The AL2 primer pair, forward (5′-TGTTTGGGATTGCAGCTCTA-3′), reverse (5′-CTTTGCATCTTTCCCTCACG-3′), was designed for

Table 1. Water sample collection metadata from Oyster Landing (public shellfish harvesting area), Huntington Beach State Park, Murrells Inlet, SC.

Sample ID	Collection Date	Time	Water Temp (C°)	Salinity (ppt)	Tide
1.1	6/7/22	9:30 a.m.	74	37	High
1.2	6/7/22	9:30 a.m.	74	37	High
2.1	6/14/22	9:30 a.m.	80	36.5	Mid
2.2	6/14/22	9:30 a.m.	80	36	Mid
2.3	6/14/22	9:30 a.m.	80	37	Mid
3.1	6/21/22	9:55 a.m.	80	38	Low
3.2	6/21/22	9:55 a.m.	80	38	Low
3.3	6/21/22	9:55 a.m.	80	38	Low
4.1	6/28/22	3:30 a.m.	81	39	Low
4.2	6/28/22	3:30 a.m.	81	38	Low
4.3	6/28/22	3:30 a.m.	81	39	Low
5.1	7/19/22	8:25 a.m.	83	36.5	Low
5.2	7/19/22	8:25 a.m.	83	36.5	Low
5.3	7/19/22	8:25 a.m.	83	36.5	Low

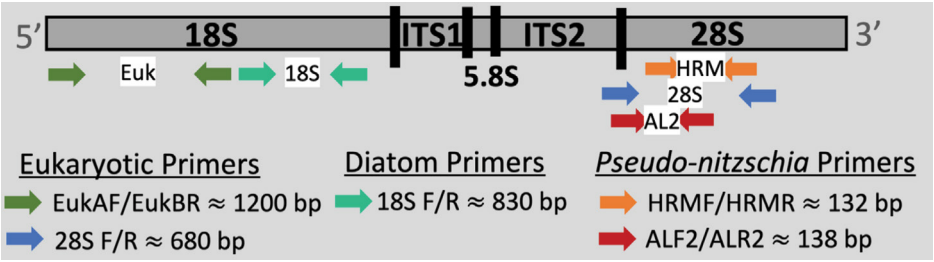

Figure 1. Primer Map showing the approximate binding sites of the primers used in this study on a eukaryotic ribosomal DNA template.

this study using Primer-BLAST (Ye et al., 2012) with a template of *P. pungens* (NCBI accession no. HQ111384.1) and examined with BLAST demonstrating *in silico* the primer pair's specificity to *Pseudo-nitzschia* targets.

The thermal cycling conditions varied for each primer set based on amplicon length and primer annealing temperatures but generally consisted of PCR profiles with an initial denaturation of 3 min. at 95°C, followed by 35–40 cycles of 95°C for 10–15 sec., an annealing step of 60–61°C for 15–30 sec. and an extension step of 72°C for 10–30 sec.; with a final 72°C extension for 7 min. as shown in Table 2.

The PCR amplicons were cleaned using AMPure XP beads (Agencourt) according to the manufacturer's instructions, visualized on a 1.5% agarose gel with SYBR® Green (Lonza Bioscience), quantified by fluorometry as described above, and pooled to normalize DNA concentrations for library construction.

Amplicon libraries were constructed with Oxford Nanopore's Ligation Sequencing Kit SQK-LSK- 110. Ligation was performed with a modified protocol using T4 ligase (New England Biolabs) to attach adaptors (motor protein with helicase activity) to the amplicons. Following library construction, a R9.4.1 Flow Cell was inserted into the MinION MkIB device connected to a MacBook Pro meeting ONT device standards. The amplicon library was then primed and loaded into the flow cell, and sequencing was started using MinKNOW software (ONT) which translates the voltage disruptions of bases passing through a nanopore saved in the FAST5 and FASTQ file formats. Post-processing removal of the Nanopore adapter sequences, filtration of low-quality reads, and base calling were performed by the recurrent neural network software Guppy version 4.2.2 (ONT) integrated with the MinKNOW program.

After base calling, EPI2ME (Metrichor Ltd., ONT), a cloud-based nanopore data platform, was used to initially thread data into a bioinformatic workflow. At the time of data analysis, eukaryotic microalgae were not supported by the WIMP (What's In My Pot) workflow. This required that taxonomic identification of sequence reads was accomplished by threading the data into NCBI's Nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al. 1990) to detect *Pseudo-nitzschia* species and other taxa of interest. As the sequence runs generated variable numbers of sequence reads, comparative subsamples (200 reads) were collected from each of the five runs for taxonomic identification. Identifications were assigned based on BLASTn sequence identity values above 90% with sequence coverage values of at least 90%. In cases with conflicting species-level matches, taxonomic assignments were restricted to the genus-level. The subsampled Nanopore sequencing data analyzed in this study have been deposited in the NCBI BioSample database with accession numbers SAMN34493767, SAMN34493768, SAMN34493769, SAMN34493770.

RESULTS

The total number of Nanopore sequence reads from MinION runs of amplicon libraries generated from broad spectrum primer sets are as follows: Euk (2,757), 28S (2,680), and18S (995). The influence of specific library and flow cell preparation factors in producing the observed variance in the total number of sequenced reads recovered from each amplicon library, however, remains undetermined, as each amplicon library was subjected to a single sequence run on the MinION device. For the broad primer sets, analyses using the EPI2ME WIMP bioinformatic workflow indicate that, proportionally, the Euk primer set produced the greatest percentage of off-target prokaryotic sequences (52.8%), followed by 28S (16.8%) and 18S (11.8%) as shown in Figure 2. The Euk primer set library recovered chlorophyte (green algae) sequences most frequently (41.7%), while diatom (subphylum Ochrophytina) sequences constituted 28.4% of the analyzed subsample, as shown in Figure 3. Amplicons indicating the presence of the DA producer, *Pseudo-nitzschia delicatissima*, were found in low abundance (2%) within the subsampled Euk primer set library. The analyzed 28S primer set library sequences were found to amplify a greater proportion of ochorophytes (66.7%) relative to the Euk primers, yet like the Euk library, *P. delicatissima* was the only *Pseudo-nitzschia* taxa represented (6%) in the subsample.

Table 2. Primer specific Thermal-cycling PCR conditions.

Primer Set	Annealing Temp. (C°)	Annealing Time (Sec.)	72 C Extension Time (Sec.)
18SF/18SR	60	30	30
28SF/28SR	60	30	30
AL2F/AL2R	61	15	10
EukF/EukR	61	30	30
HRMF/HRMR	60	15	10

Interestingly, the diatom specific 18S primer set library did not recover any *Pseudo-nitzschia* sequences, despite generating sequence data representing 21 diatom genera which comprised 74.2% of the library's analyzed sequence data.

The use of *Pseudo-nitzschia*-specific primer sets yielded a total of 14,069 quality filtered sequence reads; 8,600 from the HRM amplicon library and 5,469 from the AL2 amplicon library. For the HRM amplicon library, 72% of the total recovered sequences were of eukaryotic origin, and 28% of those sequences were identified as belonging to the genus *Pseudo-nitzschia*. The most abundant sequences recovered using the HRM primers belong to *P. pungens* (43%), *P. calliantha* (14%), and *P. cuspidata* (13%), all of which are identified as potential DA producers (see Table 3). In contrast, 61% of the AL2 amplified sequence data is of eukaryotic origin of which 67% represents *Pseudo-nitzschia* taxa. Like the HRM primers, the AL2 primers recovered both *P. pungens* (49%) and *P. cuspidata* (14%). However, the AL2 primer set did not generate sequence data identifiable as *P. calliantha* but did amplify the DA producer *P. multiseries* (15%). Although exploratory, these results indicate the taxonomic complexity of *Pseudo-nitzschia* taxa in the shellfish harvesting grounds of HBSP, which merits consideration in HAB management along coastal South Carolina waters.

DISCUSSION

The use of Nanopore third-generation technology can be an important tool in exploring HAB diversity. Nanopore sequencing is increasingly being applied in biological monitoring efforts as a portable and economically efficient method which can accommodate variable amplicon lengths in eDNA screening (Garlapati et al. 2019). For example, the Oxford Nanopore MinION device has been used both to monitor toxic *Mycrocystis* blooms in the Great Lakes and to estimate the abundance of HAB-associated taxa (Koeppel et al.,2022). This genomics-based approach was able to detect differential abundances among taxa from pre-bloom eDNA samples relative to post-bloom samples which Koeppel et al. (2022) posit may prove valuable in developing HAB forecasting models.

All amplicon sequencing approaches, however, are subject to several challenges, including degraded eDNA (Deiner et al., 2017), and primers amplifying off-target and artifact fragments (Valentini et al. 2016; Vecherskii et al. 2021). Given that the dissimilar taxonomic profiles recovered from sequence data amplified using multiple variably designed primer-sets was anticipated (van der Loos and Nijland 2021), it was nevertheless surprising that the diatom-specific primer set did not recover *Pseudo-nitzschia* species. This result indi-

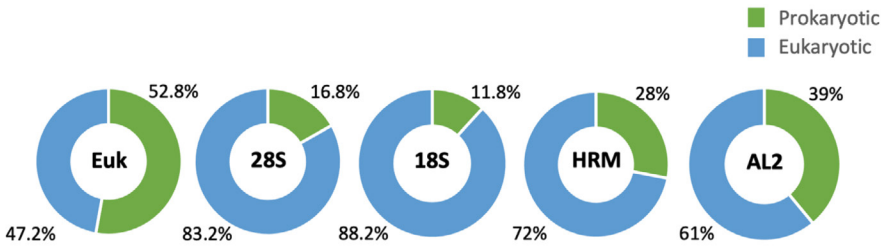

Figure 2. Percentage of Eukaryotic and Prokaryotic identified nucleotide reads recovered from Nanopore sequencing of primer specific amplicon libraries.

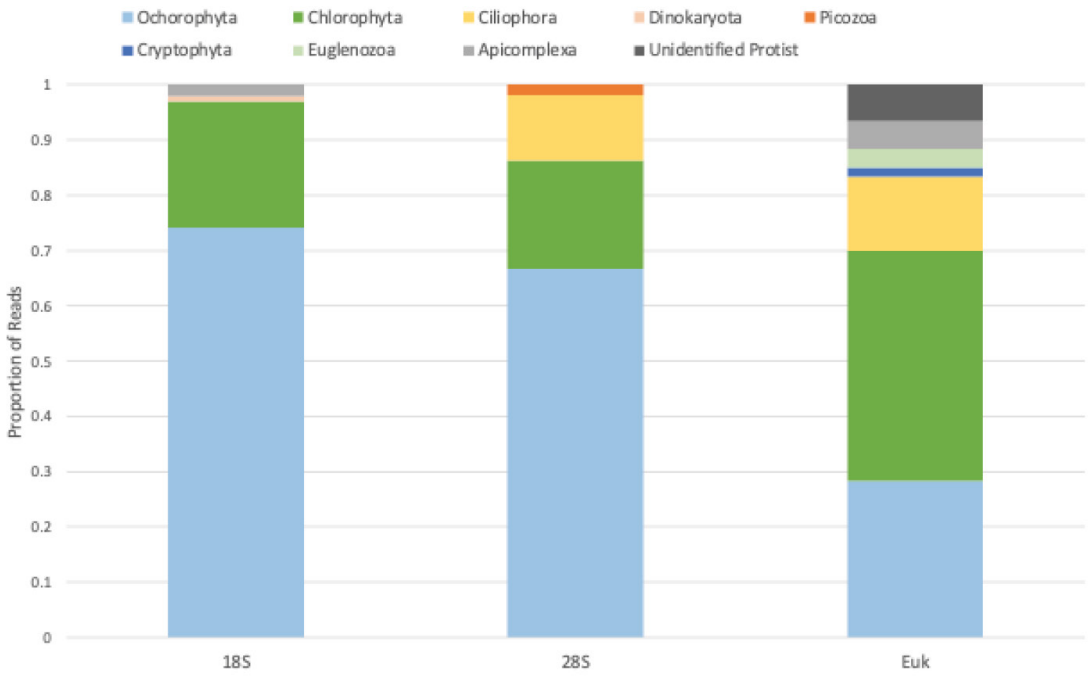

Figure 3. Stacked taxa barplot displaying the proportion of reads from the Euk, 28S, and 18S primer belonging to phyla within Protista as designated by NCBI Blast identity.

Table 3. Most common Pseudo-nitzschia taxa identified in subsampled Nanopore sequences across libraries generated with four primer sets used to amplify eDNA collected from the public shellfish harvesting grounds at HBSP, SC. Blue filled rows indicate DA producing taxa with proportional representation in subsamples of over 10%.

***Pseudo-nitzschia* taxa**	**HRM Primers**	**AL2 Primers**	**28S Primers**	**Euk Primers**	**DA Production**
P. pungens	43%	49%	—	—	Yes
P. calliantha	14%	—	—	—	Yes
P. cuspidata	13%	14%	—	—	Yes
P. limii	10%	—	—	—	No
P. limea	10%	—	—	—	No
P. multiseries	—	15%	—	—	Yes
P. fraudulenta	—	10%	—	—	No
P. australis	—	2%	—	—	Yes
P. mulstriata	—	4%	—	—	Yes
P. delicastissima	—	—	6%	2%	Yes

cates the need to design primer sets targeting additional loci and that a multi-primer approach may be needed to address both taxonomic breadth and specificity. Although none of the primer sets used in this study captured all of the *Pseudo-nitzschia* species present in eDNA samples, they do improve the taxonomic resolution and thus our understanding of *Pseudo-nitzschia* populations in South Carolina coastal environments.

As the prevalence of high-throughput sequencing in aquatic environment monitoring efforts continues to increase, so too does the importance of testing the robustness of these methods to generating useful data (van der Loos and Nijland 2021). To this end, the read length flexibility of Nanopore sequencing allowed for the application of multiple ribosomal primer sets including broad spectrum eukaryotic, diatom targeted, and *Pseudo-nitzschia*-specific primers that

produced results consistent with, but at an improved level of, resolution relative to previously used methods of *Pseudo-nitzschia* detection. These results indicate both the need for additional target loci and further expansion of reference databases to improve bioinformatic resolution. Future development of this approach into a useful monitoring tool will require establishing methodological standards to produce comparable results among collection locations. Moreover, these exploratory sequence data can be used in combination with ELISA and culture data from multiple South Carolina coastal water locations to develop a simple and cost-efficient primer assay (e.g., qPCR) sufficient for assessing the diversity of *Pseudo-nitzschia* species. The relatively low-cost sequencing offered by the MinION, allows for the simultaneous detection of co-occurrence with other target taxa (e.g., enterobacteria) key to water management. The development of a *Pseudo-nitzschia* taxonomic assay tailored to South Carolina populations has the potential to serve as an important tool for water managers to mitigate the impact of HABs on shellfish harvesting areas by improving the resolution of information regarding the presence and relative abundance of DA-producing taxa in HAB conditions.

ACKNOWLEDGMENTS

This research is supported by grant P20GM103499-20 (SC INBRE) from the National Institute of General Medical Sciences, National Institutes of Health and was conducted by undergraduate students (A. Lawson, Z. Padgett, and E. Renshaw) and the PI (M. Cevasco) as part of the CCU SCoRE (SC INBRE) summer research program.

REFERENCES

Ajani PA, Verma A, Kim JH, Woodcock S, Nishimura T, Farrell H, Zammit A, Brett S, Murray SA. Using qPCR and high-resolution sensor data to model a multi-species Pseudo-nitzschia (Bacillariophyceae) bloom in southeastern Australia. Harmful algae. 2021;108:102095. https://doi.org/10.1016/j.hal.2021.102095.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of Molecular Biology. 1990;215:403–410. https://doi.org/10.1016/ S0022-2836(05)80360-2.

Bates SS, Hubbard KA, Lundholm N, Montresor M, Leaw CP. Pseudo-nitzschia, Nitzschia, and domoic acid: New research since 2011. Domoic Acid 30 Years On. 2018;79:3–43. https://doi.org/10.1016/j.hal.2018.06.001.

Berdalet E, Fleming L, Gowen R, Davidson K, Hess P, Backer L. Marine harmful algal blooms, human health and wellbeing: Challenges and opportunities in the 21st century. Journal of the Marine Biological Association of the United Kingdom. 2015;96:61–91.

Clark S, Hubbard KA, McGillicuddy DJ, Ralston DK, Shankar S. Investigating Pseudo-nitzschia australis introduction to the Gulf of Maine with observations and models. Continental Shelf Research. 2021;1;228:104493.

Deiner K, Bik HM, Mächler E, Seymour M, Lacoursière-Roussel A, Altermatt F, Creer S, Bista I, Lodge DM, de Vere N et al. Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Molecular Ecology. 2017;26(21):5872–5895. https://doi.org/10.1111/mec.14350.

Dong HC, Lundholm N, Teng ST, Li A, Wang C, Hu Y, Li Y. Occurrence of Pseudo-nitzschia species and associated domoic acid production along the Guangdong coast, South China Sea. Harmful Algae. 2020;98(101899):101899.

Garlapati D, Charankumar B, Ramu, K. Madeswaran P, Ramana Murthy, MV. A review on the applications and recent advances in environmental DNA (eDNA) metagenomics. Reviews in Environmental Science and Biotechnology. 2019;18:389–411. https://doi.org/10.1007/s11157-019-09501-4.

Grant KS, Burbacher TM, Faustman EM, Gratttan L. Domoic acid: Neurobehavioral consequences of exposure to a prevalent marine biotoxin. Neurotoxicology and Teratology. 2010;32(2):132–141. https://doi.org/10.1016/j.ntt.2009.09.005.

Koeppel AF, Goodrum WJ, Steffen MM, Wurch LL, Turner SD. Environmental DNA sequencing dataset from Lake Erie algal blooms using Oxford Nanopore MinION. Data Brief; 2022. 45:108688 https://doi.org/10.1016/j.dib.2022.108688.

Kelchner H, Reeve-Arnold KE, Schreiner KM, Bargu S, Roques KG, Errera RM. Domoic Acid and Pseudo-nitzschia spp. Connected to Coastal Upwelling along Coastal Inhambane Province, Mozambique: A New Area of Concern. Toxins. 2021;13(12):903. https://doi.org/10.3390/toxins13120903.

Liu Q, Zhang Y, Wu H, Liu F, Peng W, Zhang X, Chang F, Xie P, Zhang H. A review and perspective of eDNA application to eutrophication and HAB control in freshwater and marine ecosystems. Microorganisms. 2020;8(3):417. https://doi.org/10.3390/microorganisms8030417.

McCarthy M, Bane V, García-Altares M, van Pelt FNAM, Furey A, O'Halloran J. Assessment of emerging biotoxins (pinnatoxin G and spirolides) at Europe's first marine reserve: Lough Hyne. Toxicon. 2015;108:202–209.

Mason CE, Elemento O. Faster sequencers, larger datasets, new challenges. Genome Biology. 2012;13:314.

Medlin L, Elwood HJ, Stickel S, Sogin ML. The characterization of enzymatically amplified 16S-like rRNA-coding region. Genetica. 1988;71:491–499.

Moore SK, Cline MR, Blair K, Klinger T, Varney A, Norman K. An index of fisheries closures due to harmful algal blooms and a framework for identifying vulnerable fishing communities on the U.S. West Coast. Marine Policy. 2019;110:103543.

Nishimura T, Murray JS, Boundy MJ, Balci M, Bowers HA, Smith KF, Harwood DT, Rhodes LL. Update of the planktonic diatom genus Pseudo-nitzschia n Aotearoa New Zealand coastal waters: Genetic diversity and toxin production. Toxins. 2021;13(9):637. https://doi.org/10.3390/toxins13090637.

Penna A, Galluzzi L. The quantitative real-time PCR applications in the monitoring of marine harmful algal bloom (HAB) species. Environmental Science and Pollution Research. 2013;20:6851–6862.

Pillet L, de Vargas C, Pawlowski J. Molecular identification of sequestered diatom chloroplasts and kleptoplastidy in foraminifera. Protist. 2011;162(3):394–404.

Pugliese L, Casabianca S, Perini F, Andreoni F. Penna A. A high resolution melting method for the molecular identification of the potentially toxic diatom Pseudo-nitzschia spp. in the Mediterranean Sea. Scientific Reports. 2017;7(1):4259. https://doi.org/10.1038/s41598-017-04245-z.

Rousseau F, Leclerc MC, de Reviers B. Molecular phylogeny of European Fucales (Phaeophyceae) based on large subunit rDNA sequence comparisons. Phycologia. 1997;36:438–446.

Singer GAC, Fahner NA, Barnes JG, McCarthy A, Hajibabaei M. Comprehensive biodiversity analysis via ultra-deep patterned flow cell technology: a case study of eDNA metabarcoding seawater. Scientific Reports. 2019;9(1):5991. https://doi.org/10.1038/s41598-019-42455-9.

Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E. Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular Ecology. 2012;21:2045–2050.

Tenorio C, Álvarez G, Quijano-Scheggia S, Perez-Alania M, Arakaki N, Araya M, Álvarez F, Blanco J, Uribe E. First report of domoic acid production from Pseudo-nitzschia multistriata in Paracas Bay (Peru). Toxins. 2021;13(6):408. https://doi.org/10.3390/toxins13060408.

Valentini A, Taberlet P, Miaud C, Civade R, Herder J, Thomsen PF, Bellemain E, Besnard A, Coissac E, Boyer F et al. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Molecular Ecology. 2016; 25(4):929–942. https://doi.org/10.1111/mec.13428.

Van der Loos LM, Nijland R. Biases in bulk: DNA metabarcoding of marine communities and the methodology involved. Molecular Ecology. 2021;30(13):3270–3288. https://doi.org/10.1111/mec.15592.

Van Meerssche E, Greenfield DI, Pinckney JL. Coastal eutrophication and freshening: impacts on Pseudo-nitzschia abundance and domoic acid allelopathy. Estuarine Coastal and Shelf Science. 2018;209:70–79.

Vecherskii MV, Semenov MV, Lisenkova AA, Stepankov AA. Metagenomics: A new direction in ecology. Biology Bulletin. 2021;48(3):107–S117. https://doi.org/10.1134/S1062359022010150.

Wang Y, Zhao Y, Bollas A, Wang Y, Fai Au K. Nanopore sequencing technology, bioinformatics, and applications. Nature Biotechnology. 2021;39(11): 1348–1365.

Wright JLC, Boyd RK, de Freitas ASW, Falk M, Foxall RA, Jamieson WD, Laycock MV, McCulloch AW, McInnes AG, Odense P et al. 1989. Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. Canadian Journal of Chemistry. 1989;67(3):481–490.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics. 2012;13:134. https://doi.org/10.1186/1471-2105-13-134.