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## Detecting Potentially Neurotoxic *Pseudo-nitzschia* Species in the Grand Strand Area

Chesney Price

### Introduction

Diatoms within the genera *Pseudo-nitzschia* are capable of inducing harmful algae blooms (HABs) in coastal waters (Ziyan et al., 2021). Some of these HABs produce domoic acid, a neurotoxin responsible for causing amnesic shellfish poisoning (ASP) in humans. In 1987, the first documented ASP (amnesic shellfish poisoning) episode occurred in eastern Canada affecting 107 individuals and causing three deaths (Pulido, 2008). ASP resulted in gastrointestinal distress, as well as, negatively impacting both the central nervous and cardiovascular systems of those affected. Specifically, exposure to diatom-produced domoic acid (DA) is a known cause of memory loss, seizures, coma, and death in mammals (Pulido, 2008).

Compounding the issue of harmful algal blooms, is bioaccumulation. Bioaccumulation is the gradual uptake and absorption of substances in an organism at a rate faster than the substance can be catabolized and excreted. (“Bioaccumulation”, 2022). Because shellfish are filter feeders, they process the organic matter and chemicals in the water through a filtering mechanism. Domoic acid can be stored within shellfish, leading to the bioaccumulation of neurotoxins up the food chain. With increasing abundance of *Pseudo-nitzschia* blooms in parts of the US, growing concern for the bioaccumulation of domoic acid has led to research being conducted regarding the growth conditions needed for these algal blooms (Clark et al., 2019). For example, populations of *Pseudo-nitzschia* are increasing in the Gulf of Maine, as is the risk for ASP in many parts of the US, although no definitive cause for this increase has been concluded (Clark et al., 2019).

Currently the total number of known *Pseudo-nitzschia* species is 52. Of these 52 species, 26 were found to be toxigenic DA producers (Bates et al., 2018). In addition, recent studies have found that zooplankton grazers are able to induce the production of domoic acid in *Pseudo-nitzschia* and that bacteria interact with *Pseudo-nitzschia* through certain signaling pathways (Bates et al., 2018). Using molecular techniques to study the population genetic structure of these diatoms, coupled with comparative transcriptomic approaches, it has been suggested that *Pseudo-nitzschia* can survive under a broad range of environmental conditions, such as iron limitation (Bates et al., 2018).

Knowledge regarding *Pseudo-nitzschia* blooms, and the potent neurotoxin domoic acid that they produce, have advanced greatly in recent years. Research conducted by Trainer et al. (2012) indicates that DA's effects are most detrimental in upwelling systems although *Pseudo-nitzschia* has recently been detected in the open ocean's low-chlorophyll and high nitrate regions, as well as in fjords, gulfs, and bays. This shows the adaptive ability of *Pseudo-nitzschia* and its ability to survive and reproduce in harsh environments. Previously believed to only thrive in supporting environments, this research suggests that *Pseudo-nitzschia* is able to thrive in low nutrient areas as well (Trainer et al., 2012). Changes in *Pseudo-nitzschia* reproduction and production of DA are monitored globally and elevated levels of bioaccumulated DA have been detected in both marine sediments and in tissue of shellfish, crustaceans, echinoderms, zooplankton, worms, marine mammals, and birds. This demonstrates that DA can readily move through marine food webs as well as abiotically accumulate in benthic substrates. Domoic acid production in relation to nitrogen availability, trace metal uptake, and salinity may suggest that its production is dependent upon environmental factors present in certain regions (Trainer et al., 2012).

The research of Mauriz and Blanco (2010) demonstrated domoic acid bioaccumulation within the scallop species *Pecten maximus*, in multiple organ systems. Recently biopsies were conducted to confirm domoic acid in the glands of a certain species of scallop (Blanco et al., 2020). The king scallop *Pecten maximus* accumulates domoic acid. In order to determine the binding capabilities of domoic acid in the cells of digestive glands, size exclusion chromatography, centrifugation, and ultrafiltration were used to measure these parameters. It was concluded that domoic acid is free in the cytosol of digestive glands of *Pecten maximus*. The prolonged retention time of domoic acid present in the digestive glands of *Pecten maximus*, proposes the idea that there is a lack of membrane transporters in this species of scallop (Mauriz and Blanco, 2010).

Domoic acid was first isolated from the red algae species *Chondria armata*. Kainoid-producing red algae contain kainic acid, a molecule related to the biosynthesis of domoic acid (Steele et al., 2022). Through the use of gene transfer and neofunctionalization, domoic acid is capable of inducing itself into different taxonomic groups of algae (Steele et al., 2022). Domoic acid is also produced in species of red algae. It was previously proposed that the biosynthetic pathway for DA was geranyl diphosphate and L-glutamate, but due to insignificant correlation was not proven. Maeno et al. (2018) determined the structure of six DA related compounds isolated from the red algae taxa *Chondria armata*. A pyrrolidine ring formation could be formed by the dehydration and electron transfer from an internal olefin compound 7 (N-geranyl-3(R)-hydroxy-L-glutamic acid). Compounds two and three are proposed to be the cyclized products of seven. A possible terminal methyl group of the side chain connecting two and three is predicted to be oxidized to hydroxymethyl, then to carboxylic acids, and lastly forming isodomoic acids.

The terminal olefin of isodomoic acid A is thought to be isomerized forming DA. *Pseudo-nitzschia* multiseriis was incorporated into DA, proving that 4 (7'-methyl-isodomoic acid A) is the precursor to DA (Maeno et al., 2018). As DA resembles a conformationally restricted form of L-glutamate, it has the capability to work against ionotropic glutamate receptors by binding to their substrates. This conformation allows for the potent neurological effects seen in those who ingest acute amounts of DA. The structural features present in domoic acid are responsible for its capability to bind in the brain, being an agonist to glutamate (Maeno et al., 2018).

Gaps remain in the knowledge of health effects of chronic exposure to low levels of DA and how DA can be removed from shellfish. The risk of human exposure to high and low concentrations of DA are of great concern to the scientific community (Zabalgo et al., 2016). After the eastern Canada ASP episode, a regulatory limit was set at 20µg/g DA and has been adopted by four countries (Schmidt, 2020). When ingested, DA induces long-lasting depolarization leading to the accumulation of excess calcium, neuronal swelling, production of reactive oxygen species, neurological dysfunction, mitochondrial damage, and DNA damage among other things. Symptoms among humans exposed to DA vary depending on the amount of toxin to which they are exposed. The first 24 hours post-exposure resulted in nausea, diarrhea, vomiting, and abdominal pain, while the 48-hour marked neurological deficits such as confusion, memory loss, seizures, and coma (Pulido, 2008). The direct effects of consuming acute levels of DA are well known and studied, but the long-term effects of sub-acute level exposure to DA remain unknown (Zabalgo et al., 2016).

Grant et al. (2010) highlight the effects of DA, the causative agent of amnesic shellfish poisoning and its effects on the human body. As *Pseudo-nitzschia* is the primary microalgal

source of DA, and HABs are increasing globally the importance of monitoring and researching *Pseudo-nitzschia* populations becomes increasingly important (Clark et al., 2021). The acute, mid-dose, and sub-acute-dose effects of DA on animal models are highlighted in Grant et al. (2010) using monkeys and rodents. As domoic acid primarily affects the central nervous system, studies have shown that DA exposure primarily affects the hippocampal regions of the brain, causing seizures and other cognitive defects. The neurobehavioral impacts of DA exposure resemble human anterograde amnesia, including transient as well as permanent changes in memory function. Studies conducted on non-human candidates support the idea that DA, and its effects, are dose-dependent producing neuropathological changes consistent with excitotoxicity. The first symptoms displayed involve scratching and hyperactivity in monkeys and rodents. Mid-dose effects include memory impairment, behavioral hyperactivity, and emotional discourse. At acute levels, DA exposure caused seizures and death in rodents (Grant et al., 2010). In addition, the means of exposure to toxic levels of DA suggests that intraperitoneal and intravenous exposure produce signs of poisoning at much lower rates than oral exposure. It is also suggested that DA exposure can enter the placenta and cause neurological deficits such as hippocampal damage, seizure disorders, and persistent behavior changes in offspring (Grant et al., 2010).

Twenty years ago, it was discovered that DA can cause adverse cardiovascular effects. DA has caused morbidity and mortality along the west coast of the United States in marine mammals and seabirds (Moriarty et al., 2021). This study monitored DA exposure and fatal cardiac disease in sea otters off the coast of California during the years 2001-2017. A sea otter with acute levels of DA exposure was found to have a 1.7-fold increased risk for fatal cardiomyopathy compared to otters with sub-acute exposure to the toxin. Otters who consumed

excessive amounts of crab and clam had a 1.2-2.5 times greater risk of death due to cardiomyopathy than otters who consumed low amounts of crab and clam (Moriarty et al., 2021). This was the first study to determine that DA exposure affects the risk of cardiomyopathy more effectively in prime-aged adults rather than older adults. An otter who was four years old had a 2.3 times greater risk for fatal cardiomyopathy than a 10-year-old otter who only showed 1.2 times greater risk, both being exposed to acute levels of DA exposure. Prime aged adults were most affected by DA exposure suggesting that DA may impact the long-term viability of otters, due to its impact detrimental effects during reproductive years (Moriarty et al., 2021).

Hogberg and Bal-Price (2011) concluded that DA has shown increased sensitivity in rat neonates, leading to prenatal exposure associated with decreased brain GABA levels, along with increased glutamate levels (Hogberg and Bal-Price, 2011). Rat cerebellum was used in Hogberg and Bao-Price (2011) study to distinguish between the effects of DA on immature and mature primary cultures in neurons and glial cells. mRNA levels were evaluated in selected genes, assessing the induced toxicity by activation of the AMPA/KA and/or the NMDA receptors. Neurological deficits were shown in both mature and immature cultures; however, the mature test subjects were more impacted by DA exposure. Mature worm subjects were also used and were determined to be more sensitive to DA exposure than immature subjects, due to their effects being observed at much lower concentrations than immature worm strain cultures. DA effects were abolished by the glutamate receptor AMPA/KA (NBQX), as the antagonist of the NMDA receptor (APV) only partially blocked the effects of DA exposure. This glutamate receptor initiates the influx of sodium and potassium ions which mediates synapses and memory function. Neurotransmitter GABA also prevented the effects of domoic acid in mature cycles. These DA-

induced effects were reduced by the addition of NBQX, APV, and GABA (Hogberg and Bal-Price, 2011).

As the cuttlefish plays a huge role in the marine food web, it is a key food source for top predators in the food chain, increasing its ability to spread DA throughout the entire ecosystem. Costa et al. (2005) studied the accumulation of domoic acid in the common cuttlefish, *Sepia officinalis*. Consistent amounts of DA were found in the digestive glands of cuttlefish during the years 2003-2004. Concentrations of up to 241.7 microg DA g<sup>-1</sup> (Costa et al., 2005). The highest accumulation of DA in cuttlefish was found during the spring and summer months, when *Pseudo-nitzschia* occur in plankton. In fact, *Pseudo-nitzschia* blooms resulted in the highest amounts of DA concentrations in cuttlefish. Tissue distribution showed the highest concentrations of DA in digestive glands and branchial hearts. Degradation and biotransformation were shown in branchial hearts. This uptake of this neurotoxin into cuttlefish tissue presents a high risk to human health as more marine life is being threatened with the potent neurotoxin domoic acid (Costa et al., 2005).

Dietary exposure to toxic levels of DA due to shellfish consumption was studied by Grattan et al. (2018) in order to determine its relevance to mild memory problems in humans. ASP has led to memory deficits in humans, leading scientists to study if there is a correlation between repeated exposure to low levels of DA throughout a long timeline and everyday memory (EM). Everyday memory involves the frequency of memory failure in everyday life. A sample of men and women from the Pacific Northwest was taken in order to determine the effects of dietary consumption of DA in razor clams. Age, sex, and education were considered in their study, along with their normal intake of razor clams. Studies determined that Native Americans



who consumed presumably safe levels of DA throughout previous weeks and years were at high risk for clinically significant memory problems (Grattan et al., 2018).

As multiple *Pseudo-nitzschia* species distributed worldwide in marine environments can be sources of domoic acid production molecular techniques can assist in identifying neurotoxic species (Costa et al., 2014). Because the genus *Pseudo-nitzschia* contains many cryptic species, molecular primers can be used to target and amplify specified DNA regions that can distinguish among isolates such as *P. calliantha*, *P. delicatissima*/*P. arenysensis complex*, and *P. pungens* (REF). Using PCR amplification in correlation with the high-resolution melting (HRM) assay Pugliese et al. (2017) were able to identify cryptic species within isolates of the genus *Pseudo-nitzschia* strains from the Mediterranean Sea. This HRM technique was able to accurately identify three distinct *Pseudo-nitzschia* taxa. Using a similar approach Pugliese et al. (2017) environmental DNA (eDNA) was collected from local public shellfish harvesting waters to screen for the presence of domoic acid producing *Pseudo-nitzschia* taxa.

## Methods

### DNA Collection

Environmental DNA (eDNA) collection can be used to assess biodiversity, specific organisms, or populations in a given area. Collection, isolation, and identification of this DNA can be used to quantify occupancy of specific organisms present in an area. qPCR, PCR, and DNA sequencing are tools that can be utilized to accurately identify a specific genus or species present within a given area through the use of eDNA. This detection of DNA would indicate that the target species is present in a given area (Buxton et al., 2021). In order to optimize environmental DNA collection and detection, it is imperative that the sample collection and

laboratory analysis remain consistent. The eDNA collection and analysis can experience error at two levels. These levels reflect both eDNA errors occurring during collection as well as during analysis. Depending on the collection timing, DNA concentration within the water, strategy of collection, volume of the sample, and the DNA amplification protocols, the data can be skewed. It is important that these factors are taken into consideration in order to reduce error. Refining collection strategies or amplification methods can reduce error but cannot abolish all sources of error. However, through using calibrated equipment and proper laboratory technique, the margin for error should be reduced. False negatives (target DNA is present, but not detected) and false positives (target DNA is detected, but it is actually absent: misidentification) can still occur, even when implementing consistent strategies (Buxton et al., 2021).

On June 11, 2021, at 9:00 am EST, the first water collection was made at an oyster collection site in Murrells Inlet, SC (33.5240316, -79.0624130). The water sample was 81°F (feels like 87° F) and a salinity of 37.5%. On June 22, 2021 at 9:00 am EST, the second water sample was taken at the same location as the first collection. The water collected had a temperature of 79.7° (feels like 84° F) and salinity of 36%. Upon collection, the water samples were distributed by sediment exposure and placed into labeled tubes. The first extraction group were labeled OL 1.1, 1.2, 2.1, and 2.2. The OL 1.1 tube was considered to be non-sediment. OL 1.2 was considered to be non-sediment. The OL 2.1 tube contained sediment and OL 2.2 contained sediment. The second collection group was labeled OL 1.3, 1.4, 2.3, 2.4 and left on a heat block at 65° C. Tubes OL 2.1 and 2.4 samples were purified and their concentrations were reevaluated. The third extraction was conducted on July 7, 2021 at the same oyster landing in Murrells Inlet. The salinity was measured at 36.5%. The first tube was labeled OL 3., the second tube OL 3.2, the third tube OL

3.3, and the fourth tube was labeled as OL 3.4. The fourth collection was made at the same location on 7/26/21. The salinity was measured at 37%. The tubes were labeled as OL 4.1, OL 4.2, OL 4.3, and OL 4.4. The fifth DNA collection was observed on August 2, 2021 at the same oyster landing. The salinity was calculated to be 35%. The tubes were labeled as OL 5.1, OL 5.2, OL 5.3, and OL 5.4. On October 8, 2021 a sixth collection was made at the same oyster landing. The tubes were labeled as OL 6.1, OL 6.2, OL 6.3, and OL 6.4. Vacuum filtration was used to remove any unwanted sediment from the eDNA. Collections were made five times throughout the summer and once during the fall on different days to ensure that the water collected was an accurate representation of the current environmental DNA.

#### DNA Extraction

The quantity of DNA recovered during eDNA collection is influenced by several factors. The size of the water collection and amount of DNA available for recovery can affect the quantity of DNA available for extraction and quantification. Before the DNA can be identified, several things must occur. The eDNA must be maintained at suitable environments, the proteins must undergo denaturation and hydrolysis, the denatured proteins must be removed, the DNA has to be purified, and then the DNA can undergo quantification. In order to for denaturation and hydrolysis of the proteins to occur, the DNA must be exposed to a buffer solution which will dissociate the histone proteins attached to the DNA. Hydrolysis occurs when an enzyme activates during SDS and isolates the DNA.

For the extraction, four 1.5 microliter microcentrifuge tubes were labeled, and one quadrant of filter paper was placed in each tube. A Qiagen RNeasy Micro Plant Kit was used in order to extract DNA from the water samples. 400 microliters of AP1 and 4 microliters of RNase A

solution were added to each tube. The solution was vortexed in order to thoroughly mix the solution. The tubes were then placed on a heat block at 65°C, to allow for the unannealing of the DNA. 130 microliters of P3 buffer was added to each tube, mixed, and then incubated on ice for several minutes. The tubes were centrifuged at the highest speed for 5 minutes and the supernatant was removed and placed into the QIAshredder then centrifuged. The flow through was transferred to a new tube without disturbing the pellet. 1.5 volumes of AW1 buffer was added into the flow through and the entire volume was transferred to a DNeasy mini spin column and centrifuged. Discarding the flow-through and centrifuging the remaining lysate was repeated. AW2 buffer is added to the solution and centrifuged. The tube continued to be centrifuged and flow through discarded until there is no excess buffer. Once the DNA was properly extracted and purified, it was stored in the refrigerator until further use.

#### DNA Purification

Purification is done to ensure that the DNA is isolated. Small molecules are removed, which could be inhibitors of catalytic activity. A common method used to purify DNA is through the use of AMPure XP magnetic beads. The addition of 0.8  $\mu$ L AMPure beads for each 1.0 $\mu$ l of sample is mixed and incubated for approximately five minutes at room temperature. Next, sample tubes are placed into the magnet plates for two minutes in order to separate the beads from the DNA solution. After the solution has cleared, the solution should be aspirated and discarded. Additionally, 200  $\mu$ L of 70% EtOH will be added and incubated for a minimum of 30 seconds at room temperature. These steps can be repeated as necessary. The tubes containing beads should be dried for at least three minutes at room temperature and 40  $\mu$ L of elution buffer (Qiagen) should be added and incubated for a minimum of five minutes at room temperature.

The tube containing the sample should be placed onto the magnet plate for two minutes in order to separate the beads from the solution (Repseqio. (2017, March 21)). This will remove any unwanted DNA or impurities.

## DNA Quantification

Once the DNA is purified, the quantification process can begin (Baechtel, Samuel, F. (n.d.)). There are four common methods for quantifying DNA. The first method involves the use of ultraviolet radiation to measure absorbency. These absorbency readings will determine if there are any remaining components in the solution other than DNA. The second method uses fluorescent measurements, in accordance with dyes, as a more accurate quantification method than ultraviolet radiation. The third strategy implements a gel procedure. By running a gel, it is possible to measure the molecular weight of the DNA. The final method to quantify DNA is a dot blot with the use of a probe. This strategy uses DNA standards, is highly sensitive, and is only semi-quantitative (Baechtel, Samuel, F. (n.d.)).

For the DNA quantification, a working solution was created. A Qubit 2.0 fluorometer was used in the eDNA quantification process. The solution was a 1:200 dilution and was calculated based on how many tubes +2, because two standards were being made. 5 mL tubes were used to create the working solution. 1  $\mu$ L of dye was used per tube. Creating the standards, it was decided that 190  $\mu$ L of working solution and 10  $\mu$ L of DNA standard would be used in each tube. Two standard tubes were created, the first being for DNA standard#1 and the second being for DNA standard #2. After both components were added the solutions were vortexed. Creating the DNA samples was done using a Qbit reader. The first step involves choosing DNA, then selecting high sensitivity DNA, next choosing “yes” in order to create new standards, and lastly reading each

standard to prepare for the DNA samples. In each tube, 195 $\mu$ L of working solution and 5 $\mu$ L of DNA sample were added. To record the concentrations, one must select the “stock concentration” button and record in ng/mL. In order to purify the eDNA, Agencourt AMPure XP was used, which utilizes magnetic beads to eliminate any unwanted DNA.

### Amplification through PCR

PCR is a tool used to amplify certain regions of DNA. In a typical amplification, there is a target set of DNA, a thermodynamically stable polymerase, oligonucleotide primers, dNTPs, and a reaction buffer. Once this solution is made, it is placed in a thermocycler programmed to keep the solution at a set of different temperatures for certain amounts of time. One cycle of these programmed temperatures is considered to be one cycle of amplification. Theoretically, each cycle doubles the amount of targeted DNA sequence in the solution. This would indicate that with smaller amounts of DNA, more cycles would need to be employed, while larger segments of DNA would require less cycles. The thermocycler contains a denaturation, primer annealing, and primer extension temperature setting. Denaturation targets the DNA by heating it to 94 degrees Celsius or higher for at least 15 seconds to two minutes. The two strands of DNA will fall apart yielding a single-stranded piece of DNA needed for replication by DNA polymerase. Next, the temperature is reduced to between 40 and 60 degrees Celsius in order for the primers to anneal to the denatured ends of the DNA strand. This step usually lasts 15 to 60 seconds. In order for the new DNA to be synthesized, the temperature is raised to the optimum temperature for DNA polymerase to replicate DNA. This step usually lasts one to two minutes at 70 to 74 degrees Celsius. Typically, each PCR will be running between 20 and 40 cycles, depending on

how much original DNA was present in the solution. PCR is a very practical tool for DNA amplification (*PCR Amplification*. Promega. (n.d.)).

Throughout the course of this research, many PCR's were conducted. On June 28, 2021, the first PCR was conducted. The primers used in this PCR were HRMF (PLA) and HRMR (Oxy). The mixture in each tube contained 5.5 microliters of nuclease water, 1.0 $\mu$ L HRMF, 1.0 $\mu$ L HRMR, 12.5 $\mu$ L GoTaq, and 5 $\mu$ L of template DNA. The first lane was used as a control, and therefore no DNA was added. The second lane consisted of DNA from the 1.1 tube, the third lane contained DNA from the 2.1 tube, the fourth lane contained purified DNA from the 2.1p tube, the fifth lane contained DNA from the 1.1 tube, the sixth lane contained DNA from the 2.1 tube, and the seventh lane contained DNA from the 2.1p tube. The PCR reaction was run for 3 minutes at 95 degrees Celsius, 30 seconds at 95° C (49x, or 50 cycles), 30 seconds at 53° C for 50 cycles, 30 seconds at 72° C for 50 cycles, 72° C for 3 minutes, and 10° C on hold. On July 30, 2021, a second PCR was run. The mixture contained 1 $\mu$ L of SSOF (forward primer), 1 $\mu$ L of SSUR (reverse primer), 12.5 $\mu$ L GoTaq, 10.5 $\mu$ L of water (control) or 5.5 $\mu$ L of water (DNA lanes), and 5 $\mu$ L of DNA. The first lane was a control lane and contained no DNA. The PCR was conducted at 95°C for 3 minutes, 95°C for 30 seconds (34x), 53°C for 40 seconds (34x), 72°C for 30 seconds (34x), 72°C for 5 minutes, and held at 12°C infinitely. On August 4, 2021 a third PCR was run to reamplify the DNA from the fourth collection. The Mastermix contained 10 $\mu$ L of GoTaq, 1.5 $\mu$ L SSUF, 1.5 $\mu$ L SSUR, 7 $\mu$ L of water, and 1 $\mu$ L of DNA template. The PCR was run at 94°C for 3 minutes, 94°C for 25 seconds (24x), 56°C for 25 seconds (24x), 72°C for 25 seconds (24x) and 72°C for 3 minutes. Tube 1 was the control and used GoTaq, tube 2 contained DNA from 4.1 and used GoTaq, tube 3 contained DNA from 4.2 and used GoTaq, tube 4 was a control

and used qGoTaq, tube 5 contained DNA from 4.1 and used qGoTaq, and tube 6 contained DNA from 4.2 and used qGoTaq. On August 27, 2021, another PCR was run with a culture of *Pseudonitzschia arctica*. The first sample had a concentration of 340 ng/mL and the second had a concentration of 190 ng/mL. The Mastermix was made with 3  $\mu$ L of DNA sample, 1  $\mu$ L of HRMF primer, 1  $\mu$ L HRMR primer, 12  $\mu$ L of GoTaq, and 3  $\mu$ L of water times four. The first tube contained a full DNA sample from OL 2.2, the second tube was a 1:10 dilution of OL 2.2, the third tube contained a cleaned sample from 2.4p, and the fourth tube contained only water and was used as the control. The PCR reaction was run at 94°C for 3 minutes, 94°C for 30 seconds (44x), 57°C for 25 seconds (44x), 72°C for 30 seconds (44x), 72°C for 5 minutes, and at 12°C for holding infinitely. On September 1, 2021, a Reamplification PCR check was conducted. The Mastermix contained 1 $\mu$ L of HRMF, 1 $\mu$ L of HRMR, 12 $\mu$ L of qGoTaq, and 5 $\mu$ L of water all times 5. Tube 1 contained 19 $\mu$ L of the Mastermix and 1 $\mu$ L of PCR product. Tube 2 contained 19 $\mu$ L of Mastermix and 1 $\mu$ L of PCR product. Tube 3 contained 19 $\mu$ L of Mastermix and 1 $\mu$ L of PCR product. Tube 4 contained 18 $\mu$ L of water, 1 $\mu$ L of HRMF, and 1 $\mu$ L of HRMR. Tube 5 contained 18 $\mu$ L of Mastermix and 2 $\mu$ L of water. Tube 6 contained 17 $\mu$ L of Mastermix and 3 $\mu$ L of DNA. The PCR was run at 94°C for 3 minutes, 94°C for 30 seconds (34x), 57°C for 25 seconds (34x), 72°C for 30 seconds (34x), 72°C for 5 minutes, and held at 12°C infinitely. On September 2, 2021 a reamplification of *P.arctica* was conducted. The Mastermix contained 1 $\mu$ L of HRMF, 1 $\mu$ L of HRMR, 12 $\mu$ L of qGoTaq, 1.5 $\mu$ L of *P.arctica* DNA, and 4.5 $\mu$ L of water. The control groups contained 8 $\mu$ L of qGoTaq and 10 $\mu$ L of water, due to their lack of DNA and need to have the same volume. The reaction was conducted at 94°C for 3 minutes, 94°C for 30 seconds (24x), 57°C for 25 seconds (24x), 72°C for 25 seconds (24x), and 72°C for 3 minutes. On September



9,2021 a PCR reamplification was conducted in order to visualize reamplified environmental DNA from a previous PCR as well as *P.arctica* template DNA. 1.5µL HRMF, 1.5µL HRMR, 8µL of qGoTaq, and 9µL of water were used for the negative control. 2.0µL of reamplified DNA, 1.5µL of HRMF, 1.5µL of HRMR, 10µL of qGoTaq, and 5µL of water were used in the reamplified group. The *P. arctica* lane consisted of 4.0µL of *P. arctica* DNA, in order to ensure that the DNA could be visualized, 1.5µL of HRMF, 1.5µL of HRMR, 10µL of qGoTaq, and 3µL of water. This reaction was conducted at 94°C for 3 minutes, 94°C for 25 seconds (34x), 57°C for 20 seconds (34x), 72°C for 20 seconds (34x), and 72°C for 3 minutes. On September 19, 2021, a PCR was run to visualize two of the reamplifications from 8/27 and the reamplification from 9/9. The Mastermix contained 10µL of PCR product, 18µL of AmPure, and 25µL of AE buffer. The first lane contained a reamplification of OL 2.1, the second lane was a reamplification of 2.4p, and the last lane was the reamplification from September 9<sup>th</sup>. On September 21, 2021, a PCR was run from collecting DNA from colonies on a petri-dish. This petri-dish was cultured and kept in the fridge in order to preserve the environmental DNA. Tubes 1-3 contained secondary colonies and the fourth tube contained DNA from the original plate (tube 1). Tubes 5-7 contained secondary colonies and the eighth tube contained DNA from the original plate. A Mastermix was made for tubes 1-4 and contained 8µL of water, 1µL of M13F (forward primer), 1µL of M13R (reverse primer), and 10µL of GoTaq, all x4. The Mastermix for tubes 5-8 were made with the same components, except qGoTaq was used in place of GoTaq. This reaction was run at 95°C for 10 minutes, 95°C for 30 seconds (29x), 57°C for 30 seconds (29x), 72°C for 30 seconds (29x), 72°C for 5 minutes, and held infinitely at 12°C. Another PCR from the colonies was conducted on September 23, 2021. This reaction contained the same

Mastermix as the previous reaction, using GoTaq. Tubes 1-3 came from the subcloning plate and tube 4 came from the stock plate. Tubes 1-4 contained DNA from tube 2 (petri-dish #2). Tubes 5-7 contained DNA from the subcloning plate while tube 8 originated from the stock plate. Tubes 5-8 contained DNA from tube 3 (petri-dish #3). This PCR reaction used the same temperature scheme as the previous colony PCR reaction. A second PCR reaction was run on September 23, 2021. This reaction used the same Mastermix mentioned in the two previous PCR's and was set for the same temperatures. Tubes 1-4 contained DNA from plate 1, and tubes 5-8 contained DNA from plate 2. On September 30, 2021 another PCR was conducted from the colony DNA. A Mastermix was made for the M13 primers which included: 8 $\mu$ L of water, 1 $\mu$ L M13F, 1 $\mu$ L M13R, and 10 $\mu$ L GoTaq x4. The second Mastermix contained 8 $\mu$ L water, 1 $\mu$ L T3, 1 $\mu$ L T7, and 10 $\mu$ L of GoTaq x4. T3 and T7 were the forward and reverse primers used in this particular reaction. Tube 2 from the second plate and subclone were used in these reactions. Reaction tubes 1 and 2 were used with the M13 primers with the original plate. Tubes 5 and 6 were used with the M13 primers and the subclone plate. Tubes 3 and 4 were used with the original plate and T3/T7 primers. Tubes 7 and 8 were used with the original plate and T3/T7 primers. This reaction was run at a temperature of 95°C for 10 minutes, 95°C for 30 seconds (29x), 57°C for 30 seconds (29x), 72°C for 30 seconds (29x), 72°C for 5 minutes, and held infinitely at 12°C. The next PCR was conducted on October 5, 2021. This Mastermix contained 8 $\mu$ L water, 1 $\mu$ L M13F, 1 $\mu$ L M13R, and 10 $\mu$ L GoTaq x4. Reaction tubes 1 and 2 included DNA from the original plate, while tubes 3 and 4 included DNA from the subclone plate. All tubes contained DNA from tube 3 (plate #3). This reaction was run at 95°C for 10 minutes, 95°C for 30 seconds (29x), 57°C for 30 seconds (29x), 72°C for 30 seconds (29x), 72°C for 5 minutes, and held infinitely at 12°C. On

October 14, 2021 another PCR was ran with DNA extracted from colonies on a plate. The same Mastermix was used as in previous colony PCR's. The temperatures for the reactions also stayed the same. Tube 1 (plate 1) DNA was used in reaction tubes 1-4 and tube 3 (plate 3) DNA was used in reaction tubes 5-8.

### Amplification through qPCR

The first qPCR was conducted on October 8, 2021. The Mastermix contained 10 $\mu$ L of qGoTaq, 0.5 $\mu$ L of HRMF, 0.5 $\mu$ L HRMR, 4 $\mu$ L water, and 5 $\mu$ L of DNA template. The first lane contained environmental DNA from OL 3.1, the second lane was from OL 3.2, the third lane was *P. arctica* DNA, and the fourth lane was negative. This qPCR was run at 95°C for 2 minutes, 95°C for 10 seconds (64x), 59°C for 20 seconds (64x), and held infinitely at 10°C.

### Cloning

Topoisomerase TA cloning uses Taq polymerase, an enzyme responsible for adding a single 3' A overhang on the end of each PCR product. This allows for linear cloning vectors containing a single 3' T overhang. Taq polymerase does not leave a blunt-end PCR product, which makes it a good candidate for cloning. Through the use of DNA-topoisomerase I, which is a restriction enzyme and a ligase, it is possible to access already linearized DNA that allows for direct ligation of the PCR product (*Ta and topo-ta cloning*. TA and TOPO®-TA cloning – Protein Expression and Purification Core Facility. (n.d.)). Once this plasmid is constructed, it will be inserted into bacteria such as *E. coli*, and if it is taken up by the *E. coli*, then the DNA has been successfully cloned.

In order to sequence, plates containing *E. coli* were plated with the eDNA. Scratch plates were This experiment utilized blue-white cloning along with TA cloning. White colonies contain transformed a vector with a plasmid, while blue colonies contain vectors only.

### Sanger Sequencing

Sanger Sequencing is a method used to identify nucleotide sequences of DNA through the use of electrophoresis and the utilization of random chain-terminating dideoxynucleotides (ddNTPs) during in vitro DNA replication. DNA polymerase synthesizes a primer that incorporates radiolabeled nucleotides requiring an additional two polymerizations. With only a single nucleotide present, base and minus reactions are used to sequence up to the position before the next missing nucleotide. As a “chain-termination” sequence method, radiolabeled or fluorescently labeled nucleotides prevent further extension and are incorporated into DNA polymerizations. This sequencing method identifies the lane in the gel which presents a band matching the 3' terminating labelled ddNTP (Heather, J., M., & Chain, B., 2016).

### Analysis of Sequence Data

The analysis of the sequence data was completed using a multiple alignment software (MUSCLE 3.0) and RAxML 8 was utilized to generate a phylogenetic tree illustrating genetic relationships between eDNA and known species of *Pseudo-nitzschia*. The MUSCLE 3.0 alignment software creates multiple alignments of a given protein sequence in order to determine genetically similar sequences. MUSCLE functions in three stages. The first stage constructs a progressive alignment, the second stage improves the accuracy of the tree using fractional identity computed from mutual alignments, and the third stage refines the alignment using a tree-dependent variant with restricted partitioning. This new profile to profile alignment receives a

sum-of-pairs (SP) score after the completion of stages two and three. If the score increases, the refined alignment is retained, but if the scores decreases or remains the same it is discarded (Edward, R., C., 2004). This alignment software functions to improve the accuracy of protein alignments between different species in order to portray the most closely related species.





RAxML 8 (Randomized Axelerated Maximum Likelihood) is a program used for phylogenetic analysis of large amounts of DNA under maximum likelihood confinements. It is fast and accurate measurement of likelihood tree search algorithms that results in trees with high scores in similarity. This software was used to construct a genetically accurate tree illustrating relationships between different species of the same genus (Stamatakis, A.,2014) .

## Results

The collected eDNA was found to be highly similar to *P. nitzschia delicatissima*, *P. nitzschia pungens*, *P. nitzschia simulans*, and *P. nitzschia HAB 231 B*. This was determined through sequencing. *P. nitzschia pungens* was sequenced through utilizing a pure culture in the lab. *P. nitzschia delicatissima*, *P. nitzschia HAB 231 B*, and *P. nitzschia simulans* were sequences obtained through NCBI's domain. These sequences were comparatively analyzed alongside the sequences obtained from the eDNA. Their relationships are displayed through the phylogenetic tree which was constructed using a General Time Reversible model with a discreet gamma distribution. Sequences of the eDNA are approximately 300 base pairs in length. The bootstrap values used are 1000 replicates, with over 50% shown. The similarity in genetic makeup between these known species of *Pseudo-nitzschia* compared to the eDNA collected illustrate their likely similarity in structure and function. It is highly likely that the collections made on June 22, 2021 are domoic acid producers as they fall within the clades of four DA producing *Pseudo-nitzschia*



Table 1. eDNA collection dates including concentration values (ng/mL), color-coding clade presence corresponding to the phylogenetic tree, and likelihood of DA production.

Collection Dates	Average DNA Concentration (ng/mL)	Location Within Tree (clades)	Likely Domoic Acid Production
June 22, 2021	$1.67 \times 10^4$	 A, B, C, D	+++
June 23, 2021	$2.16 \times 10^4$	 A, B, C	+
July 7, 2021	$1.64 \times 10^4$	 A, B, C	+
July 26, 2021	$4.9 \times 10^3$	 A, B, C	++
August 2, 2021	$1.42 \times 10^3$	N/A	N/A

## Discussion

### *P. nitzschia delicatissima*

*Pseudo-nitzschia delicatissima* is a known domoic acid producer. It is native to the Atlantic Ocean, Gulf of Maine, Gulf of Mexico, and Pacific Ocean. As a cryptic species, *P. nitzschia-delicatissima* contains both toxic and non-toxic strains (Loureiro, S. et al., 2009). Toxin production is thought to be associated with nutrient status. Through organic nutrient uptake, this *P. nitzschia* species is capable of overcoming low-nutrient affinity areas. By supplementing their typical inorganic nutrient diet with organic nutrients, this species utilizes a well-known survival strategy which increases their ability to introduce DA into the food chain. *P. nitzschia-delicatissima* utilizes organic nutrients as sources of nutrition and survival strategy. This versatile *P. nitzschia* species, considered to be an r-selected diatom, is capable of inducing HABs in upwelling systems. As an r-selected diatom, this cryptic species possesses a fast growth rate and

cell yield furthering its ability to survive and reproduce in nutrient-rich areas (Loureiro, S. et al., 2009).

### *P. nitzschia pungens*

*Pseudo-nitzschia pungens* is a known producer of domoic acid. As a native to the Atlantic Ocean, Gulf of Maine, Gulf of Mexico, Pacific Ocean, and North Carolina (USA). Three closely related lineages of *P. nitzschia-pungens* have been analyzed and labeled as separate clades in order to distinguish them based on differences in their cingular band structure. Clade I includes *P. pungens var. pungens*, clade II includes both *P. pungens var. pungens* and *P. pungens var. cingulate*, and clade III is represented by both *P. pungens var. pungens* and *P. pungens var.* (Kim, J., H. et al., 2020). Clade I features a global distribution, clade II is native to the eastern North Pacific coasts, and clade III is most commonly found in tropical environments. The sexual compatibility between different clades of *P. pungens* has been studied in order to better understand the gene flow between genetically diverged populations. These boundaries and evolutionary strategies that assist *P. pungens* in maintaining genetic diversity are described by their ability to reproduce between different clades. Clades I and II are genetically distanced by 1.2%, clades I and II at 2.8%, and clades II and III were only separated by 3.1% (Kim, J., H. et al., 2020). Sexual reproduction between clades I and III and clades I and II was established as possible. However, sexual events between clades II and III did not occur due to geographic restraints. The capability of reproduction between *P. pungens* clades allows for genetic diversity and the expansion of DA producers. This allows for population expansion globally. Because certain *P. pungens* species are capable of DA production, this reproductive capability allows for a more widespread DA presence.



*P. nitzschia simulans*

*Pseudo-nitzschia simulans* is a known producer of DA. It is native to the East China Sea, South China Sea, and the Yellow Sea. *P. nitzschia* blooms are commonly found in China, including strains that are potentially toxic; however, none have ever tested positive for the presence of DA (Li, Y. et al., 2017). *P. simulans sp. nov.*, which belongs morphologically to *P. delicatissima*, is determined to be the first DA producer in Chinese waters. *P. simulans sp. nov.* was analyzed through liquid chromatography tandem mass spectrometry in stationary growth stages. This analysis resulted in the detection of DA at concentrations ranging from 1.05 fg cell<sup>-1</sup> to 1.54 fg cell<sup>-1</sup> (Li, Y. et al., 2017). This *P. simulans* species is the first toxigenic diatom species reported in Chinese waters.

As a potentially neurotoxic diatom, certain species of *Pseudo-nitzschia* produce domoic acid. eDNA collected from the Huntington Beach State Park was sequenced in order to determine the clades of known *Pseudo-nitzschia* species that this eDNA would fit into. It was determined, that the collected eDNA fell into the clades of four known species of *Pseudo-nitzschia* species. These four species were selected because they shared a very similar genetic makeup with the collected eDNA. Due to their similarity in genetic makeup, these species are expected to share many similarities in structure and function. This research suggests, that because all four of the known *Pseudo-nitzschia* species are known producers of domoic acid, that the collected eDNA that fell within these clades are likely producers of DA as well. Each eDNA as well as the date of collection were observed in each clade and their likeliness to produce DA was recorded.

Due to the ASP episode occurring in 1987 Canada, the potential for domoic acid epileptic disease was recognized. Domoic acid epileptic disease is defined by recurrent seizures experienced throughout the passing weeks to months post DA exposure (Ramsdell, J., S., & Gulland, F., M., 2006). A human case study was conducted after the ASP incident that characterized the disease as a chronic epileptic syndrome through several DA cases between 1998 and 2006. Sea lions were used in this study to observe abnormal behaviors, brain pathology, and epidemiology. Rat models were also implicated in order to predict the progression of this epileptic state and its relation to neural systems. This delayed manifestation of DA poisoning is believed to trigger dendrodendritic synapses in the olfactory bulb and manifests in the olfactory cortex (Ramsdell, J., S., & Gulland, F., M., 2006). An exposed male from the 1987 ASP incident experienced initial GI tract distress, confusion, and coma. After three days his symptoms progressed from focal seizures to partial status epilepticus. He was not responsive to the mild doses of anti-seizure medication, but large doses resulted in resolving his seizures after three weeks. A CT scan was done, resulting in normal results; however, months after his discharge he began to experience severe anterograde memory deficits. Upon the eight-month mark, after continued high dosing of phenobarbital, he received a normal EEG. A year after the initial poisoning event, the man exhibited an acute episode of focal seizures which responded to medication, but later died from pneumonia almost a year and a half later (Ramsdell, J., S., & Gulland, F., M., 2006).

In southern California, DA concentrations during the years 2003, 2006, 2007, 2011, and 2017 were considered to be comparable to some of the highest recorded values (Smith, J. et al., 2018). Because DA has been found in such high concentrations nationally, it becomes

increasingly important to monitor the presence and environmental conditions favorable of these HABs. Pseudo-nitzschia blooms were present predominately in the spring with variability between years. Due to the tropical nature of the climate experienced along the coast of southern California, HABs experience an environment similar to the Mediterranean, which is most favorable for *P. nitzschia* blooms. This region alone is home to an estimated 18 million people, as of 2015 (Smith, J. et al., 2018). With a population this large, it is necessary that the conditions most favorable for DA production are recognized in order to maintain public health in times of high DA production. In 1991 central California, a seabird mortality event occurred due to DA poisoning, which subsequently led to the death of many marine mammals. Between the years of 2003 to 2016, California's Department of Public Health detected DA in shellfish tissues virtually every year; however, the amount of toxin detected has varied considerably each year (Smith, J. et al., 2018). The highest concentrations and most frequent occurrences of DA have been observed in central and northern California. In southern California, during the year 2002, a marine mammal mortality event occurred that was determined to have been due to domoic acid (Smith, J. et al., 2018). This event led to more studies being conducted regarding the presence of DA in the waters of southern California. As DA detection is becoming increasingly abundant in the US, it becomes of greater concern to those that live on the coast or consume shellfish regularly. Due to its ability to impact human health, domoic acid is a toxin that should be regularly screened for in all coastal waters.

Domoic acid is a mussel neurotoxin that is responsible for inducing ASP in humans. Of those who experienced mortality due to the Canadian ASP episode, many were found to have lesions in parts of the brain such as the hippocampus, amygdala, thalamus, and cerebral cortex

(Stewart, G. et al., 1990). Of the survivors, one was determined to be globally demented, while others displayed continued memory impairment and experienced deficits in cognition. All victims experienced GI distress, blood pressure instability, agitation, confusion, and disorientation. Some consider DA neurotoxicity to be a dementing process, while others consider it to be amnesic (Stewart, G. et al., 1990).

Domoic acid is a counterpart of excitotoxic glutamate and kainic acid. DA binds to the excitatory glutamate receptors in the brain. DA has neurotoxic effects on the hippocampus and striatum regions of the brain (Stewart, G. et al., 1990). Following the Canadian ASP episode, DA was isolated and purified in order to determine its neuroexcitatory and neurotoxic effects. Hippocampal neurons and retinas from chick embryos were used in vitro in order to determine DA's electrophysical and neurotoxicological effects. Additionally, adult rats were used to distinguish behavioral and neuropathological effects of domoic acid through in vivo experimentation. Currents induced by DA in hippocampal neurons resulted in typical responses from kainic acid meaning there was no evidence of desensitization (Stewart, G. et al., 1990). Regarding the chick retina, DA resulted in swelling of neuropil processes and cytoplasmic swelling that affected neurons in the inner nuclear layer. Rat behavioral responses to DA resulted in repetitive head scratching, shaking, immobile staring, and seizures, all typical of kainic acid treatment. Continuous seizure activity was experienced (epilepticus). Brain damage such as neural and glial swelling leading to neurodegeneration was observed in the rats exposed to varying concentrations of DA (Stewart, G. et al., 1990). The cortical mantle, hippocampus and transitional regions, amygdala and associated structures, and thalamus experienced varying degrees of acute damage following DA administration. It is well supported that DA imposes a

kainic acid like neurotoxic syndrome in rats. However, DA neurotoxic activity, both in-vivo and in-vitro, was five to eight times greater than kainic acid, making DA the most potent excitotoxin studied to date (Stewart, G. et al., 1990). Rats have been established as good model organisms when studying the toxicology of DA. Rats can be used to develop antidotes in order to protect humans from DA poisoning and ultimately neurodegeneration.

## Conclusion

Through the development of this research and the results observed, it can be assumed that there are several species of *Pseudo-nitzschia* present in the Grand Strand Area. According to the results gathered from this study, several of the *Pseudo-nitzschia* species detected in the Grand Strand Area are likely producing domoic acid (DA). Due to the genetic similarity between eDNA collected from the Huntington Beach State Park and sequences of known *Pseudo-nitzschia* species, it is highly likely that the DNA collected on June 22, 2021 is a producer of DA. Because of this newfound information, it becomes increasingly important to discern the environmental conditions optimal for HABs survival. During times in which HABs are present, and actively producing DA, it is imperative that shellfish consumption be inhibited. In order for humans to experience ASP, they must consume shellfish containing DA. This can be prevented if the tissues of shellfish are screened for the presence of DA. As HABs are increasing globally, it is imperative that research is able to discern what conditions influence the amount of DA that is produced by HABs and how it can be reduced. As DA poisoning primarily affects the central nervous system, it is crucial to reduce the amount of shellfish that is consumed during times in which toxic amounts of DA are present. In conclusion, more research should be conducted in order to determine if DA is being produced in the Grand Strand area. In confirming the presence

of Pseudo-nitzschia in the water, great steps have been made; however, determining if DA is actively being produced is of great concern to the public health in this area. Once the presence of DA is confirmed or denied, further steps can be taken concerning the safety of the Grand Strand population.

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