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# The effects of nutrient limitation on toxin production for the harmful algal bloom species, Pseudo-nitzschia, in the Gulf of Maine

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# Acknowledgements

I often joke that I was lucky my semester at Bigelow Laboratory coincided with the first toxic bloom of *Pseudo-nitzschia* in the Gulf of Maine's history; otherwise I wouldn't have had the material to fill the pages of this thesis. But attributing it all to luck wouldn't be giving enough credit to the people at Bigelow who carried me through the project from start to finish. So, to give credit where it's due, I'd like to thank Pete Countway, my advisor, who has worked tirelessly with me from the start, willing to answer my every question and always encouraging me to *keep asking* questions, pushing me beyond what I thought I was capable of. I'd like to thank Nicole Poulton, my mentor during the Bigelow semester program, whose patience and support helped keep me calm through the most chaotic days in the lab. I want to thank everyone else at Bigelow, who was willing to drop what they were doing and give me a hand. Over the past two years, Bigelow has become something of a second home for me, and that wouldn't have been possible without the wonderful people who made it that way.

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# 1. ABSTRACT

*Pseudo-nitzschia* is a pennate marine diatom that produces the neurotoxin domoic acid (DA). Domoic acid accumulates in the digestive tract of filter feeders and becomes concentrated at higher trophic levels. When DA concentrations reach critical levels, consumption of toxic shellfish can induce a condition known as Amnesic Shellfish Poisoning (ASP), which has been responsible for the deaths of marine mammals, seabirds and humans. This study worked to respond to a toxic *Pseudo-nitzschia* bloom in the Gulf of Maine during the fall of 2016, with the goal of quantifying and identifying *Pseudo-nitzschia* spp. in the bloom region and to determine the role of macronutrients (N, P, Si) in cell growth and toxin production. Cell densities were quantified using quantitative PCR targeting the 18S rDNA gene for samples collected from 29 sites along the coast of Maine. The average gene copy number per cell in *Pseudo-nitzschia pungens* was determined to be 79.4 ± 10 18S rDNA genes. Cell concentrations were compared to nutrient and domoic acid concentrations at each bloom site. The relationship between individual nutrient concentrations and toxin concentrations was inconclusive regarding the role of individual nutrients limiting *Pseudo-nitzschia* growth, but examining nutrient stoichiometry in relation to the Redfield ratio offered evidence that silica limitation was the primary driver of toxicity in the bloom. To further examine this theory, three nutrient limitation experiments were conducted, subjecting cultures of *Pseudo-nitzschia pungens* to phosphate and silicate limitation to examine how cell growth and domoic acid production were affected. While macronutrient limitation halted cell growth in all experiments, DA production was not detected in any lab-cultivated *Pseudo-nitzschia pungens.* Further experimentation should repeat nutrient limitation experiments with the confirmed toxin-producer *P. australis*, a species known to be present in high concentrations during the 2016 bloom event, but not obtained in culture.

# 2. INTRODUCTION

Harmful algae have been a persistent problem in the coastal Gulf of Maine for the past several decades with official reports of their presence dating back to 1945 (Townsend et al., 2001) and further evidence suggesting their presence as early as 1889 (Ganong, 1889; Townsend et al., 2001). The term "harmful algae" refers to a taxonomically diverse range of eukaryotic microalgal species that produce toxic or noxious compounds, or cause other detrimental impacts to the ecosystem during a bloom, such as hypoxia or anoxia. Historically, the primary harmful algal bloom (HAB)-forming organisms in the Gulf of Maine have been dinoflagellates belonging to the genus *Alexandrium*, whose blooms turn the ocean a reddish color due to their production of the photopigment peridinin. Other potentially harmful species that occur in the Gulf of Maine include the dinoflagellates *Dinophysis* and *Karenia* and the diatom *Pseudo-nitzschia*.

Toxic *Pseudo-nitzschia* blooms have been recorded globally (Kotaki et al., 1999; Almandoz et al., 2007, Almandoz et al., 2008; Quijano-Scheggia et al., 2010; Pugliese et al., 2017), but historically, within North American coastal waters, toxic blooms have been primarily restricted to the west coast (Fryxell et al., 1997; Trainer et al., 1998; Schnetzer et al., 2007; Hubbard et al., 2013; Du et al., 2016; Z. Zhu et al., 2017; Grattan et al., 2018) and the Gulf of Mexico (Bargu et al., 2016), where they have led to massive closures of shellfish harvesting, in terms of areal extent and duration. These blooms had not been a major concern in the Northeastern coastal region of the United States until recently.

*Pseudo-nitzschia* is a diatom that is found throughout the Gulf of Maine including the inshore waters of the Damariscotta River estuary. Some species of *Pseudo-nitzschia* are known to product the neurotoxin domoic acid (DA) (Bates et a., 1993; Hasle, 1994; Bates & Trainer, 2006; Trainer et al., 2012; Fernandes et al., 2014). When *Pseudo-nitzschia* are consumed by shellfish, DA accumulates in the digestive tract of those filter-feeding organisms (Lesser et al., 2016). When other organisms consume these toxic shellfish, they absorb the DA into their own systems (Bates et al., 2006; Trainer et al., 2012).

This toxin has been known to affect marine mammals (Frady, 2004), sea birds (Robinson, 2009) and humans (Grattan et al., 2018). The most notable case of this occurred on Prince Edward Island, Canada in 1987, where over 107 people were diagnosed with amnesic shellfish poisoning (ASP), and several died as a result (Lesser et al., 2016). In the time since this event, technology and shellfish monitoring programs have greatly improved so that DA concentrations are easily detected, largely preventing further poisoning in humans. Consequently, whenever DA is detected at an established safety threshold, the affected shellfish farms and harvesting regions are forced to close and dispose of impacted shellfish, resulting in significant economic loss (Trainer, 2012).

The focus of this study is the initial toxic bloom that occurred during October of 2016, hereafter referred to as "the fall 2016 bloom". The Gulf of Maine and the Damariscotta River are home to at least nine *Pseudo-nitzschia* species and experience regular non-toxic blooms (Fernandes et al., 2014), but the region had not been subject to ASP closures until the fall of 2016, when the Gulf of Maine experienced its first toxic bloom of *Pseudo-nitzschia* forcing closures on shellfish harvesting along the coast of New England and resulting in the recall of 5 tons of softshell clams, mussels and quahogs (McGuire, 2016). Two more toxic blooms occurred in October and December, 2017, expanding to the Casco Bay Region, which had not been affected by the toxic bloom of 2016. Officials from the National Oceanographic and Atmospheric Administration (NOAA) and Maine's Department of Marine Resources state that they will expand monitoring efforts in the coming years to understand toxic bloom dynamics in the Gulf of Maine and to prevent future recalls on shellfish (NOAA, "Improving the Gulf of Maine HAB Forecast with Environmental Sample Processors," 2017; McGuire, 2017a). The Department of Marine Resources has announced that they have adopted a cautionary protocol for closures on shellfish harvesting, prohibiting harvest at the first sign of domoic acid in the water, a threshold far below the federal detection limit of 20 ppm of DA in shellfish tissue (McGuire, 2018).

## 2.1 Changes in the Gulf of Maine

The Balch lab at the Bigelow Laboratory for Ocean Sciences has been conducting the Gulf of Maine North Atlantic Time Series (GNATS) since 1998. They provide an overview of physical, chemical and biological characteristics of the Gulf that have changed over that time-period (Balch et al., 2012). They identify eight major changes:

1. Decreased salinity and density in surface waters of W. GOM [Driven by temp.]
2. Reduced temperature and vertical temperature gradients in upper 50m.
3. Increased colored dissolved organic matter (CDOM) in W. GOM.
4. Increased nitrate and phosphate concentrations everywhere but E. GOM.
5. Increased silicate, especially Si:N, in W. GOM.
6. Sharp decrease in carbon fixation by phytoplankton.
7. Moderately decreased chlorophyll, POC and PIC in central GOM.
8. Decreased POC- and PIC-specific growth rates.

Many of these changes were significantly correlated with river discharge. Balch et al. (2012) assert that increased precipitation and river discharge are having significant effects on the chemical, physical and biological composition of the GOM. These observations have important implications for phytoplankton blooms and may point to why we are beginning to see a pattern of toxic blooms of *Pseudo-nitzschia* in the GOM for the first time in the region's history. Colored dissolved organic matter (cDOM) decreases light transmission in the water. Balch et al. (2012) found that increases in cDOM significantly decreased primary productivity in the Gulf of Maine. This change in population structure has the potential to open a niche that is ideal for the growth of *Pseudo-nitzschia.* Studies have shown that *Pseudo-nitzschia* able to persist under low light conditions due to its ability to grow at rates much lower than other diatoms ($\leq$0.1 $day^{-1}$; (Pan et al., 1996). One study showed that *Pseudo-nitzschia* was the twelfth most common of 80 genera found in a "very" low light estuarine region (>5 µmol $m^2$ $s^{-1}$ of PAR) Goa, India (Ramakrishnan et al., 2018), supporting this theory. Studies have also shown that *Pseudo-nitzschia* produce higher

concentrations of DA under low light conditions (Pan et al., 1996), which aligns with higher DA levels found in the Gulf of Maine following an increase in cDOM, as reported by Balch et al. (2012).

## 2.2 History

While the fall 2016 bloom was the first toxic *Pseudo-nitzschia* bloom on record in the Gulf of Maine (GOM) (McGuire, 2016), *Pseudo-nitzschia* have long been present in the region. They were recorded for the first time in the 1920s and 30s in Georges Bank region and around Cape Cod (Fish, 1925; Bigelow et al. 1926; Gran 1933; Fernandes et al., 2014). Other studies have reported the presence of *Pseudo-nitzschia* in the Gulf of Maine in the time since, but none described domoic acid levels high enough to require closures of shellfish harvesting (Fernandes et al., 2014).

The Bay of Fundy, located north of the Gulf of Maine, has previously experienced toxic blooms associated with *Pseudo-nitzschia*, including one in 2003 that was suspected of playing a role in the deaths of multiple humpback whales in the region – the first recorded mammalian deaths to occur on the East Coast of the US as a result of domoic acid poisoning (NOAA, 2004; Fernandes et al, 2014).

## 2.3 Known species of *Pseudo-nitzschia* in the Gulf of Maine

One study reported the presence of fourteen species of *Pseudo-nitzschia* in Gulf of Maine waters, nine of which were collected from samples in 2007 and 2008 including; *P. americana, P. calliantha, P. cuspidata, P. delicatissima, P. fraudulenta, P. hasleana, P. heimii, P. multiseries, P. pseudodelicatissima, P. pungens, P. seriata, P. subpacifica, P. turgidula* and *P*. sp. GOM (Fernandes et al., 2014). The results of the present study revealed that *P. australis* was present in the Gulf of Maine, as of 2016.

## 2.4 2016 Bloom populations

The two species of *Pseudo-nitzschia* identified in this study during the fall 2016 bloom were *P. australis* and *P. pungens*. These two species of *Pseudo-nitzschia* differ in their capacity for toxin production, morphology and ease of laboratory-based cultivation.

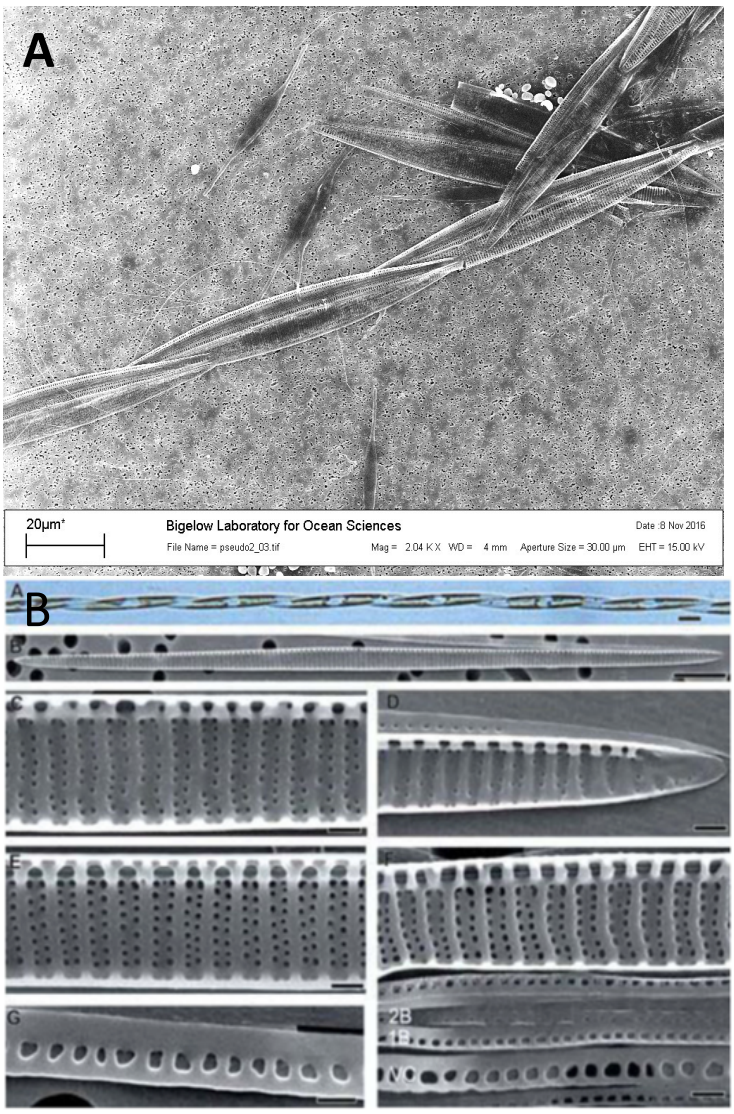


**FIGURE 1 LIGHT MICROSCOPY AND SEM IMAGES OF A) PSEUDO-NITZSCHIA AUSTRALIS, IMAGED FROM THE FALL 2016 BLOOM AT BIGELOW LABORATORY FOR OCEAN SCIENCES AND B) PSEUDO-NITZSCHIA PUNGENS IMAGED FROM A PREVIOUS GULF OF MAINE STUDY (FERNANDES ET AL., 2014).**

*Pseudo-nizschia australis* was found in samples collected from the site of the bloom, where toxin levels were highest. This species has been reported as a primary toxin producer in many blooms since 1991, especially on the West coast of the US (Bates et al., 1998; Trainer et al., 2012; Adams et al., 2017).

*P. pungens* is characterized as having 15-19 striae per 10 µm in the valvocopula (Fernandes et al., 2014). They tend to appear narrower under light microscopy than *P. australis*. *Pseudo-nitzschia australis* tend to appear wider under light microscopy compared to *P. pungens*.

*Pseudo-nitzschia pungens* was the species identified in multiple samples collected from the Damariscotta River Estuary, and area that was unaffected by the 2016 bloom. *P. pungens* is a species that is often non-toxic in the environment and has been shown to produce low concentrations of domoic acid in culture (Bates et al., 1993; Casteleyn et al., 2008; Fernandes et al., 2014). A review of select case studies marking major DA toxin events found patterns of succession of *P. pungens* to *P. australis* (Monterey Bay, 1991; Oregon and Washington coast, 1994) and *P. pungens* to other high-toxin-producing *Pseudo-nitzschia* species (Prince Edward Island, 1987; Bay of Fundy, 1998) following increases in runoff and temperature (Bates et al., 1998). More recently, Klein et al. (2010) observed the seasonal succession of six *Pseudo-nitzschia*

species over one year in Baie de Veys, Normandy, France, and found that toxin events coincided with the appearance of P. australis in early fall during high chlorophyll *a*, high temperature and high silicate concentrations. Temperature and runoff conditions in the Gulf of Maine preceding the fall 2016 DA toxin event mirrored those described in these studies.

## 2.5 Economic importance of the Damariscotta River Estuary

The Damariscotta River estuary is a vital component to the success of the Maine shellfish industry. The estuary houses more oyster farms than any other region in Maine across 100 acres of surface and bottom waters (Thompson et al., 2006; Brady, 2016; DMR, "Aquaculture Division Aquaculture Map," 2018). At this scale, any factors that affect the shellfish populations and the success of these operations have substantial economic and ecological consequences, as oyster aquaculture offers diverse ecosystem services to the surrounding area, such as filtering particles from the water column (Higgins et al., 2011).

Shellfish are sessile filter feeders, dependent on the natural phytoplankton populations in the surrounding environment as a source of food and nutrition (Thompson et al., 2006; Trainer et al., 2012; Fernandes et al., 2014; Lesser et al., 2016). Understanding the phytoplankton dynamics within the Damariscotta River, such as when and under what conditions phytoplankton blooms occur, can provide shellfish farmers with a better understanding of the most productive times to farm and when their shellfish are at risk of encountering HABs. HABs are not generally lethal to shellfish because of their toxins, but can cause death due to hypoxia or feeding suppression, as is the case with Brown Tide alga *Aureococcus anophagefferens* (Gobler et al., 2005). Once they have accumulated toxins, filter feeders must be purified in toxin-free water before they are safe for consumption. If shellfish are harvested before toxins can be purged, they are not safe to use as food product and must be destroyed. These events can result in great economic loss to fisheries from the recalled shellfish and generating fear that shellfish is not safe to eat, even after toxic conditions have subsided.

## 2.6 PREDICTING *PSEUDO-NITZSCHIA* BLOOM CONDITIONS

*Pseudo-nitzschia* is a cosmopolitan genus, able to persist across a wide range of physical conditions. They have been grown in culture at salinities ranging from 6 – 48 PSU and temperatures from 5 – 30˚C (Trainer et al., 2012; Zhu et al., 2017). *Pseudo-nitzschia* are described as r-strategists because of their opportunistic tendency to produce blooms whenever nutrients are available and, as a result, are found naturally in the environment all over the world's oceans (Bates & Trainer, 2006; Trainer et al., 2012), including the Southern Ocean near Antarctica (Countway, personal communication), where surface water temperatures are approximately -1˚C.

It is difficult to predict exactly when and where a toxic bloom will occur, but analyzing the environmental conditions that have preceded past ASP events provides some insight into the physical and chemical conditions that could act as potential triggers. Dense *Pseudo-nitzschia* blooms have been shown to occur in areas with low temperature, high salinity and high nutrient availability (Trainer et al., 2012). These are conditions common to eastern boundary upwelling systems (EBUS; Trainer et al., 2012), including the California, Humboldt, Canary and Benguela Current Systems (Barth et al., 2015), where currents carry deep-water nutrients to the surface, providing conditions suitable for algal bloom formation. The Gulf of Maine is not an EBUS, so it experiences different upwelling patterns.

Blooms also tend to occur in coastal areas, near river plumes where nutrient loading is high because of anthropogenic inputs from agriculture and sewage, in addition to higher temperatures and lower salinities (Trainer et al., 2012). Toxic conditions are often found near riverine inputs. One study showed that toxin production is highest at high salinities (30-40 PSU) and lower at low at salinities (10-20 PSU), suggesting that toxin production is lower when more energy is expended maintaining an osmotic balance in low salinity environments (Doucette et al., 2008).

Increased runoff following a period of drought has often preceded toxic *Pseudo-nitzschia* blooms (Bates et al., 1998; Trainer et al., 2012). This is consistent with runoff conditions in the Gulf of Maine

prior to the 2016 bloom, when *Pseudo-nitzschia australis* appeared in the region for the first time. Varying seasonal and environmental conditions likely contribute to seasonal succession of different species of *Pseudo-nitzschia* in a region. However, it is still unknown which of these factors encouraged toxic strains to appear in the Gulf of Maine.

## 2.7 TRIGGERS OF TOXIN PRODUCTION

There are various theories behind the triggers for domoic acid (DA) production in *Pseudo-nitzschia,* more than one of which likely contributes to toxic conditions in any given bloom. Prominent theories posit that DA could be used as an allelopathic agent to inhibit other microbes competing for nutrients in the environment, used as a trace metal chelator and used to defend against predation (Fehling et al., 2004; Maldonado et al., 2002; Wells et al., 2005; Trainer et al., 2012).

### *2.7.A DOMOIC ACID AS AN ALLELOPATHIC AGENT IN RESPONSE TO MACRONUTRIENT LIMITATION*

Studies have shown an inverse correlation between macronutrient concentrations and domoic acid concentrations, suggesting that domoic acid (DA) might act as an allelopathic agent (Fehling et al., 2004). This correlation has been shown primarily with phosphate and silicate limitation in cultured experiments (Fehling et al., 2004). The macronutrient nitrate is required for growth and DA biosynthesis, so nitrogen limitation is not generally conducive to toxin production (Fehling et al., 2004; Trainer et al., 2012). In theory, DA might be produced to inhibit the growth of other microbes competing for the same nutrient sources, but studies subjecting other organisms to toxic *Pseudo-nitzschia multiseries* or synthetic DA in culture have not supported this theory (Lundholm et al., 2005).

### *2.7.B DOMOIC ACID AS ESSENTIAL TO TRACE METAL UPTAKE*

The structure of domoic acid (DA) enables it to act as a copper chelator (Wells et al., 2005). *Pseudo-nitzschia* requires copper for a multi-copper iron oxidase that facilitates the oxidation of $Fe^{2+}$ to

$Fe^{3+}$, so that this trace metal can travel through a high affinity iron transporter (Wells et al., 2005). Studies have supported this theory by showing that low-iron, high-copper conditions triggered DA production in *Pseudo-nitzschia* (Maldonado et al., 2002; Wells et al., 2005).

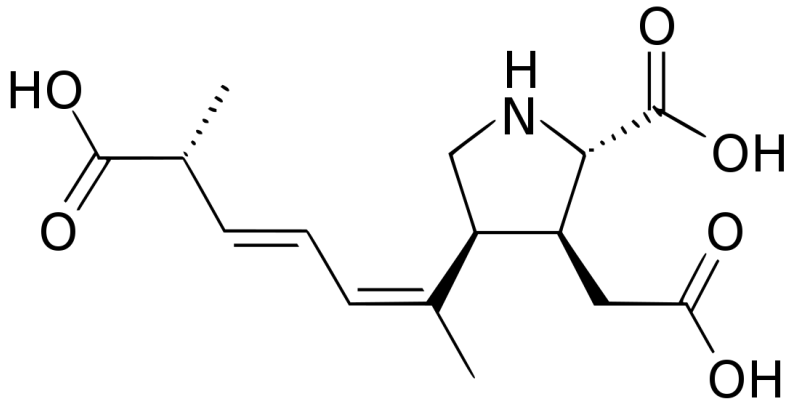


**FIGURE 2 A MOLECULE OF DOMOIC ACID (DA)**

This has been replicated in the environment in the context of iron fertilization experiments, which are often followed by toxic blooms of *Pseudo-nitzschia* (Trainer et al., 2012).

### *2.7.C DOMOIC ACID AS A DEFENSE AGAINST PREDATION*

One study found evidence that the neuroexcitatory properties of DA have a negative effect on krill grazing at high toxin concentrations (Bargu et al., 2006). Bargu (2016) showed that the grazing rate of krill (*Euphausia pacifica*) was significantly lower in the presence of domoic acid than in the presence of glutamic acid, an amino acid whose structure is similar to that of DA. This suggests that DA could act as a defense mechanism against grazing.

## 2.8 IDENTIFICATION OF *PSEUDO-NITZSCHIA* STRAINS

Multiple methods are available for the identification of *Pseudo-nitzschia* at the genus and species levels. Historically, *Pseudo-nitzschia* spp. have been identified according to their ultrastructural morphology, using microscopy. More recently, molecular methods have been developed to achieve more accurate identification at the species and subspecies levels.

### *2.8.A CLASSIFICATION AND MORPHOLOGY*

*Pseudo-nitzschia* is a pennate, chain-forming diatom, with cells of about 40 µm in length. Chains of individual cells range from two cells to up to dozens of cells in length. The cells attach end to end in stepped colonies (Hasle, 1994). *Pseudo-nitzschia* was first categorized as a genus in 1900 by Peragallo et al. (Peragallo & Peragallo, 1900; Hasle, 1994). Debate followed this characterization over whether *Pseudo-nitzschia* constituted its own genus, or only a section within the genus *Nitzschia* (Hasle et al. 1994). In the time since, it has been established as its own genus and distinguished from the *Nitzschia* genus according to various morphological traits: 1) a weakly silicified cell, 2) a shallow, flattened valve, 3) an eccentric raphe, 4) raphe canal walls without poroids, 5) no conopea 6) a strip of nonporoid silica between the valve face and the distal mantle, 7) rows of circular poroids along the valve striae, and 8) perforated girdle bands (Hasle, 1994). These characteristics vary slightly between species within the genus and are used to identify *Pseudo-nitzschia* at the species level (Hasle, 1994; Amato et al., 2007; Fernandes et al., 2014).

Light microscopy is capable of identifying *Pseudo-nitzschia* at the genus level and can distinguish some characteristics between species but is not a reliable method for species level identifications (Amato et al., 2007). Electron microscopy (Transmission EM or Scanning EM) is able to capture morphological details that distinguish *Pseudo-nitzschia* species.

### *2.8.B MOLECULAR TECHNIQUES*

Various molecular methods have been developed to identify *Pseudo-nitzschia* at the species level that have revealed cryptic species and clades that could not be identified by microscopy alone (Trainer et al., 2012). These methods require analysis of gene sequences following Polymerase Chain Reaction (PCR) analysis of a DNA sample. The 18S region has been identified as a reliable target region for genus-level identification of *Pseudo-nitzschia,* but does not have enough variability within species to offer sufficient identification at lower taxonomic levels (Fitzpatrick et al, 2010).

Identification of *Pseudo-nitzschia* at the level of species requires analysis of genomic regions with higher genetic variability compared to the variability within the 18S rDNA gene. Various studies have focused on the rbcL, LSU, ITS1, 5.8S and ITS2 regions of ribosomal DNA (Amato et al., 2007; Trainer et al., 2012). The ITS1-5.8S-ITS2 sequence is most popular because sequential differences in the ITS2 region align best with species delineations that have been determined according to electron microscopy and mating compatibility (Amato et al., 2007). In contrast, according to the biological species concept, the ITS1, rbcL and LSU regions do not act as good indicators for species discrimination because strains with different genotypes in these regions were still able to interbreed and produce viable daughter cells (Amato et al., 2007).

The development of species-specific primers for *Pseudo-nitzschia* has posed challenges to researchers. Even within the ITS1-5.8S-ITS2 region that is used for species-level identification, certain species of *Pseudo-nitzschia* vary by only one or two base pairs (Andree et al., 2011). This similarity allows for cross-reactivity with primers that are meant to target a single *Pseudo-nitzschia* species, potentially confounding the results. It would be advantageous to have a species-specific primer for the species known to be present during the fall 2016 bloom, as such a primer could be used to analyze bloom composition and determine whether the bloom community was comprised of a single *Pseudo-nitzschia* species or multiple species that contributed to toxic conditions.

Species-specific primers have been developed for some species of *Pseudo-nitzschia*, including *P. brasiliana, P. calliantha, P. delicatissima, P. arenysenis, P. fraudulenta, P. galaxiae, P. multistriata,* and *P. pungens* (Andree et al., 2011). To design these primers, the research group paired one primer in a region of high variability, specific to a given species, in either the ITS1 or ITS2 regions with a genus-specific primer in the 5.8S region – either sense or antisense depending on which region was used.

Other methods are also available for molecular identification at the species level. One accurate method for identification is through PCR amplification of the ITS1-5.8S-ITS2 region using genus-specific

primers combined with DNA and sequencing (Fitzpatrick et al., 2010). While effective and accurate, this method can be time consuming both in terms of sample preparation and in the time that it takes to send samples out for sequencing. More recently, novel methods have been developed to allow for cost- and time-effective species-level identifications. One of these methods is known as automated ribosomal intergenic spacer analysis (ARISA), which works by determining the species-specific variation in length of a portion of the ITS1 region (Hubbard et al., 2008; Hubbard et al., 2014). This method can detect the difference of a single nucleotide and can thus create a distinct profile for each species. The researchers used a genus specific primer (PnAll F/R) to amplify the target region, which is then analyzed for length variation. ARISA can identify individual species in a sample, but it is not capable of quantifying the species present, an advantage of quantitative PCR (qPCR) based analyses. Each method has its advantages and drawbacks, but it is likely that alternative genomic regions will need to be targeted if a reliable species-specific qPCR assay is to be designed for *Pseudo-nitzschia australis*.

## 2.9 Domoic Acid

### *2.9.A Traditional medicine*

Domoic acid was first discovered in Japan in 1958, where it was found to be present in the red alga, *Chondria armata* (LaBarre et al., 2014). The domoic acid in this alga was used in traditional medicine to treat ringworm infestations and as an insecticide (LaBarre et al., 2014; Daigo, 1959). Because the toxin was used in relatively low concentrations, neurological effects in humans were not detected until much later.

### *2.9.B Amnesic Shellfish Poisoning*

The first reported human deaths resulting from domoic acid poisoning occurred in 1987 following a shellfish festival on Prince Edward Island, where over 100 people were affected by the toxin and four died (LaBarre, Trainer et al., 2012). Blue mussels (*Mytilus edulis*) acted as the vector in this case study. In response to the event, scientists dissected toxic mussels and cultured the diatom present in their digestive

tract. They identified the responsible diatom as *Pseudo-nitzschia* sp. The term Amnesic Shellfish Poisoning (ASP) was coined to describe this intoxication because of the impairment of short-term memory and other neurological capacities of affected individuals.

ASP is characterized both by gastrointestinal (nausea, vomiting, gastric bleeding and diarrhea) and neurological symptoms (dizziness, confusion, weakness, lethargy, trouble sleeping, short-term memory loss, seizure, coma and death). It is especially dangerous for the elderly and those with compromised renal function (Anderson et al., 2001).

To reach levels high enough to induce ASP in secondary consumers, domoic acid concentrates in the digestive tract of sessile filter feeders, such as shellfish. From here, domoic acid travels up the food web (Bates et al., 1998; Trainer et al., 2012). Many marine mammals and sea birds consume planktivorous fish, which can also accumulate high concentrations of domoic acid in their digestive tracts (La Barre et al., 2014). The first known case of non-human vertebrate intoxication occurred in 1991 in Monterey Bay, CA, where Pelicans and Brandt's cormorants consumed anchovies that had accumulated high levels of domoic acid produced by *Pseudo-nitzschia australis* (Bates et al., 1998).

### *2.9.C CHEMICAL PROPERTIES*

Domoic acid (DA) is a water-soluble amino acid that belongs to the kainic acid (KA) family. KAs act as excitatory neurotransmitters, which facilitate nerve signal transmission (Maloney, 1999). DA has a strong affinity to glutamate receptors in the central nervous system and has a glutamate receptor binding ability three times stronger than kainic acid (Bates et al., 1998; La Barre et al., 2014; Anderson et al., 2001). It has the chemical formula $C_{15}H_{21}NO_6$, and a molecular weigh of 311. Its structure enables it to depolarize and rupture neuron cells located in the hippocampus, which is primarily dedicated to memory retention (Bates et al., 1998; La Barre et al., 2014; Anderson et al., 2001).

There are twelve compounds that belong to the DA family, nine of which are DA isomers and two of which are DA analogs (La Barre et al., 2014). DA and its diastereoisomer 5'-epi-Domoic acid act

as “Very Potent” and “Potent” ASP toxins respectively (La Barre et al., 2014). DA and three other isomers also act as potent insecticides, but the three isomers only act as “Weak” ASP toxins (La Barre et al., 2014). DA and six of its isomers have been synthesized in a lab setting for research purposes (La Barre et al., 2014).

At this point, the biosynthesis pathway for DA in *Pseudo-nitzschia* is unknown. One study used cDNA microarray analysis to monitor *Pseudo-nitzschia multiseries* cells during toxin production (Boissonault et al., 2013). Their aim was to determine which genes were responsible for the production and release of domoic acid. They found 12 candidate transcripts for genes that were up-regulated during toxin production, which provides a stepping-off point for future genetic studies, but no definitive pathways were revealed. A better understanding of DA biosynthesis would be extremely beneficial to studies of toxic bloom populations.

## 2.9.D MONITORING & MANAGEMENT

Since the 1987 ASP event on Prince Edward Island, monitoring for dangerous levels of domoic acid (DA) has successfully prevented more human deaths due to DA poisoning (Bates et al., 1998). The Food and Drug Administration advises that the safe consumption limit for DA is less than 20 ppm in fish and shellfish tissue (FDA, 2011; Lefebvre, 2017). Above this level, closures are enacted on shellfish harvesting and enforced by local monitoring officials. At this dose, a single exposure can have severe and long-lasting impacts on cognitive function. Recent studies have shown, though, that chronic exposure to DA below this threshold can have significant impacts on cognitive activity (Lefebvre, 2017). At low doses, effects on cognitive function are reversible after DA exposure has been eliminated, but the toxin may go unnoticed in the environment because it is not flagged by monitoring programs when it is below a concentration of 20 ppm and there is no current standard for free DA levels in seawater. This has implications for coastal residents, including subsistence farming indigenous communities in the Pacific Northwest, who are chronically exposed to low levels of DA due to frequent consumption of razor clams and other toxic shellfish (Grattan et al., 2016; Lefebvre et al., 2017). This also poses a danger to marine

mammals and vertebrates whose primary food sources may carry low levels of DA (Grattan et al., 2016). DA also adsorbs into sediments, where it can remain for long periods of time and can be taken up by benthic organisms (Trainer et al., 2012).

The volatility of toxicity in the water also poses problems in shellfish monitoring. Toxicity can increase and decrease drastically over short time periods, so there can be significant discrepancies in toxin concentrations between the time of sampling and the time the toxin is tested (Hall, 2012). Because DA is hydrophilic in nature, it does not bioaccumulate in the tissue of filter feeders. Instead, it concentrates in their digestive tract, and can dissipate once the toxin is no longer present the digestive tract region. The retention period of DA varies between a few hours in the blue mussel, *M. edulis*, to over a year in the scallop, *Pecten maximus,* suggesting that DA can accumulate for long periods of time in tissue (Lefebvre 2017). This quality also contributes to the volatility of toxin concentrations in shellfish tissue.

## *2.9.E ASSAYS AND ANALYSES*

### 2.9.E.i Aquaculture management

Algal biotoxins pose a problem to remediation in that they are hard to remove from shellfish through sanitation or sterilization the way that certain pathogens can be eliminated (Hall, 2012). Instead, shellfish can either remain in the water until toxins have filtered out – this can take days to over a year, depending on the concentration of toxin in the shellfish gut and tissue and the type of shellfish or crustacean that is affected – or, if they have already been harvested, they must be recalled and disposed of (Hall, 2012). Both of these methods can pose great economic losses to farmers and harvesters because recalled products cannot be remediated and sold, and shellfish left in the water for long periods of time can grow to the point that they are no longer ideal for selling to consumers.

The costs associated with biotoxin assays often prevent monitoring programs from conducting frequent and comprehensive testing (i.e. testing for all potential biotoxins in a given area). Maine is praised for its monitoring efforts against Paralytic Shellfish Poisoning (PSP) – a condition caused by the

consumption of seafood with high levels of saxitoxin, a neurotoxin produced by the dinoflagellate, *Alexandrium*, which has posed a persistent problem to Gulf of Maine aquaculture for decades (McGillicuddy et al., 2014). These efforts include PSP forecasting programs and the deployment of autonomous Environmental Sampling Processers, which provide near real-time reports of the presence of saxitoxins around the Gulf (NOAA, "Gulf of Maine Red Tide Monitoring Season Begins for NOS," 2016). Farmers and monitoring programs were less prepared for the *Pseudo-nitzschia* bloom that occurred in the fall of 2016, which resulted in the recall and destruction of about 5 tons of shellfish, some of which had reached toxin levels of 129 ppm, more than six times the warning limit (McGuire, 2016). Now that awareness has increased, more efforts and funding are being directed towards Amnesic Shellfish Poisoning (ASP) monitoring, but this serves as an example of how challenging it can be to proactively screen for unfamiliar biotoxins. The most recent bloom to affect the Gulf of Maine involved the dinoflagellate *Karenia mikimotoi*, a species that had never before been observed in the region (Blank, 2017). This species does not adversely affect human health, but has been responsible for fish kills due to hypoxia worldwide, and may have been responsible for softshell clam mortality in Freeport, Brunswick and Harpswell, ME during the bloom. Monitoring and management efforts in the Gulf of Maine must adapt to account for multiple new species threatening fisheries and aquaculture.

### 2.9.E.ii Toxin analysis

There are various methods used to quantify domoic acid concentrations in shellfish tissue and algal culture samples. A mouse bioassay was the first method used to detect DA following the ASP event in PEI 1987, and is still used around the world today (Anderson et al., 2001). This method involves intraperitoneal injection of a shellfish extract, which may or may not contain DA, to mice, followed by observation of injected mice for up to 18 hours for signs of a loss of motor skills, convulsions and death. The detection limit for this method (40 ppm) is too high to detect DA at the level required for shellfish monitoring (20 ppm by weight).

More recently, high-pressure liquid chromatography (HPLC) methods have been developed that are more accurate and have much lower detection limits than the mouse bioassay (Anderson et al., 2001). They work by separating DA from its isomers, which are also present in environmental samples, but do not contribute to ASP (Anderson et al., 2001). Bigelow Analytical Services, based at the Bigelow Laboratory for Ocean Sciences uses two forms of HPLC for biotoxin detection: HPLCPCOX (Post-Column Oxidation) and LC-QQQ-MS (Liquid Chromatography triple-Quadrupole Mass Spectrometry). The latter is more sensitive and has a lower detection limit of 0.1 ppm ("Biotoxin Analysis," Bigelow Analytical Services).

The enzyme-linked immunosorbent assay (ELISA) test kit was developed as a more cost-efficient, rapid and accessible method for DA detection. Where HPLC methods require time and specialized equipment, the ELISA method requires only a testing kit and a plate reader. A 96-well plate coated with horseradish peroxidase-conjugated primary antibodies binds DA from a sample in addition to a DA HRP enzyme conjugate that is pipetted into each well ("Domoic Acid Plate Kit"; Kleivdal et al., 2007). Once the reaction is complete, the concentration of DA can be read on a plate reader. This method has a dynamic working range of 0.1-250 ppm and a minimum detection limit of 0.003 ppm (Kleivdal et al., 2007). Studies have shown that it does not exhibit cross-reactivity with DA isomers and is therefore a reliable method for DA detection.

## 2.10 Purpose of Study

The purpose of this study was to analyze the biological and chemical characteristics of the fall 2016 toxic *Pseudo-nitzschia* bloom that occurred in the Gulf of Maine. Using data collected during the bloom as part of a NOAA-funded Rapid Response effort, macronutrient concentrations in the seawater were compared to cell and toxin concentrations to get a better understanding of the influence of nutrients on the bloom community. Methods were developed for relating quantitative PCR results to cell densities, which aided in our bloom analysis. These conditions were then replicated in a laboratory setting, looking at how cultured *Pseudo-nitzschia pungens* responded to nutrient stress. Various nutrient limitation

scenarios were explored. The findings of this study can be used to aid future studies in the area, ultimately contributing to management efforts for toxic blooms of *Pseudo-nitzschia* in the Gulf of Maine.

# 3. CALIBRATING GENE COPY NUMBER TO CELL COUNT

Results from qPCR offered quantification of *Pseudo-nitzschia* in terms of 18S rDNA gene copy number, but this information does not translate directly to cell counts without determination of a "gene copies per cell" conversion factor. To determine approximately how many copies of the 18S rDNA there are per cell, and thereby get an estimate of *Pseudo-nitzschia* concentrations in our environmental samples, gene copy numbers determined using qPCR were calibrated to the number of cells present in the same sample of cultured cells that were enumerated by microscopy.

## 3.1 DETERMINATION OF BEST COUNTING METHOD

*Pseudo-nitzschia* cells are pennate, forming long chains that do not lend themselves to counting. The cells, especially when degraded, tend to have little pigment. Stains can be used to improve visibility. A first attempt at staining followed a protocol using 2.5x SYBR Gold to stain the *Pseudo-nitzschia* cells and view them with epifluorescence microscopy (Noble and Fuhrman, 1998). This method did not increase pigment to the desired extent. Lugol's Iodine solution (1 drop Lugol's/1 ml sample) provided better staining and did not require the use of an epifluorescence microscope. Imaging was performed using an inverted microscope at 20x magnification, as it provided the clearest focus for cells that had settled to the bottom of a gridded counting chamber.

Next, the appropriate counting chamber was determined to use for cell counting. Because of their oblong shape and chain-forming tendencies, *Pseudo-nitzschia* cells do not distribute evenly in many counting chambers. In a haemocytometer, traditionally used for counting blood cells and optimally used for other cells with a similar size and round, symmetrical morphology, *Pseudo-nitzschia* chains clustered around the edges rather than distributing evenly across the gridded counting cell. Two other counting

chambers tested included the Palmer-Maloney (round chamber; 100 µl) and Sedgewick-Rafter (rectangular chamber; 1212 µL). The Sedgewick-Rafter worked best because it appeared to allow for even distribution of cells. Its higher surface area also worked better for the counting scheme: rather than count all cells present in the chamber, forty fields of view were captured with a camera attached to the microscope. The counting chamber was shifted between captures to attain forty randomly chosen fields of view. Individual cells were counted from the pictures taken.

A stage micrometer was used to calibrate the volume of the camera field of view. A length of 528 pixels was equal to 100µm. Using this conversion factor, the field of view area was converted from pixels squared to millimeters squared (36,152,320 pixels$^2$ = 1.297mm$^2$). The volume of the chamber was calibrated by subtracting its dry mass from its mass when filled with water to find an approximate volume of 1.212 ml. The surface area of our field of view was 0.129% of the surface area of the chamber, so calculated the field of view volume was also 0.129% of the chamber volume, or 0.1567 ml. This information was used to estimate the number of cells per ml that were counted.

## 3.2 Cell counting and qPCR

A culture of *Pseudo-nitzschia pungens* collected from the East Boothbay Dock in East Boothbay, Maine during the fall of 2016 was transferred into four 250 ml Erlenmeyer flasks to grow for six days in L1 medium (Guillard & Hargraves, 1993). After six days, 5 ml aliquots were collected for cell counts and preserved with five drops of Lugol's solution in 5 ml Lo-Bind Eppendorf centrifuge tubes for later counting. Counts were performed on 40 random fields of view captured in a Sedgewick-Rafter counting chamber and extrapolated to cell concentration per ml of sample, as described above.

Immediately after taking aliquots for cell counts, 100 ml of each culture were vacuum filtered onto GF/F filters and frozen for later analysis using qPCR. The filtered samples were amplified using *Pseudo-nitzschia* genus-specific 18S rDNA primers using the methods outlined above (Fitzpatrick et al., 2010). qPCR returned counts for 18S gene copies present in our starting volume, which were then

compared to cell concentrations determined using microscopy. Taking the average across our four replicates DNA samples, it was estimated that there are 79.4 ± 10 18S rDNA gene copies per cell of *Pseudo-nitzschia pungens*.

## 3.3 Discussion of Calibration

Figure 4 shows each replicate used for calibration compared to the theoretical ratio for gene copy number to cell count of 79.4:1. The biplot shows evidence of an outlier, which may be skewing the data, suggesting that it may be valuable to repeat this calibration method with more replicates to understand whether these deviations are a result of uneven sampling. Despite the presence of an outlier in the data, the estimate used in this study aligns exceptionally well with the results of another study that determined that there are approximately 80 18S gene copies per cell of *Nitzschia closterium* (F. Zhu et al., 2005), a close relative of the *Pseudo-nitzschia* genus.

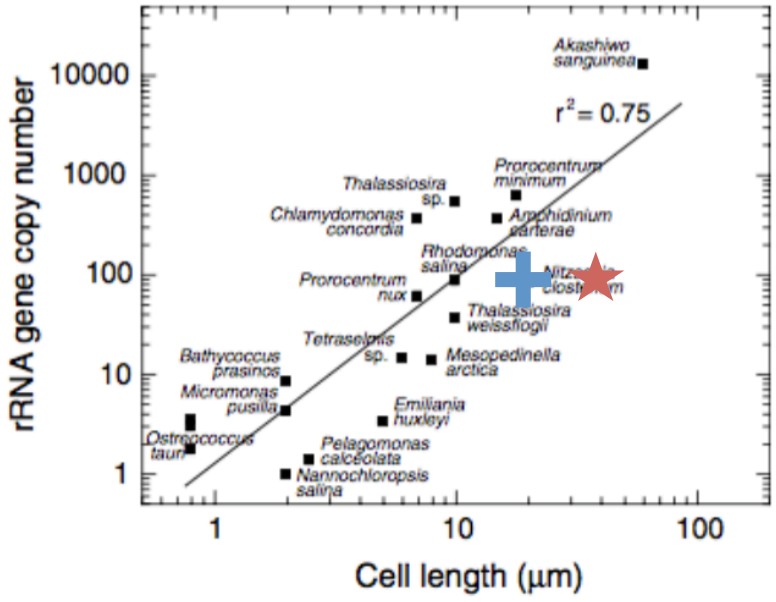


**FIGURE 3 A SCATTER PLOT SHOWING RRNA GENE COPY NUMBER FOR VARIOUS MARINE PHYTOPLANKTON, ACCORDING TO CELL LENGTH (ZHU ET AL., 2005). ANNOTATED TO SHOW *NITZSCHIA CLOSTERIUM* (BLUE CROSS; DETERMINED BY ZHU ET AL., 2005) AND *PSEUDO-NITZSCHIA PUNGENS* SHOWN (RED STAR; DETERMINED IN THIS STUDY USING QPCR) (FIGURE MODIFIED FROM ZHU ET AL., 2005).**

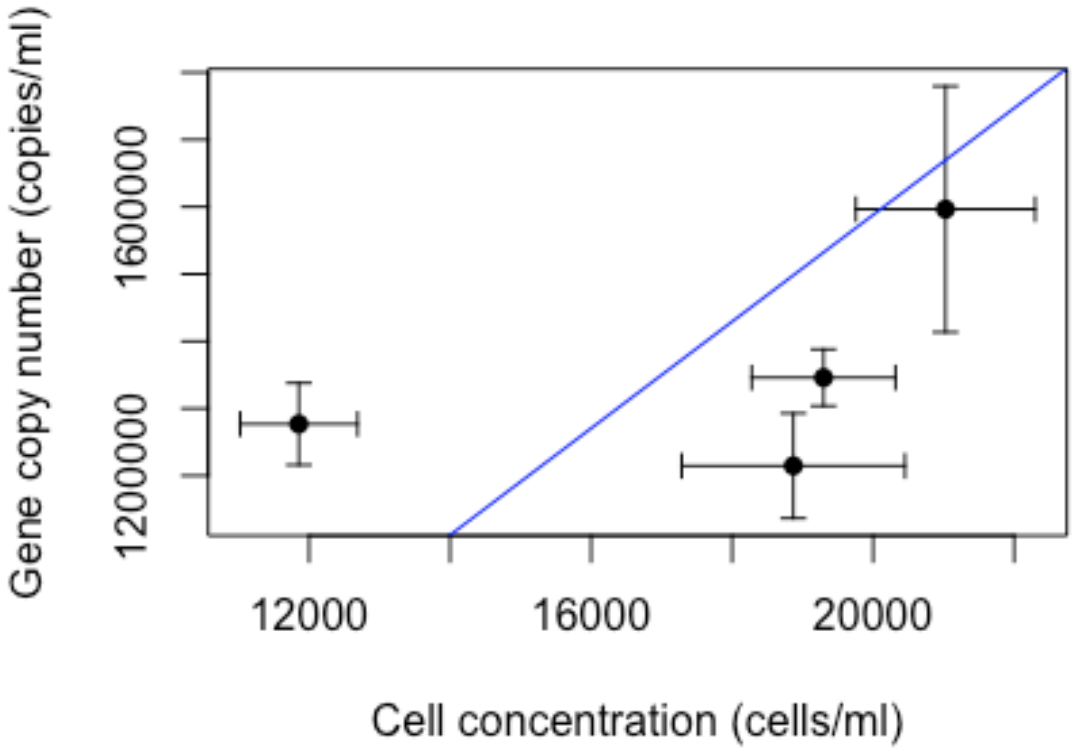


**FIGURE 4 A BIPLOT SHOWING CELL COUNTS PER ML DETERMINED USING MICROSCOPY AND GENE COPY NUMBERS PER ML DETERMINED USING QUANTITATIVE PCR FOR FOUR REPLICATES USED FOR CALIBRATION. THE THEORETICAL RATIO FOR GENE COPY NUMBER TO CELL COUNT OF 79.4:1 IS SHOWN BY THE BLUE LINE.**

This approach can be used to rapidly determine cell abundance during bloom conditions. *Pseudo-nitzschia* are known for reaching relatively low cell concentrations, even in peak bloom periods, on the order of $10^5$ or $10^6$ and occasionally $10^7$ cells $L^{-1}$ (Trainer et al., 1998; Du et al., 2016), compared to other toxic algal bloom species, such as *Alexandrium*, which regularly reach concentrations on the order of $10^6$ and $10^7$ cells $L^{-1}$ (Estrada et al, 2009; Robinson et al., 2009). Because of this, it is important to be able to tell what concentrations constitute a potentially toxic bloom efficiently, using methods like this cell-calibrated qPCR method.

# 4. PRIMER DESIGN

## 4.1 METHODS FOR PRIMER DESIGN

This study aimed to develop species-specific primers for multiple species known to be present in the Gulf of Maine, including one known to be present during the bloom, *Pseudo-nitzschia australis.* Sequences from the ITS1, 5.8S and ITS2 regions of *Pseudo-nitzschia* were downloaded from GenBank for species identified in the Gulf of Maine during a survey conducted in 2007, including *P. americana, P. fraudulenta, P. subpacifica, P. heimii, P. pungens, P. seriata, P. delicatissima, P.* sp. GOM and *P. turgidula* (Fernandes et al., 2014), in addition to the species identified during the fall 2016 bloom, *P. australis*. These sequences were aligned using MEGA and BioEdit and used Primer3Plus to identify potential regions for primer design. For the purposes of this study, the development of species-specific primers was focused on *P. pungens* and *P. australis*. AlleleID v7 (Primer Biosoft) was used to assess primer specificity.

## 4.2 RESULTS FOR PRIMER DESIGN

All primers that were assessed in AlleleID had poor species specificity in that a primer set targeted for one species usually showed potential for cross-reactivity with another non-target species. Given the limited genetic information available for multiple species of *Pseudo-nitzschia*, it was impractical, within the scope of this project, to expend further effort to design novel primers. Successful species-specific primer design will require new DNA sequences for multiple isolates of *Pseudo-nitzschia*. To date, there is only one species of *Pseudo-nitzschia* whose complete genome has been sequenced, *P. multiseries* CLN-47 (Yuan et al., 2016), which poses challenges when designing primers for other *Pseudo-nitzschia* species and limits our knowledge of alternative gene targets.

# 5. BLOOM ANALYSIS

## 5.1 METHODS OF BLOOM ANALYSIS

### *5.1.A 2016 BLOOM ANALYSIS*

For the first time in recorded history, the Gulf of Maine experienced a toxic bloom of *Pseudo-nitzschia* that reached domoic acid (DA) concentrations above the shellfish harvesting closure limit of 20 ppm of DA in shellfish tissue. In response, the National Oceanic and Atmospheric Administration (NOAA) funded a rapid response effort to investigate the physical, biological and chemical conditions surrounding the bloom. Responders from Bigelow Laboratory for Ocean Sciences and Woods Hole Oceanographic Institute (WHOI) sampled from twenty-nine stations along the coast of Maine, ranging from Penobscot Bay to Machiasport, which was the area identified as the origin of the bloom. Samples were collected at two depths (2 m and 10 m) for quantitative polymerase chain reaction (qPCR), Automated Ribosomal Intergenic Spacer Analysis (ARISA), nutrient analysis, and toxin analysis.

In addition to samples collected as part of the NOAA-funded rapid response cruise, samples were collected at three depths across four stations along the Damariscotta River as part of the Changing Oceans

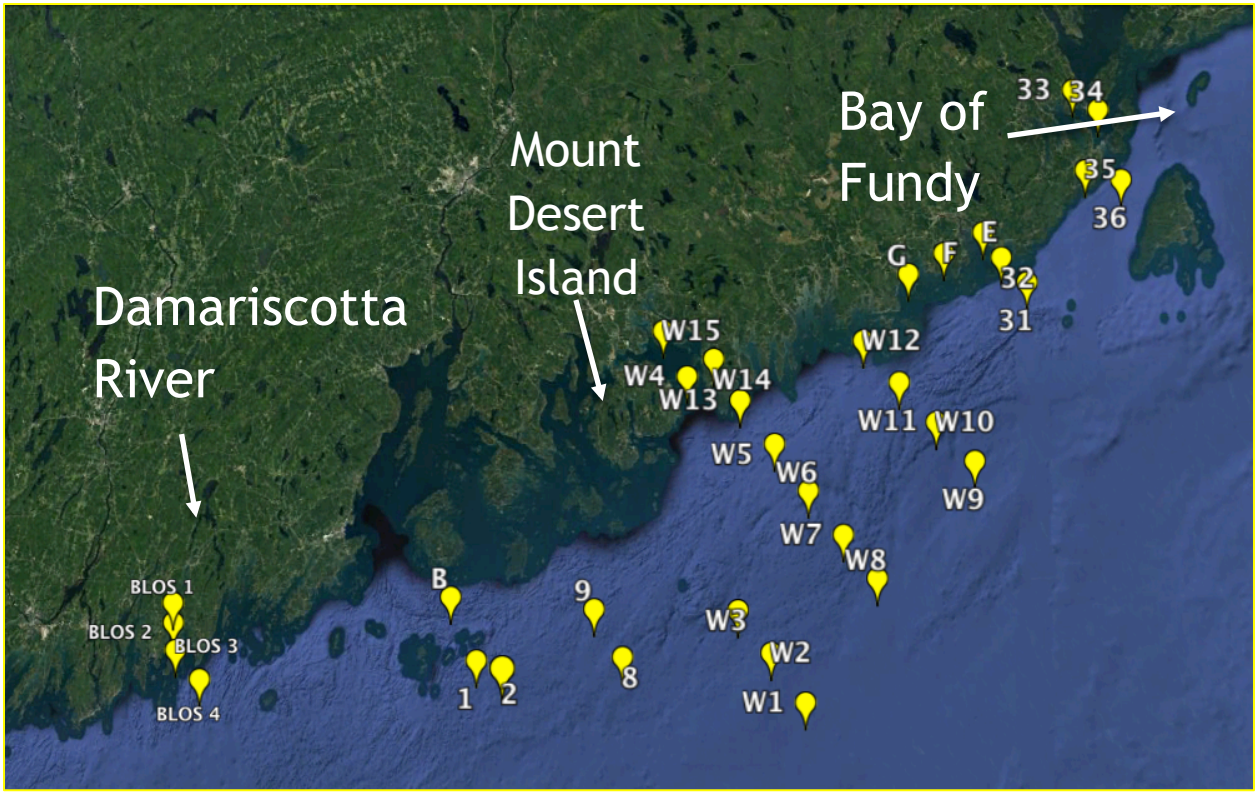


**FIGURE 5 A MAP SHOWING THE LOCATION OF STATIONS SAMPLED DURING THE NOAA-FUNDED RAPID RESPONSE TO THE FALL 2016 TOXIC PSEUDO-NITZSCHIA BLOOM. SAMPLES FROM STATIONS BLOS1-BLOS4 WERE COLLECTED ON RESEARCH CRUISES AS PART OF THE CHANGING OCEANS SEMESTER PROGRAM. SAMPLES FROM STATIONS W1-W15 WERE COLLECTED BY THE TEAM FROM WOODS HOLE OCEANOGRAPHIC INSTITUTION. SAMPLES FROM ALL OTHER STATIONS WERE COLLECTED BY THE TEAM FROM BIGELOW LABORATORY FOR OCEAN SCIENCES.**

Semester Program: Colby at Bigelow Laboratory for Ocean Sciences. Sampling occurred biweekly from Sep. 9, 2016 to Nov. 1, 2016, with two essential cruises occurring before (Oct. 4, 2016) and after (Oct. 19, 2016) the peak of the toxic bloom.

### 5.1.A.i Quantitative Polymerase Chain Reaction (qPCR) for bloom samples

Quantitative PCR was performed for all samples on the 18S rDNA region following the methods of Fitzpatrick et al. (2010) for *Pseudo-nitzschia* genus-level quantification (Fitzpatrick et al., 2010). Whole water samples were collected and vacuum filtered onto GF/F filter and then frozen at -20 ˚C until extraction. Two-hundred and fifty µl zirconia/silica beads and 2 ml 2X Lysis buffer (40 mM EDTA, pH 8; 100mM Tris, pH 8; 100 mM NaCl; 1% SDS) were added to the frozen sample tubes, which were then thawed in a dry bath at 70 ˚C. The samples were then subjected to a cycle of heating at 70 ˚C and bead beating at 20/sec on a Retsch Mixer Mill MM 400 according to similar lysis procedures previously described in the literature (Countway and Caron 2006). The filter and lysate were then transferred to a 10 ml syringe and compressed in the syringe barrel to extract as much lysate from the filter as possible, collecting the lysate in a new 2 ml Lo-Bind Eppendorf tube. Lysates were stored at -20 ˚C until further use.

Crude cell lysates were diluted 1:100 with nuclease-free water prior to qPCR analysis. Five µl of diluted lysate was mixed with 10 µl of 2X Perfecta® SYBR® Green FastMix® (Quanta Biosciences) and 500 nM (final concentration) of the primers PnGenusFwd 5'- CTGTGTAGTGCTTCTTAGAGG -3' and PnGenusRev 5' – AGGTAGAACTCGTTGAATGC – 3' (Fitzpatrick et al., 2010) plus enough molecular-grade water to make a final volume of 20 µl per reaction. Samples were run in triplicate along with five standards created from cloned *Pseudo-nitzschia* 18S rDNA of known concentrations ranging from 3.99 to 3.99 x $10^4$ gene copies per µl. Samples were analyzed on a BioRad CFX96 with the following thermal protocol: one initial 30 second cycle at 95 ˚C, then 45 cycles consisting of: 5 seconds at 95 ˚C, 15 seconds at 61 ˚C and 10 seconds at 72 ˚C.

### *5.1.B CELL CULTURING*

Prior to the bloom, samples were collected from the Bigelow Laboratory Dock on the Damariscotta River in East Boothbay, ME using a 20 µm plankton net. Individual cells were picked from these samples using a microcapillary and washed in particle-free salt water to ensure unialgal isolation. Isolated cells were transferred into fresh L1 medium, and grown in an incubator at 18 ˚C, 100 µE $m^{-2}$ $s^{-1}$. Cultures were transferred every two to three weeks to keep cells actively growing. Fifteen cultures were isolated from dock samples ("PN1-15") in addition to one collected from a Changing Oceans Semester Program cruise at Station 4 offshore ("Tow 4").

Cells collected from bloom samples in Machiasport area were not amenable to cultivation, despite multiple attempts, potentially due to degraded condition of samples upon arrival at the lab and storage on ice, which is typically colder than the ideal temperature for these cells.

### *5.1.C PSEUDO-NITZSCHIA IDENTIFICATION*

#### 5.1.C.i DNA extraction of Damariscotta River samples

*Pseudo-nitzschia* cultures were isolated by picking single chains of cells from samples collected off of the Bigelow Laboratory dock, located on the Damariscotta River in East Boothbay, Maine. Once the unialgal cultures were dense, a few drops of each culture was transferred to a petri dish with a disposable pipette. Excess water from the sample in the petri dish was removed using a 10 µl pipette – small enough to prevent clumps of *Pseudo-nitzschia* chains from being pulled into the pipette tip. The remaining clumps of *Pseudo-nitzschia* were transferred from the petri dish to a 0.5 ml Lo-Bind Eppendorf tube with 100µl zirconia/silica beads. The tubes were then subjected to three cycles of heating in a dry bath at 70 ˚C for three minutes followed by vortexing to form a crude cell lysate. To separate cell waste from the lysed DNA, the lysates were centrifuged at 14,000 xg and the supernatant was transferred to a fresh 0.5 ml Lo-Bind Eppendorf tube after which the lysed DNA was stored them at -20 ˚C until further analysis.

### 5.1.C.ii Molecular identification of Damariscotta River samples

Endpoint Polymerase Chain Reaction (PCR) was used to amplify the ITS region of the *Pseudo-nitzschia* genome from the Damarascotta River Estuary sample crude cell lysates (CCLs). The CCLs were diluted 1:100 with nuclease-free water (Millipore) to reduce PCR inhibitors. Five µl of each 1:100 diluted CCL was added to 15 µl of master mix containing 2X GoTaq® G2 Hot Start Green Master Mix (Promega); B-rc primer (5'–GTAGGTGAACCTGCAGAAGGATC – 3'); NLR-204 primer (5'–ATATGCTTAARTTCAGCGGGT – 3'); and nuclease-free water). A touchdown thermal protocol was performed on a BioRad C1000 thermal cycler using the following conditions: one cycle of 2 min at 95 ˚C, then ten cycles of: 30 sec at 95 ˚C, 30 sec at 65-55 ˚C (-1˚C per cycle) and 90 sec at 72 ˚C, followed by 25 cycles of 30 sec at 95 ˚C, 30 sec at 55˚C and 90 sec at 72˚C. A final extension of 7 min at 72 ˚C was added at the end of the thermal protocol to ensure A-tailing of PCR products (for molecular cloning) before holding the reactions at 12˚C until further processing or transfer to the freezer.

PCR products were separated on a 1.2% agarose gel (SeaKem) for 60 minutes at 100 V, stained for visualization on a blue light Dark Reader (Clare Chemical) with 1x SYBR Gold (ThermoFisher), then band-isolated and purified using a Wizard® SV Gel and PCR Clean-up System (Promega). Once the DNA was purified from the gel, it was quantified using a Qubit fluorometer and the double-stranded DNA quantification reagents (ThermoFisher).

PCR products were cloned with the TOPO TA Cloning kit for Sequencing (ThermoFisher) following the kit protocol for chemically competent TOP10 *E. coli.* PCR products were diluted to 5 ng/µl, mixed with 1 µl of salt solution, 1 µl of cloning vector/DNA ligase solution (pCR4-TOPO), and molecular water for a total of 6 µl of ligation reaction volume. Transformation of ligated PCR products into TOP10 chemically competent E. coli was accomplished via heat-shocking at 42 ˚C. Transformed cells were grown on LB + Kanamycin (50 µg/ml) selective plates overnight prior to selection of individual bacterial colonies of transformants that carried the cloned *Pseudo-nitzschia* genes. Plasmid DNA was extracted and purified from overnight cultures of the selected bacterial colonies. This DNA

served as a template for Sanger sequencing of the cloned *Pseudo-nitzschia* ITS DNA using T7 and T3 sequencing primers that flank the cloning region of the TOPO vector. Plasmid DNA was extracted from bacterial cell pellets with the Zyppy plasmid purification kit (Zymo Research), diluted to approximately 100 ng/µl and shipped out for Sanger DNA sequencing to Eurofins Genomics (Louisville, KY).

#### 5.1.C.iii SEM identification of bloom sample

A sample was received from Bass Harbor, one of the areas most affected by the Fall 2016 bloom that did not yield cultivated cells or extractable DNA for molecular identifications. In lieu of these methods, scanning electron microscopy (SEM) was used to identify the species present.

A sample was gently vacuum-filtered onto a 0.8 µm, 25mm diameter black polycarbonate filter (Nuclepore). The filter was mounted onto a stub and allowed to dry in a desiccator over the weekend before sputter coating with gold particles. The cell preparation was imaged on the Zeiss Supra25 SEM at Bigelow Laboratory for Ocean Sciences at a magnification between 2,040x and 13,810x with assistance from Amy Wyeth. Images were sent to Dr. Carmelo Thomas at UNC Wilmington, who identified the cells as *Pseudo-nitzschia australis*, a known toxin-producing species.

### *5.1.D METAPOPULATION ANALYSIS OF BLOOM SAMPLES*

Metapopulation analysis can be used to analyze microbial communities, identify relationships between key bloom species and assess whether multiple species of a target organism are present. For these reasons, next-generation DNA sequencing was used to analyze the eukaryotic metapopulation in the Fall 2016 bloom samples (Comeau et al., 2011).

A full DNA extraction was performed on filtered samples collected from Station F at depths of 2 m and 10 m during the Fall 2016 bloom Rapid Response cruise, following established methods (Countway, 2005). DNA was measured on a Qubit fluorometer, as described above and shipped out for Next-Generation Sequencing to the Integrated Microbiom Resource within the Centre for Comparative

Genomics and Evolutionary Bioinformatics (Dalhousie University, Halifax). The results were analyzed using Mothur v 1.39.

### *5.1.E FLOWCAM ANALYSIS OF DAMARISCOTTA RIVER SAMPLES*

The FlowCam is an instrument that images microplankton in whole water samples. It can be used to identify and enumerate larger (>10 µm) planktonic species present in a sample. As part of the *Colby at Bigelow: Changing Oceans Semester Program*, biweekly samples were collected from four stations along the Damariscotta River using Niskin bottles deployed from a CTD at a depth of 2 m for FlowCam analysis. Samples were processed on the FlowCam and analyzed by creating image libraries to sort images into group, genus or species. *Pseudo-nitzschia* biomass was calculated using ABD volume using equations from Menden-Deuer & Lessard (pgC/mL = 0.288 x volume(µm3)^0.811; Menden-Deuer & Lessard, 2000).

### *5.1.F NUTRIENT ANALYSIS*

Nutrient samples collected at each station were filtered using a 0.2, 25mm Whatman® Hydrophilic Nuclepore Polycarbonate Filter Membrane into polycarbonate nutrient bottles and stored at -20˚C until analysis using a Lachat system (Bigelow Laboratory for Ocean Sciences).

### *5.1.G STATISTICAL ANALYSIS*

To understand the role of macronutrient limitation in toxin production, concentrations of nitrogen (nitrate + nitrite), phosphorous (phosphate) and silica (silicate) were compared to cell density and domoic acid concentrations at each bloom station.

#### 5.1.G.i Correlation of individual nutrient concentrations to toxin concentration

During preliminary analysis, correlations were tested between log-transformed cell densities, log-transformed DA concentrations, nitrogen (nitrate+nitrite), phosphorous (phosphate) and silica (silicate) concentrations using a Pearson product-moment correlation (Appendix A). This analysis was limited to

stations with domoic acid concentrations greater than 0 ng $ml^{-1}$ to reduce confounding stations not affected by the bloom. Nutrient concentrations were treated as predictor variables throughout the experiment. Cell densities and DA concentrations were treated as response variables.

This preliminary analysis showed a correlation between cell density and domoic acid concentrations (r = 0.55) in addition to strong correlations between phosphate and nitrate (r = 0.97), between phosphate and silicate (r = 0.95) and between nitrate and silicate (r = 0.99) (Appendix A). Because all predictor variables were extremely collinear, this study attempted to use dimension reduction via various ordination techniques, such as principal components analysis (PCA; Wold et al., 1987), canonical correspondence analysis (CCA; ter Braak & Verdonschot, 1995)add citation to works cited) and non-metric multidimensional scaling (NMDS; Kenkel & Orloci, 1986). All are statistical tools that can be used cluster data along eigenvectors or gradients defined by the principal components affecting the data. Environmental variables (nutrients) did not distinguish themselves within the principle component axes for any technique used. CCA was also used to analyze the data from this study, but did not produce any conclusive results. Because of this, linear regression was a more appropriate method for determining relationships between predictor (nutrients) and response (cell densities and DA concentrations) variables.

### 5.1.G.ii Nutrient analysis compared to Redfield Ratio

In addition to individual nutrient comparisons with cell density and DA concentration, nutrient stoichiometry was analyzed at each station, comparing ratios of N:Si, Si:P and P:N to the Redfield Ratio, modified by Brzezinski to include silica, of Si:N:P equal to 15:16:1 (Redfield, 1958; Brzezinski, 1985; Ptacnik et al., 2010). A comparison of nutrient ratios at each station to the Redfield Ratio was done using a one-sided t-test with the following alternative hypotheses: N:Si and Si: P at stations affected by the bloom would be significantly greater than Redfield (16/15 or 1.0667 and 15 respectively; Redfield, 1958; Brzezinski, 1985), suggesting silica limitation in both cases; P:N at stations affected by the bloom would be significantly less P:N as defined by the Redfield Ratio (1/16 or 0.0625; Redfield 1958).

### 5.1.G.iii Comparison of nutrient ratios 'in-bloom' and 'out-of-bloom'

It is acknowledged in the literature that nutrient ratios in coastal areas diverge from nutrient ratios defined by Redfield for the open ocean due to inputs from coastal runoff, drawdown of nutrients due to primary productivity and other factors that differ from the biogeochemistry of the open ocean (Redfield, 1958; Ptacnik et al., 2010). For this reason, it is useful to analyze nutrient ratios at each station relative to those of other stations in the bloom area, as opposed to comparing them strictly to the Redfield Ratio. To apply this analysis to the 2016 bloom sample data, samples from each depth at each station were designated either as 'in-bloom' or 'out-of-bloom' based on a threshold domoic acid concentration.

### 5.1.G.iv Determining the bloom threshold

Four potential DA thresholds (1, 5, 10 and 20 ng $ml^{-1}$) were tested for bloom analysis to determine which would offer a clear delineation between 'in-bloom' and 'out-of-bloom' sites. Observations taken at both depths (2 m and 10 m) for each station were used in this analysis to increase sample size for statistical analysis. Had there been more observations available, one station would have been considered a single observation, rather than two observations – one at two meters and one at ten meters. A 1 ng $ml^{-1}$ threshold included 14 observations across 9 stations (Appendix B), a 5 ng $ml^{-1}$ threshold included 7 observations across 4 stations (Appendix C), a 10 ng $ml^{-1}$ threshold included 5 observations across 3 stations (Appendix D), and a 20 ng $ml^{-1}$ threshold included 2 observations at 1 station (Appendix E). The threshold with the least variation within groups and with the largest possible sample size, given that constraint, was chosen for analysis: 5 ng $ml^{-1}$ (Appendix F).

Nutrient ratios of N:Si, Si:P and P:N for the 'in-bloom' groups (defined using a 5 ng $ml^{-1}$ threshold) were compared to those of the 'out-of-bloom' groups using a paired t-test.

# 5.2 RESULTS OF BLOOM ANALYSIS

## *5.2.A SPECIES IDENTIFICATION*

### 5.2.A.i Damariscotta River samples

Seven cultures of *Pseudo-nitzschia* collected from the Damariscotta River were identified as *Pseudo-nitzschia pungens* using endpoint PCR and sequencing. Six of these cultures were isolated from samples collected off the dock at Bigelow Laboratory for Ocean Sciences and one was collected at BLOS station 4 on a. research cruise conducted on Oct. 4, 2016.

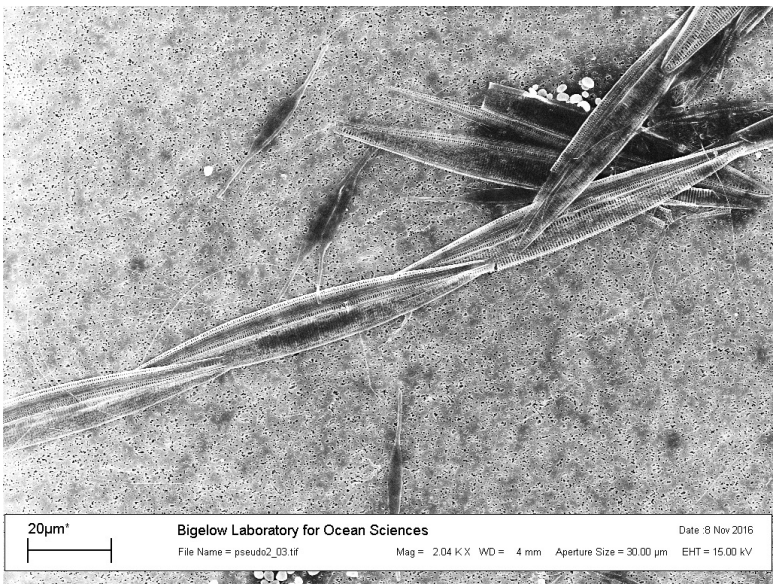


**FIGURE 6 A SCANNING ELECTRON MICROSCOPY (SEM) IMAGE OF CELLS COLLECTED FROM THE BLOOM REGION IDENTIFIED AS *PSEUDO-NITZSCHIA AUSTRALIS*.**

### 5.2.A.ii Bloom samples

As discussed above, samples collected from Bass Harbor, an area affected by the bloom, were identified using SEM as *Pseudo-nitzschia australis*, a known toxin-producing species.

## *5.2.B POPULATION DENSITIES*

### 5.2.B.i Damariscotta River samples

#### *5.2.B.i.a Quantitative Polymerase Chain Reaction (qPCR) analysis*

*Pseudo-nitzschia* concentrations were found to be higher on Cruise 3 (0ct. 4, 2016) than they were on Cruise 4 (Oct. 18, 2016) at all stations. On Cruise 3, cell concentrations were generally higher at depth offshore (BLOS3: 68 cells $ml^{-1}$ at 40m depth; BLOS4: 57 cells $ml^{-1}$ at 40m depth). In contrast, upriver on Cruise 3 (BLOS stations 1 & 2), concentrations were higher at the surface (BLOS1: 63 cells $ml^{-1}$ at 2 m depth and BLOS2 39 cells $l^{-1}$ at 10 m depth; Figure 7A). Data from Cruise 4 showed lower

cell concentrations at all stations and all depths, except for BLOS station 1 at a depth of 30m with a concentration of 72 cells $l^{-1}$ at 38m depth (Figure 7B).

*5.2.B.i.b FlowCam Analysis*

FlowCam analysis of surface samples at all Damariscotta River stations (BLOS stations 1-4) was performed to supplement qPCR analysis of river samples. This offered data for five cruise dates ranging from late September to early November to supplement the samples collected from cruises 3 and 4 for qPCR analysis.

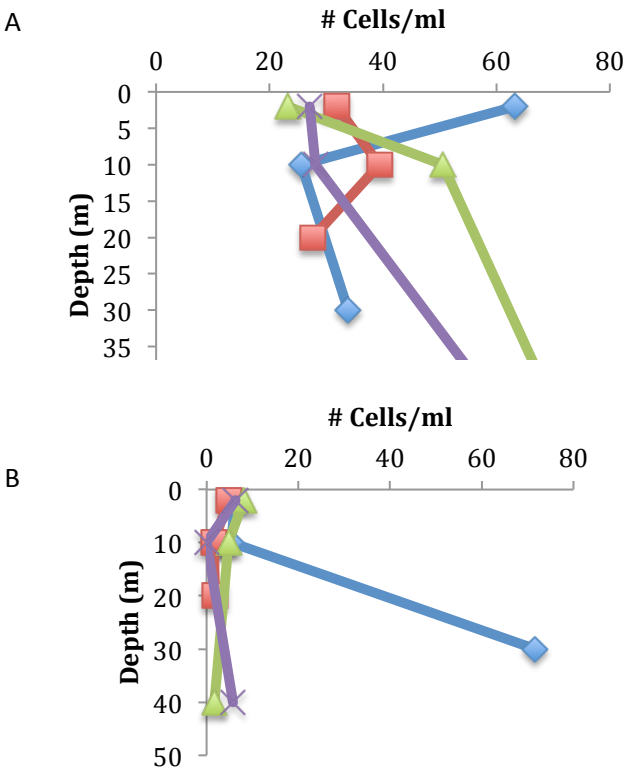


**FIGURE 7 SCATTERPLOTS SHOWING CELL CONCENTRATIONS (CELLS/ML) AT TWO DEPTHS (2 M AND 10 M) ACROSS FOUR STATIONS ON THE DAMARISCOTTA RIVER (BLOS 1, BLUE; BLOS 2, RED; BLOS 3, GREEN; BLOS 4, PURPLE) FOR TWO CRUISE DATES: A) 0CT. 4, 2016 AND B) OCT. 19, 2016.**

FlowCam analysis showed that there was a spike in *Pseudo-nitzschia* density during Cruise 1 (Sept. 8, 2016) with the highest biomass upriver at BLOS1 ($3.35 \times 10^3$ pg C $ml^{-1}$) and decreasing biomass at subsequent stations. *Pseudo-nitzschia* biomass decreased by Cruise 2 (Sept. 20, 2016), with

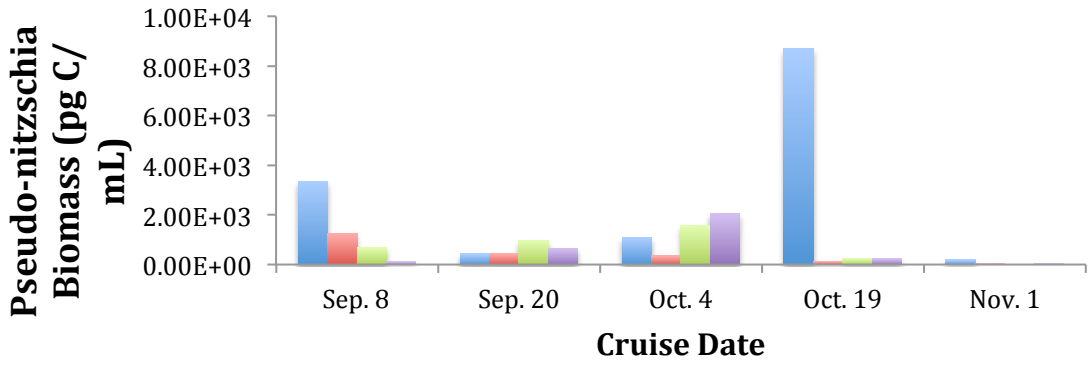


**FIGURE 8 A BAR CHART SHOWING *PSEUDO-NITZSCHIA* BIOMASS (PG C $ML^{-1}$) CALCULATED FROM FLOWCAM DATA FOR SURFACE SAMPLES COLLECTED FROM FOUR STATIONS ALONG THE DAMARISCOTTA RIVER (BLOS 1, BLUE; BLOS 2, RED; BLOS 3, GREEN; BLOS 4, PURPLE) ACROSS FIVE CRUISE DATES.**

the highest biomass at BLOS 3 ($9.69 \times 10^2$ pg C $ml^{-1}$). By Cruise 3 (Oct. 4, 2016), the start of the bloom period, *Pseudo-nitzschia* biomass began to rise again, especially offshore at BLOS3 ($1.58 \times 10^3$ pg C $ml^{-1}$) and BLOS4 ($2.06 \times 10^3$ pg C $ml^{-1}$). By Cruise 4 (Oct. 19, 2016), *Pseudo-nitzschia* biomass decreased at all stations except BLOS1, which saw a dramatic spike ($8.72 \times 10^2$ pg C $ml^{-1}$). By Cruise 5 (Nov. 1, 2016), all stations had the lowest *Pseudo-nitzschia* biomass of all cruises, with the highest biomass at BLOS1 ($1.94 \times 10^2$ pg C $ml^{-1}$).

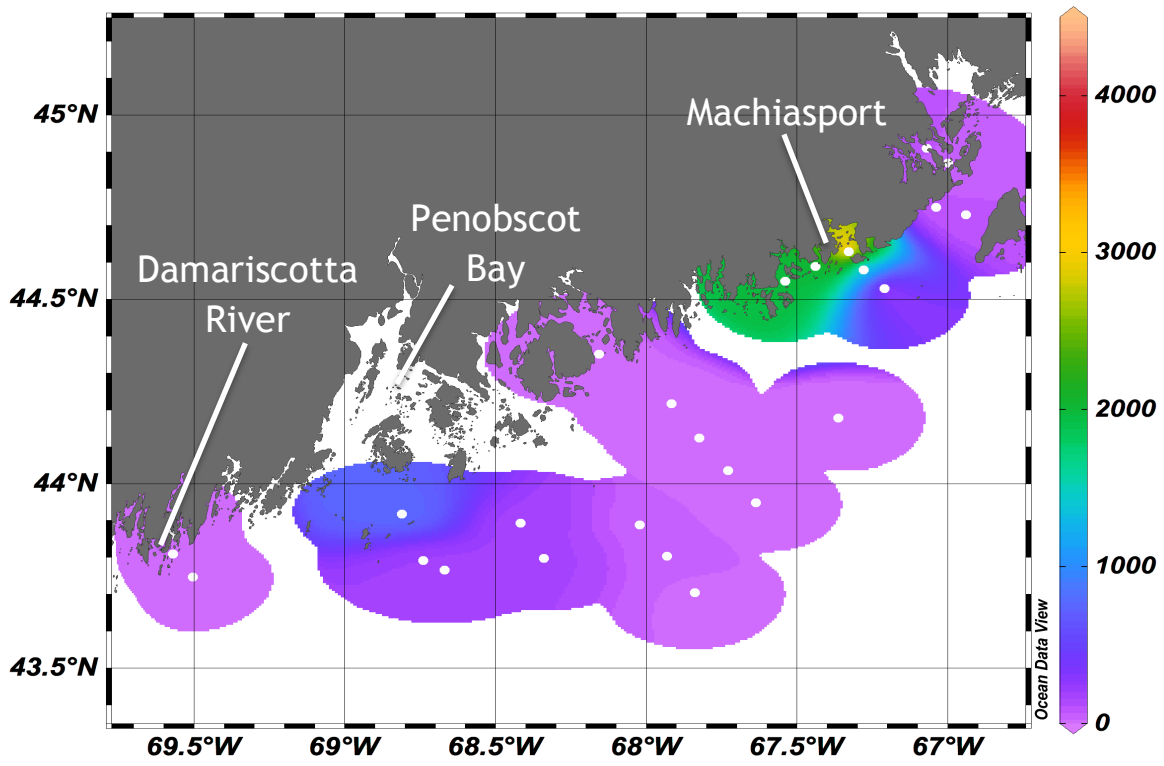


FIGURE 9 A MAP SHOWING CELL CONCENTRATIONS (CELLS $ML^{-1}$) AT 2M DEPTH, DETERMINED BY CALIBRATING GENE COPY NUMBER TO CELL CONCENTRATIONS.

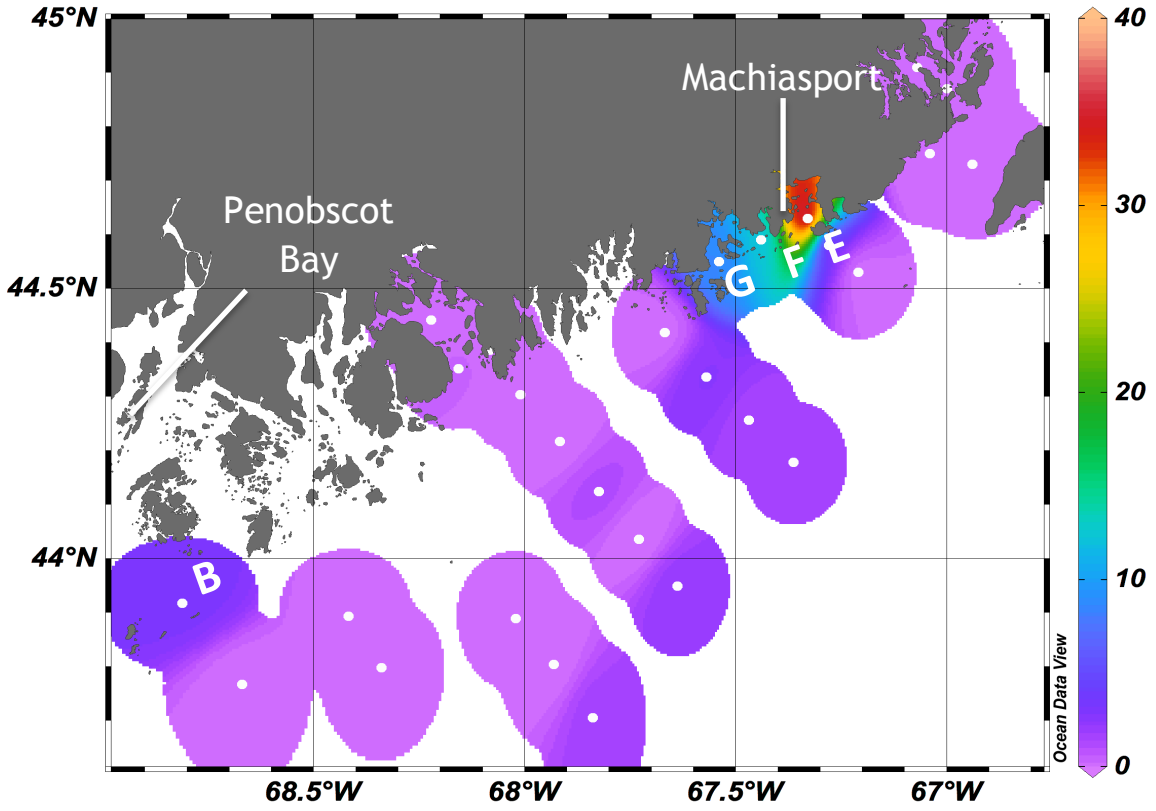


FIGURE 10 A MAP SHOWING PARTICULATE DA CONCENTRATIONS (NG $ML^{-1}$) AT 2M DEPTH.

### 5.2.B.ii Bloom samples

Cell density samples from the bloom region were orders of magnitude higher than those from the Damariscotta River. The highest cell concentrations were found in Machias Bay (Stations E, F and G) and Penobscot Bay (Station B). At 2 m depth, the highest cell concentrations were at Station E (2871 cells $ml^{-1}$), G (1885 cells $ml^{-1}$), F (1755 cells $ml^{-1}$) and B (946 cells $ml^{-1}$) (Figure 9). At 10 m depth, the highest concentrations were found at Station E (4262 cells $ml^{-1}$), F (2145 cells $ml^{-1}$), G (2056 cells $ml^{-1}$) and B (946 cells $ml^{-1}$) (not shown). The lowest cell concentrations were found near Grand Manan Island (Stations 6, 7, 8 and 9). At 2 m depth, the lowest cell concentrations were found at Station 9 (1 cell $ml^{-1}$), 6 (5 cells $ml^{-1}$), 8 (11 cells $ml^{-1}$) and 7 (13 cells $ml^{-1}$). At 10 m, depth the lowest concentrations were found at Station 31 (53 cells $ml^{-1}$), 34 (53 cells $ml^{-1}$), 35 (80 cells $ml^{-1}$) and 36 (105 cells $ml^{-1}$).

## *5.2.C TOXIN ANALYISIS*

### 5.2.C.i Damariscotta River samples

LC-QQQ-MS analysis showed that two samples collected during Cruise 3 of the Colby Changing Oceans Semester reported the presence of domoic acid (DA) at levels equal to or less than 0.1 ng $ml^{-1}$, which is the detection limit of the instrument. Additional samples were not analyzed based on these baseline detections.

### 5.2.C.ii Bloom samples

At 2 m, 20 of 28 stations were positive for particulate DA (e.g. plankton biomass that contained the toxin). At 10 m depth, 9 of 14 stations were positive for particulate DA. The highest DA concentrations at 2 m were found at Station E (37.5 ng $ml^{-1}$), F (12.2 ng $ml^{-1}$), G (8.7 ng $ml^{-1}$) and B (3.074 ng $ml^{-1}$) (Figure 10) and the highest concentrations at 10 m were found at Station E (25.0 ng $ml^{-1}$), G (11.4 ng $ml^{-1}$), F (10.9 ng $ml^{-1}$) and B (5.8 ng $ml^{-1}$) (not shown). The record high DA value was determined for a sample that was part of a previous collection event.

## *5.2.D NUTRIENT ANALYSIS*

### 5.2.D.i Damariscotta River Samples

### 5.2.D.ii Bloom samples

Macronutrient (Nitrate/nitrite, phosphate and silicate) concentrations were lowest around Stations 1, 8, 9 and B near Penobscot Bay and offshore of Machias Bay (Appendix H). They were highest off of Mount Desert Island and offshore. Linear regression testing the relationship between nutrients concentrations and cell densities showed significant negative correlations between nitrogen concentrations and cell densities (slope = -0.0176; F-statistic = 4.4; 0.04822) and between silica concentrations and cell densities (slope = -0.2419; F-statistic = 5.638; p-value = 0.02719) (Table 1). In contrast, linear regression examining the relationship between nutrient concentrations and DA concentrations found no significant correlations (Table 2).

**TABLE 2 RESULTS FOR LINEAR REGRESSION WITH N, P AND SI CONCENTRATIONS (µM) AS INDIVIDUAL EXPLANATORY VARIABLES AND CELL DENSITY AS THE RESPONSE VARIABLE**

| Predictor variable | Slope | r | F-statistic | p-value |
|---|---|---|---|---|
| **N** | -0.0176 | 0.42 | 4.4 | 0.04822 |
| **P** | -2.5878 | 0.33 | 2.605 | 0.1252 |
| **Si** | -0.2419 | 0.46 | 5.638 | 0.02719 |

**TABLE 1 RESULTS FOR LINEAR REGRESSION WITH N, P AND SI CONCENTRATIONS (µM) AS INDIVIDUAL EXPLANATORY VARIABLES AND DOMOIC ACID CONCENTRATION AS THE RESPONSE VARIABLE.**

| Predictor variable | Slope | r | F-statistic | p-value |
|---|---|---|---|---|
| **N** | -0.0028 | >0.01 | 0.001416 | 0.9703 |
| **P** | 0.53109 | >0.01 | 0.1478 | 0.7045 |
| **Si** | -0.04166 | >0.01 | 0.2001 | 0.6592 |

### *5.2.D.ii.a Comparison to Redfield Ratio*

According to the Redfield Ratio, the ratio of nitrogen to phosphorous (N:P) in plankton biomass and dissolved in the ocean should be approximately 16:1, (Redfield, 1934; Ptacnik et al, 2010; Weber &

Deutsch, 2010). Dissolved inorganic nitrogen samples from the bloom region showed N:P ratios as low as 0.88 and as high as 9.73, suggesting that N is lower than the suggested ratio at all stations and indicating depletion of nutrients from surface waters. The ratio of nitrogen to silica (N:Si) should be 16:15, or 1.067, but ratios at all stations were higher than 1.067, ranging from 1.141, suggesting silica limitation, to 2.433, also suggesting silica limitation. The ratio of silica to phosphorous (Si:P) is defined in the Redfield Ratio as 15:1, or 15 (Brzezinski, 1985; Ptacnik et al., 2010). Ratios were found between 0.97 and 13.96, suggesting that Si is lower relative to P than defined by the Redfield Ratio, and possibly limiting relative to P at all stations.

*5.2.D.ii.b Comparison of nutrient ratios 'in-bloom' and 'out-of-bloom'*

At a toxic bloom threshold of 5 ng $ml^{-1}$ DA, the 'in-bloom' and 'out-of-bloom' groups are significantly different from each other for ratios Si:P and N:Si, but not significantly different for P:N, according to a paired t-test (Si:P: t = -4.815, df = 28.306, p-value = 4.485e-05; N:Si: t = 5.3347; df = 13.329; p-value = 1.25e-04; P:N: t = -2.0288, df = 31.258, p-value = 0.051; Figure 11). For the ratio Si:P, both the 'in-bloom' and 'out-of-bloom' groups were significantly less than the Redfield Ratio value of 15 according to a one-sided, one-sample t-test (in-bloom: alternative = "less"; t = -

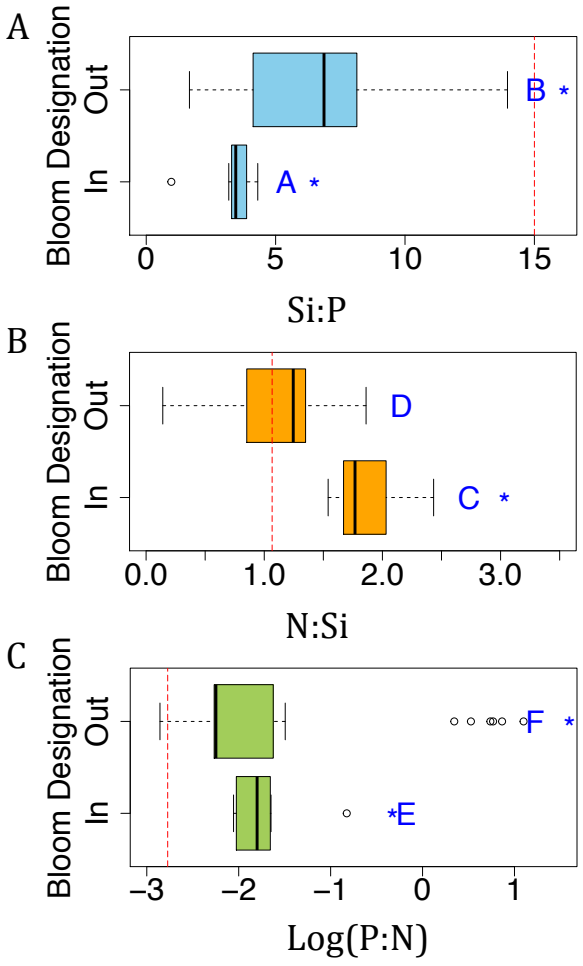


**FIGURE 11 BOXPLOTS SHOWING THE DISTRIBUTION OF NUTRIENT RATIOS SI:P (A), N:SI (B) AND P:N (C) FOR OBSERVATIONS 'IN-BLOOM' ("IN") AND 'OUT-OF-BLOOM' ("OUT"), BASED ON A PARTICULATE DA THRESHOLD CONCENTRATION OF 5 NG $ML^{-1}$. EACH BOXPLOT ALSO INCLUDES THE HYPOTHETICAL VALUE FOR THE GIVEN NUTRIENT RATIO, ACCORDING TO REDFIELD (DASHED RED LINE; REDFIELD, 1958; BRZEZINSKI, 1982), WHETHER EACH GROUP OF OBSERVATIONS IS SIGNIFICANTLY DIFFERENT FROM THE VALUE DEFINED BY REDFIELD (ASTERISK) AND WHETHER 'IN-BLOOM' AND 'OUT-OF-BLOOM' GROUPS ARE SIGNIFICANTLY DIFFERENT FROM EACH OTHER (LETTERS).**

27.988; df = 6, p-value = 6.88e-08; out-of-bloom: alternative = “less”, t = -14.462; df = 28; p-value = 8.965e-03; Figure 11A). For N:Si, the ‘in-bloom’ group was significantly greater than the theoretical value, 1.0667 as defined by the Redfield Ratio (Brzezinski, 1985), according to a one-sided, one-sample t-test (t = 6.3862; df = 6; p-value = 3.467e-4; Figure 11B). For the ratio P:N, both the ‘in-bloom’ and ‘out-of-bloom’ groups were si gnificantly greater than the theoretical value, 0.0625, as defined by the Redfield Ratio (Redfield, 1934; Redfield, 1958), according to a one-sided, one-sample t-test (in-bloom: t = 3.2291; df = 6; p-value – 3.965e-03; out-of-bloom: t = 2.9379, df = 28, p-value = 3.273e-03; Figure 11C).

## 5.3 DISCUSSION OF BLOOM ANALYSIS

### *5.3.A IDENTIFICATION*

#### 5.3.A.i Damariscotta River Samples

All seven of the samples that were successfully isolated from the Damariscotta River and sequenced were identified as *Pseudo-nitzschia pungens*. This identification supports the relative non-toxic nature of *P. pungens*, in that toxin samples collected from the Damariscotta River for this study reported concentrations of DA of ≤0.1 ng $ml^{-1}$. The detection of low levels of DA suggests that there was either another species of *Pseudo-nitzschia* present in the Damariscotta River at the time of sampling, contributing to these low toxin concentrations, or that *Pseudo-nitzschia pungens* was responsible for producing some toxin in the river during this period.

The *Pseudo-nitzschia* present in the Damariscotta River persisted at concentrations that were orders of magnitude lower than those present at the center of the bloom. These low cell abundances contributed to lower toxin concentrations in the Damariscotta River compared to the bloom region. Concentrations in the estuary did increase during the bloom period, suggesting that factors contributing to higher concentrations at the bloom site were also affecting the population in the Damariscotta River. It is possible that if concentrations of *P. pungens* reached higher numbers in the estuary, they would contribute

to substantially higher concentrations of DA. At the time of this study, the Damariscotta River has not experienced closures due to ASP in shellfish, but it is clear that the potential for this scenario exists.

### 5.3.A.ii Bloom samples

#### *5.3.A.ii.a Live sample collection methods*

Due to the degraded condition of samples that were received from the site of the bloom, cells could not be grown in culture or amplified for DNA sequencing. It would be advantageous to culture the species present at the bloom because it would be valuable to conduct toxin-induction experiments on the same species that contributed most to the bloom. To do this successfully, collected samples should be stored for the period of transportation in a cooler filled with water from the location of sample collection so that it sits at ambient temperature. Samples should also be allowed oxygen and should not be tightly sealed for long periods of time. After transportation, samples should be stored at approximately the temperature and light levels of collection (~100 $\mu E\ m^{-2}\ s^{-1}$). Additionally, cells should also be isolated as soon as possible to prevent grazing down of the phytoplankton population by microzooplankton.

#### *5.3.A.ii.b Sample identification*

As noted above, cells from environmental samples were imaged using Scanning Electron Microscopy (SEM) and sent my samples to Carmelo Tomas at UNC Wilmington for identification. Tomas, as well as a colleague in California, identified some of the cells as *Pseudo-nitzschia australis*. This identification happened concurrently with the identification of *Pseudo-nitzschia australis* in a bloom sample by the team from the Woods Hole Oceanographic Institution via DNA sequencing, ARISA and SEM analysis. This finding is especially notable because *Pseudo-nitzschia australis* has never before been detected in the Gulf of Maine. Its appearance coincides with the onset of the first major DA toxin event in the history of the Gulf of Maine. The Gulf of Maine previously experienced high-concentrations of *Pseudo-nitzschia*, suggesting *P. australis* is the species responsible for the presence of domoic acid in the region. However, there were not molecular, toxin and abundance data collected in association with previous bloom events. As discussed above, other studies have shown a species succession during

*Pseudo-nitzschia* blooms that move from non-toxin or low-toxin species like *P. pungens* moving to *P. australis*, leading to toxic blooms. The fall 2016 Gulf of Maine bloom follows a similar successional pattern.

### *5.3.B POPULATION DENSITIES*

#### 5.3.B.i Damariscotta River population densities

The data collected from the Damariscotta River for qPCR analysis on Cruises 3 (Oct. 4, 2016) and 4 (Oct. 19, 2016) as part of the Colby Changing Oceans Semester Program bracketed the peak of the fall 2016 bloom period. The toxic bloom began during late September of 2016 and ended around November 7, 2016, when the Department of Marine Resources (DMR) officially ended closures on shellfish harvesting (McGuire, 2016).

While the bloom may not have directly affected the physical, biological and chemical dynamics of the estuary, it is important that we take note of any changes during the bloom period, as the Damariscotta River accounts for over 80% of the Maine shellfish industry, and would suffer economically and ecologically if impacted by a toxic bloom.

The cruise data show that during Cruise 3, the peak of the toxic bloom, the estuary experienced high *Pseudo-nitzschia* concentrations, although the *Pseudo-nitzschia* present in the estuary were a different species (*P. pungens*) relative to those found at the site of the bloom (*P. australis*). This suggests that the estuary may have been experiencing similar environmental factors, such as changes in temperature and nutrient runoff, to those that may have triggered the toxic bloom.

By Cruise 4, cell concentrations had decreased across all stations, except for BLOS1, the station farthest upriver of the area surveyed. QPCR and FlowCam data from Cruise 4 show a spike in *Pseudo-nitzschia* present at BLOS1. This spike in *Pseudo-nitzschia* may have appeared to be an anomalous data point, however the fact that the spike shows up in both FlowCam and qPCR data offers additional confidence in this observation.

The spike may be a result of the physical characteristics of the river, which has a deep reservoir (38m) at BLOS1 compared to the shallow area at BLOS2 (20m). It is possible that BLOS1 has a higher residence time than the other stations surveyed, so that cells were trapped upriver while cells at other stations had dispersed. Another study showed that the upriver region of the Damariscotta River does have a longer residence time for phytoplankton than the area closer to the mouth of the river, but their study referred to

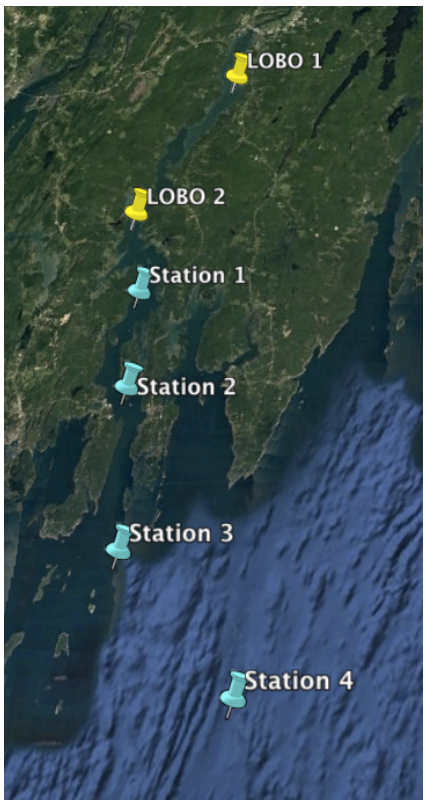


**FIGURE 13 A MAP SHOWING THE LOCATION OF LOBO BUOYS 1 AND 2 IN ADDITION TO SAMPLING LOCATIONS ("STATIONS 1-4" REFER TO BLOS 1-BLOS 4; COLBY CHANGING OCEANS SEMESTER PROGRAM, FINAL PRESENTATION)**

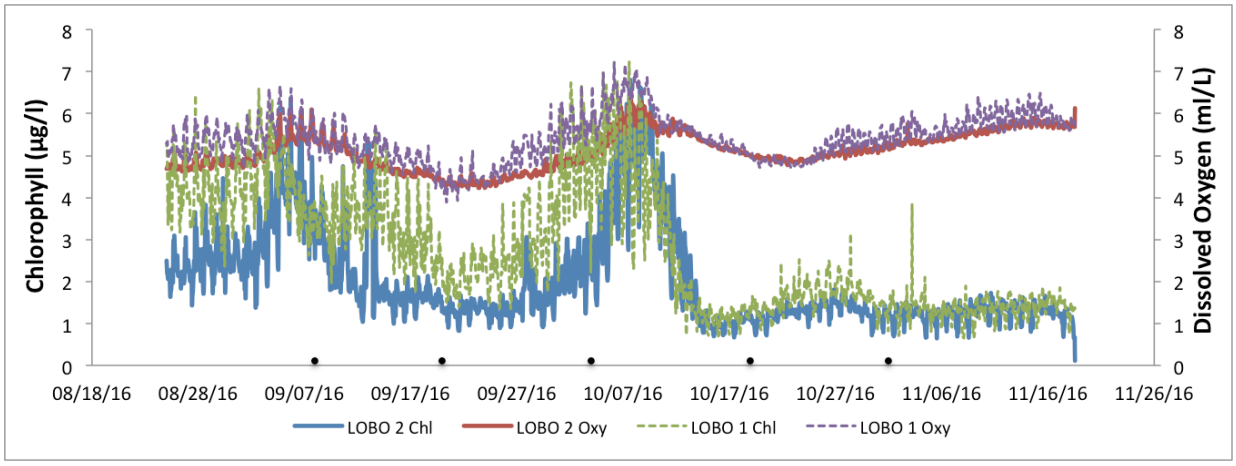


**FIGURE 12 CHLOROPHYLL *A* (µG $L^{-1}$) AND DISSOLVED OXYGEN (ML $L^{-1}$) FOR LOBO 1 AND LOBO2 OVER TIME. BLACK DOTS INDICATE COLBY CHANGING OCEANS SEMESTER PROGRAMS CRUISE DATES.**

sections of the river farther north than BLOS1 (Thompson et al., 2006).

Buoy data from the up-river region offers additional insight into the primary productivity in the estuary. During the fall of 2016, two Land/Ocean Biogeochemical Observatory (LOBO) buoys were installed at points upriver of BLOS1-4 (Figure 12). Among other measurements, these buoy records continuous data for chlorophyll-a concentrations in the river. Data ranging from late-August to mid-November, 2016 show evidence of an early September bloom, followed by a second bloom in early to mid-October, coinciding with the toxic GOM bloom (Figure 13). The buoy data do not show a spike in chlorophyll *a* at Cruise 4 to match the spike shown in the cruise data, suggesting that the uptick in *Pseudo-nitzschia* concentrations may have been unique to the area surrounding BLOS1.

### 5.3.B.ii Bloom region population densities

Highest cell densities occurred at the origin of the bloom, as reported by the Department of Marine Resources (DMR), in and around Machias Bay. It was determined that high cell concentrations correlated significantly with toxin concentrations, suggesting that if there were more species present during the bloom than the one identified (*P. australis*), they were also toxin producers. The alternative is that areas with high cell concentration and low toxin concentration might have been observed. In general, cell concentrations were higher at a depth of 10 m than at 2 m, suggesting that conditions (e.g. light and nutrients) may have been preferable to *Pseudo-nitzschia* at these depths.

## *5.3.C TOXIN CONCENTRATIONS*

### 5.3.C.i Damariscotta River samples

Liquid chromatography triple-quadrupole mass spectrometry (LC-QQQ-MS; Bigelow Laboratory for Ocean Sciences) was used to analyze the toxin concentrations of two samples collected from the research cruises on the Damariscotta River. Both samples were positive for DA, but for values equal to or less than 0.1 ng $ml^{-1}$. Despite increased concentrations of *Pseudo-nitzschia pungens* cells, toxin concentrations were still low. It is worth noting though, that DA was detected in the estuary, as *P. pungens* is not known to consistently produce DA in environmental settings. Low toxin concentrations

could be a result of the relatively non-toxic nature of *P. pungens*, or it could be a result of cell concentrations 2-3 orders of magnitude lower than those at the site of the bloom.

### 5.3.C.ii Bloom samples

*Relationship between individual nutrients and toxin production*

As discussed above, it was found that areas of high cell concentration coincided with areas of high toxin concentration in the bloom samples. This is consistent with expectations because cells must be present for DA production. Significant negative correlations between nitrogen concentrations and cell concentrations (slope = -0.176; F-statistic = 4.4; p-value = 0.048) and between silica concentrations and cell concentrations (slope = -0.242; F-statistic = 5.638; p-value = 0.02719) suggest that highest cell concentrations are correlated with low nutrient concentrations. This negative correlation may be counterintuitive, in that cells require nutrients for growth, but it suggests that at this point in the bloom, high cell concentrations had depleted most of the nutrients in the region. The lack of significant negative correlation between cell concentrations and phosphorous concentrations could suggest that phosphorous was not as thoroughly depleted by *Pseudo-nitzschia*, or it could be the product of confounding due to colinearity of explanatory variables (nitrogen, phosphorous and silica concentrations; Appendix A).

A lack of significance correlating DA concentrations to any individual nutrient concentrations in samples with DA concentration > 0.01 ng $ml^{-1}$ could suggest that nutrients are not correlated to toxin concentrations, but, more likely, this is the result of confounding due to colinearity of explanatory variables.

#### *5.3.C.ii.a Nutrient stoichiometry and toxin production*

The Redfield Ratio estimates the average nutrient composition of phytoplankton biomass in the ocean and offers a standard theoretical value to compare empirical environmental data to (Redfield 1934; Redfield 1958; Brzezinski, 1985; Ptacnik et a., 2010). As it is unrealistic to assume a universal ocean biogeochemistry, certain areas, such as coastal systems, often diverge from the Redfield Ratio due to

higher nutrient inputs from runoff and differences in primary productivity compared to offshore areas. Despite this, the Redfield ratio can be a useful tool for analyzing environmental nutrient concentrations.

Marine and terrestrial systems are often N-limited, as nitrogen must be converted by nitrogen fixing organisms so that it is available in forms that other microorganisms are able to assimilate (Vitousek & Howarth, 1991; Elser et al., 2007). Vitousek & Howarth (1991) identify various factors that compound this nitrogen limitation, including (1) energetic constraints associated with nitrogen fixation compared to nitrate reduction and ammonia assimilation for nitrogen-fixing organisms (Gutschick, 1981; Ptacnik et al., 2010), (2) limitation of nitrogen fixing organisms due to low concentrations of other macronutrients and micronutrients (Doremus, 1982; Lenton et al., 2000) and (3) ecological or physical constraints or disturbances such as the disruption nitrogen-fixing communities and anoxic microzones through ocean turbulence (Paerl & Carlton, 1988). While some studies suggest that marine and coastal areas are nitrogen-poor and phosphorous rich (Howarth 1988; Vitousek & Howarth, 1991), other studies show evidence of synergistic limitation of nitrogen and phosphorous (Elser et al., 2007). Coastal systems tend to have high levels of phosphorous due to nutrient runoff from surrounding environmental systems. My analysis aligns with this hypothesis, suggesting that there was excess phosphorous at all sites, relative to nitrogen and silica according to the Redfield Ratio (Redfield, 1934; Redfield, 1958; Brzezinski et al., 1985; Ptacnik et al,. 2010).

Nutrient stoichiometry analysis offered evidence showing that nutrient concentrations did play a role in toxin production. Si:P ratios were significantly less than the Redfield ratio (Si:P = 15; Brzezinski et al., 1985) for both the ‘in-bloom’ and ‘out-of-bloom’ groups (Figure 11A), which suggests silica drawdown was occurring due to high concentrations of *Pseudo-nitzschia* and other diatoms during the bloom. The Si:P ratio was significantly lower in the ‘in-bloom’ group than in the ‘out-of-bloom’ group (Figure 11A), showing even lower concentrations of Si relative to P in areas most affected by the bloom. This acts as evidence for silica limitation driving toxin production in the bloom region.

N:Si ratios in the 'in-bloom' group were significantly higher than the Redfield ratio (N:Si = 1.067; Brzezinski et al., 1985) and significantly different from the 'out-of-bloom' group, while N:Si ratios in the 'out-of-bloom' group were not significantly different from the Redfield ratio (Figure 11B). This further supports the theory that silica limitation was driving the bloom because silica was lower relative to nitrogen in areas affected by the bloom, but not in areas designated outside of the bloom region.

Finally, P:N ratios both 'in-bloom' and 'out-of-bloom' were significantly greater than the Redfield ratio (P:N = 0.0625; Redfield, 1934), but the groups were not significantly different from each other. This suggests that both groups were N-limited, relative to P. This N-limitation occurred across all stations sampled, so N-limitation probably was not the primary factor driving toxin production. Instead, evidence points to Si-limitation as the primary driver of toxin production during the fall 2016 bloom.

# 6. NUTRIENT LIMITATION EXPERIMENTS

## 6.1 METHODS FOR NUTRIENT LIMITATION

### *6.1.A EXPERIMENT 1: LIMITED SILICATE AND PHOSPHATE*

Studies have shown that macronutrient limitation can be a trigger for toxin production in *Pseudo-nitzschia* (Bates et al., 2006; Trainer et al., 2012; Lesser et al., 2016), and our data from the 2016 bloom support that hypothesis. To attempt to replicate these conditions in a laboratory setting, three nutrient limitation experiments were conducted, focusing on the limitation of phosphate and silica, following the methods from a similar experiment conducted in a previous study (Fehling et al., 2014).

In the first iteration of the experiment, three replicates of *Pseudo-nitzschia pungens,* isolated from samples collected from the East Boothbay Dock on the Damariscotta River (East Boothbay, ME) were grown in culture until they reached high densities then transferred into 1 liter polycarbonate bottles, filled with L1 phytoplankton growth medium (Guillard and Hargraves, 1993). Once in the larger, 1 liter bottles, the cultures were allowed to grow until they reached exponential growth phase (1-2 weeks). At this point,

75 ml of each culture was transferred into one of three nutrient conditions: (1A) replete L1 medium, (1B) L1 medium without added phosphate or silicate or (1C) L1 medium without phosphate, silicate or nitrate, using urea to replace nitrate as the nitrogen source available in the growth medium (Table 3). Each of these conditions was replicated in triplicate. The bottles were place in an incubator (18 ˚C; 100 µE $m^{-2}s^{-1}$) and allowed to grow for 11 days.

Samples were collected periodically for chlorophyll *a*, domoic acid (DA) and nutrient analysis. Chlorophyll *a* measurements were used as a proxy for cell concentration throughout the experiment. Samples were measured daily, *in-vivo* using a spectrophotometer. *In-vivo* measurements were more efficient than full extractions and provided sufficient information on relative cell abundance for the purpose of the experiment.

Nutrient samples were collected at days 0, 3, 5 and 8, filtering 60 ml of culture through 0.2 µm, 25 mm Whatman® Nuclepore filters into brown plastic HDPE nutrient bottles and stored at -20˚C until analysis for phosphate, silica and nitrate+nitrite concentrations using a Seal nutrient autoanalyzer

**TABLE 3 NUTRIENT TREATMENTS FOR ALL EXPERIMENTAL CONDITIONS. TREATMENTS 1A-C REFER TO TREATMENTS IN EXPERIMENT 1; 2A-B REFER TO TREATMENTS IN EXPERIMENT 2; 3A-B REFER TO TREATMENTS IN EXPERIMENT 3. "NITRATE" INDICATES NITRATE CONCENTRATIONS DEFINED FOR L1 MEDIUM; "+" INDICATES PHOSPHATE OR SILICATE CONCENTRATIONS DEFINED FOR L1 MEDIUM; "–" INDICATES THAT THE GIVEN NUTRIENT IS THEORETICALLY ABSENT FROM THE TREATMENT MEDIUM. IN PRACTICE, SOME "–" NUTRIENT CONCENTRATIONS WERE GREATER THAN 0 DUE TO NUTRIENT CARRYOVER FROM THE INITIAL TRANSFER INTO EXPERIMENTAL CONDITIONS.**

| Treatment | N | P | Si |
|---|---|---|---|
| **1A** | Nitrate | + | + |
| **1B** | Nitrate | – | – |
| **1C** | Urea | – | – |
| **2A** | Nitrate | + | + |
| **2B** | Nitrate | – | + |
| **3A** | Nitrate | + | + |
| **3B** | Nitrate | + | – |

(Bigelow Laboratory for Ocean Sciences, East Boothbay, ME).

Domoic acid (DA) samples were collected at days 0, 3, 5, 8 and 11. Samples were collected for both cellular and extracellular DA. Cellular DA samples were collected by filtering 100ml of each culture onto a 47 mm Whatman® glass filter, grade GF/F. Filters were rolled using forceps and stored in cryovials at -20˚C until analysis. Extracellular DA samples were collected by collecting the filtrate and storing it in polycarbonate bottles at -20 ˚C until analysis. Cellular DA samples were analyzed using an Agilent 6460 Triple Quadrupole LC/MS/MS with a detection limit of <0.1 ng (Bigelow Laboratory for Ocean Sciences, East Boothbay, ME). Extracellular DA samples were collected for the purpose of developing a method for extracellular DA analysis, but were not analyzed for the purpose of this study.

### *6.1.B EXPERIMENT 2: LIMITED PHOSPHATE*

In the second iteration of this experiment, certain aspects of the methods were modified to improve upon the first experiment. One culture of *Pseudo-nitzschia pungens*, isolated from the East Boothbay Dock, was grown in replete L1 medium and then transferred into two separate nutrient conditions: (2A) replete L1 medium and (2b) L1 medium without phosphate (Table 3) In the first experiment, excess nutrients were carried over from the initial culture to the nutrient-limited treatment so that phosphate was not drawn down completely by the end of the experiment. To improve upon this procedure in our second iteration, smaller and more concentrated aliquots of the initial culture were transferred into the experimental cultures. The cultures were allowed to grow for eight days, sampled daily for Chlorophyll *a*, sampled for nutrients on days 0, 3, 5 and 7 and sampled for cellular DA on the final day of the experiment. Instead of using a Seal nutrient autoanalyzer, phosphate concentrations were determined colorimetrically, using a Beckmen Coulter DU720 spectrophotometer. Limitations on time prevented full DA analysis, so samples were analyzed using the Scotia Rapid Test for Amnesic Shellfish Poisoning according to the protocol for algae samples with a detection limit of 50 ng/ml (Scotia). This detection limit was much higher would be expected for the cultured samples, so it was not an appropriate method to use for this study.

### 6.1.C EXPERIMENT 3: LIMITED SILICATE

In the third iteration of this experiment, two *Pseudo-nitzschia pungens* cultures were used with the aim of determining whether strains of P. pungens from two different regions had differing potentials for DA production. One culture was isolated from the Bigelow Laboratory dock and the second was isolated from the San Pedro Channel off the coast of Southern California by Dr. Avery Tatters of USC. Phosphate carryover from the initial transfer was still too high in the second iteration of our experiment, so for the third iteration, silica was limited instead of phosphate, as silica is drawn down faster by the *Pseudo-nitzschia* cells. To reduce nutrient carryover during the initial transfer, an equal volume in six parts of each culture was poured over 40 µm mesh filters, then gently back-washed the *Pseudo-nitzschia* cells, rinsing with filtered seawater, from the filters into their respective experimental bottles, filled with the appropriate nutrient condition.

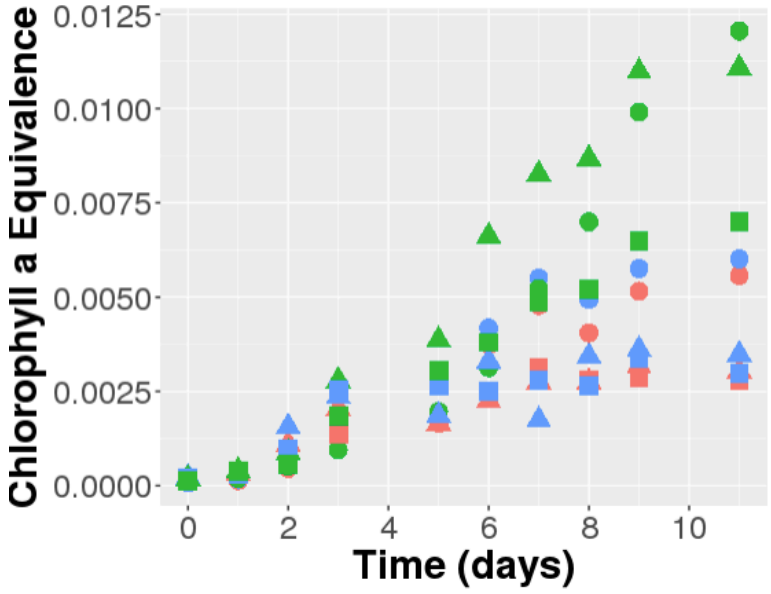


**FIGURE 14 A SCATTERPLOT SHOWING CHLOROPHYLL *A* EQUIVALENCE AS A PROXY FOR CELL GROWTH DURING EXPERIMENT 1. TREATMENT 1A IS SHOWN IN GREEN; TREATMENT 1B IS SHOWN IN RED; TREATMENT 1C IS SHOWN IN BLUE. SHAPES INDICATE *PSEUDO-NITZSCHIA PUNGENS* STRAIN USED IN THE EXPERIMENT (PN3 = CIRCLE; PN6 = TRIANGLE; PN7 = SQUARE).**

The nutrient conditions for the third experiment were triplicate cultures of (3A) replete L1 medium and (3B) L1 medium without silica (Table 3). The cultures were allowed to grow for nine days, sampling daily for Chlorophyll *a* and sampled on days 5 and 9 for nutrients and particulate DA. Silicate concentrations were determined colorimetrically using a Trilogy® Laboratory Fluorometer Silicate Snap-in Module by Turner Designs. DA was measured using liquid chromatography triple quadrupole mass spectrometry (LC-QQQ-MS).

## 6.2 NUTRIENT LIMITATION EXPERIMENT RESULTS

### *6.2.A EXPERIMENT 1: LIMITED SILICATE AND PHOSPHATE*

### 6.2.A.i Cell growth using chlorophyll a as a proxy

Each replicate in Treatment 1A (replete L1 medium) entered the exponential growth phase by day five and remained in exponential growth for the remainder of the experiment (Figure 14). PN3 showed a growth rate of 0.39 $d^{-1}$, PN6 showed a growth rate of 0.40 $d^{-1}$ and PN7 showed a growth rate of 0.26 $day^{-1}$. Replicates in Treatment 1B (L1 – P, Si) showed growth similar to that of Treatment 1A until day 5 and then plateaued (Figure 14). Replicates in Treatment 1C (L1 – P, Si, N; + Urea) largely reflected the growth of those in Treatment 1B, but showed marginally higher initial growth, prior to plateauing (Figure 14).

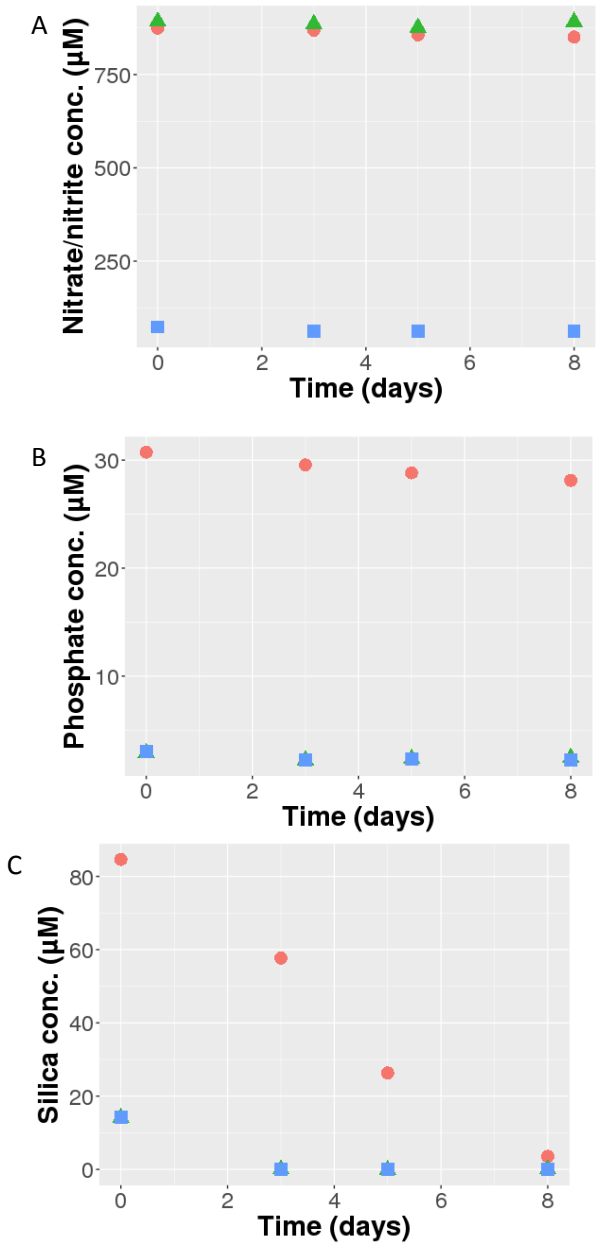


**FIGURE 15 A) NITRATE, B) PHOSPHATE AND C) SILCATE CONCENTRATIONS (µM) OVER THE COURSE OF EXPERIMENT 1. THE NUTRIENT MEASUREMENTS FOR EACH EXPERIMENTAL TREATMENT ARE SHOWN (TREATMENT 1A (REPLETE) = RED CIRCLE; TREATMENT 1B (P AND SI LIMITED) = GREEN TRIANGLE; TREATMENT 1C (UREA AMENDED) = BLUE SQUARE)**

### 6.2.A.ii Nutrient uptake

*Nitrate & Nitrite*

Nitrate and nitrite concentrations in Treatment 1A showed a steady decline, but remained abundant throughout the experiment, starting with a high of 874.48 µM on day 0 and ending with a low of 850.70 µM on day 8

(Figure 15A). In Treatment 1B, nitrate and nitrite fluctuated, starting with a high of 892.73 µM on day 0, decreasing to a low of 876.09 µM on day 5 and increasing again to 891.64 µM on day 8 (Figure 15A). Treatment 1C stayed relatively stable, showing a marginal decrease over the course of the experiment, with a high of 73.58 µM at day 0 and a low of 61.07 on day 8 (Figure 15A).

*Phosphate*

Phosphate was depleted more slowly than nitrate/nitrite. In Treatment 1A, phosphate declined from 30.73 µM on day 0 to 28.12 µM on day 8 (Figure 15B). In Treatment 1B, phosphate concentrations fluctuated, dropping from 2.92 µM on day 0 to 2.21 µM on day 3 and then increasing again to 2.46 by day 8 (Figure 15B). Treatment 1C showed a similar pattern, where phosphate began at a concentration of 3.05 µM, decreasing to 2.20 µM by day 3 and ending at a concentration of 2.22 µM on day 8 (Figure 15B).

*Silicate*

In Treatment 1A, silicate showed a rapid decrease, such that it was nearly depleted by the end of the experiment. Silicate concentrations began at 84.63 on day 0 and decreased to 3.55 µM by day 8 (Figure 15C). In Treatment 1B, silicate began at a concentration of 14.15 µM and was depleted by day 3 to a concentration of 0.02 µM (Figure 15C). Treatment 1C showed a similar pattern, beginning at 14.23 µM and decreasing to 0.01 µM by day 3 (Figure 15C).

### 6.2.A.iii Toxin Production

Liquid chromatography triple-quadrupole mass-spectometry (LC-QQQ-MS) did not detect domoic acid in any treatments (detection limit <0.1 ng $ml^{-1}$).

## *6.2.B Experiment 2: Limited phosphate*

### 6.2.B.i Cell growth determined using microscope counts

Under Treatment 2A, cell growth entered the exponential phase after day 3 and remained in the exponential growth phase for the remainder of the experiment (Figure 16A). The beginning of the

experiment, there were 1.48 x $10^3$ cells $ml^{-1}$ under Treatment 2A. By day 7, Treatment 2A had reached 3.19 x $10^4$ cells $ml^{-1}$. Treatment 2B started with 2.10 x $10^3$ cells $ml^{-1}$ and showed limited growth by day 3, reaching a low of 1.52 x $10^3$ cells $ml^{-1}$, then fluctuating and reaching a high of 2.98 x $10^3$ cells $ml^{-1}$ (Figure 16A).

### 6.2.B.ii Nutrient uptake

In Treatment 2A, phosphate showed a slow decrease from 28.273 µM on day 0 to 24.990 µM on day 7 (Figure 16B). In treatment 2B, phosphate showed a marginal increase over time from 2.364 µM on day 0 to 3.020 µM on day 7, but this increase may not be significant (Figure 16B).

### 6.2.B.iii Toxin production

The Scotia Rapid Test for Amnesic Shellfish Poisoning (SRT ASP) did not detect domoic acid in any treatments on day 7 of the experiment (detection limit: 50 ng $ml^{-1}$).

## *6.2.C Experiment 3: Limited silicate*

### 6.2.C.i Cell growth

Treatment 3A showed exponential growth beginning after day 3 and continuing through the end of the experiment. The PN2 replicates began with a cell concentration 3.47x higher than those of C9 and

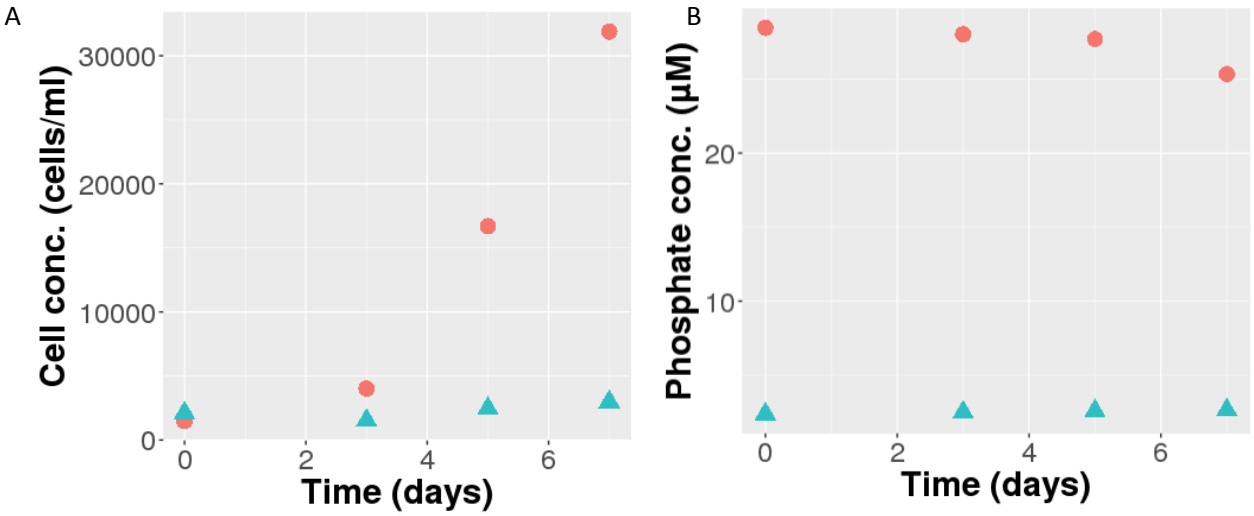


**FIGURE 16 A) CELL CONCENTRATIONS (CELLS $ML^{-1}$) AND B) PHOSPHATE CONCENTRATIONS (µM) OVER THE COURSE OF EXPERIMENT 2. THE NUTRIENT MEASUREMENTS FOR EACH EXPERIMENTAL TREATMENT ARE SHOWN (TREATMENT 2A (REPLETE) = RED CIRCLE; TREATMENT 2B (PHOSPHATE LIMITED) = BLUE TRIANGLE)**

showed earlier exponential growth than the C9 cultures. Despite this, the C9 cultures grew to nearly the same concentration as the PN2 cultures did, suggesting they may have had a higher growth rate. For treatment 3B, the PN2 cultures began to show evidence of slowed growth by day 3, reaching a plateau after that point. The C9 cultures exhibited a similar pattern at slightly lower concentrations.

### 6.2.C.ii Nutrients

In Treatment 3A, silicate showed a dramatic decrease from 75.48 µM on day 0 to >0.2 µM on day 9. In treatment 3B, silicate decreased from 2.121 µM on day 0 to >0.2 µM on day 9.

### 6.2.C.ii Toxins

LC-QQQ-MS did not detect domoic acid in any treatments on day 9 of the experiment for the three replicates tested (C9; detection limit = 0.1 ng $ml^{-1}$).

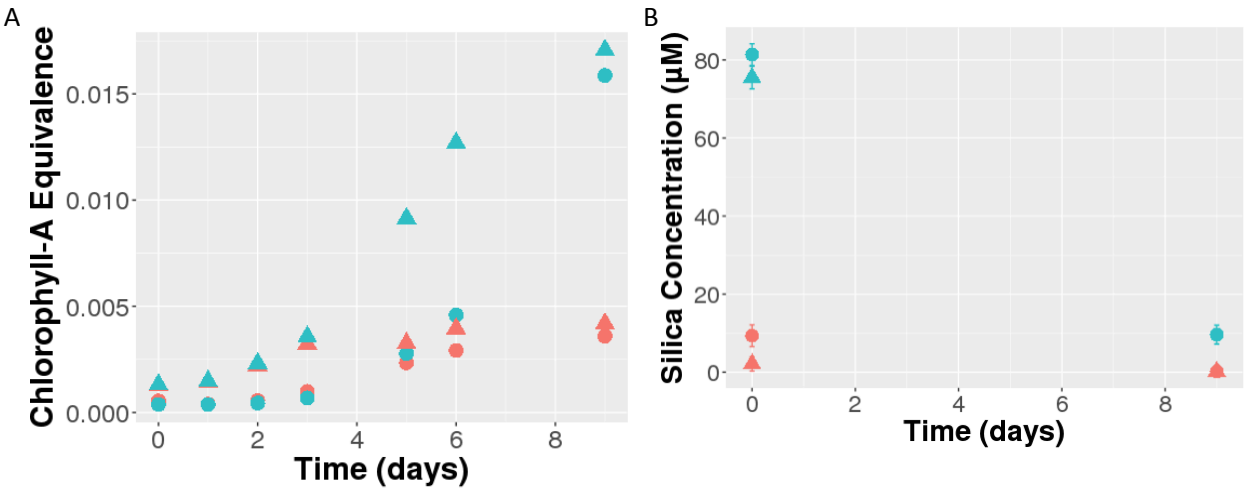


**FIGURE 17 A) CHLOROPHYLL *A* EQUIVALENCE AND B) SILICATE CONCENTRATIONS (µM) OVER THE COURSE OF EXPERIMENT 3. NUTRIENT MEASUREMENTS FOR EACH EXPERIMENTAL TREATMENT ARE SHOWN (TREATMENT 3A (REPLETE) = RED CIRCLE; TREATMENT 3B (SILICATE LIMITED) = BLUE TRIANGLE)**

## 6.3 DISCUSSION FOR NUTRIENT LIMITATION

Cell growth patterns aligned with expectations across all experiments. All cultures that were exposed to nutrient-replete conditions entered exponential growth and remained in the exponential growth phase through the end of the experiment. In cultures with limited nutrient conditions (limited P, limited Si or both), cell growth stagnated before reaching the exponential phase. Despite this, there was no evidence of toxin production in any cultures. This can be taken as evidence supporting the non-toxic nature of *P. pungens* compared to other known toxin producers such as *P. australis*.

### 6.3.A Experiment 1

#### *6.3.A.ii Nutrient uptake*

**Nitrogen uptake**

Experiment 1 was unique in that it included the substitution of urea as a nitrogen source under one experimental condition. Multiple studies have shown various results when using urea as the primary source of nitrogen for the growth of *Pseudo-nitzschia*. Some studies have shown that the use of urea in lieu of other nitrogen sources significantly increases growth rate but decreases toxin production (Auro & Cochlan, 2013). Others have shown the reverse effect; that the addition of urea significantly decreases growth rate but increases toxin production (Martin-Jézéquel et al., 2015). Still others have shown that the use of urea increases both growth rate and toxin production and that the effects vary between a lab and field setting (Armstrong et al., 2007).

The role of urea in cell growth and toxin production for *Pseudo-nitzschia* is not definitive. The results of this study are inconclusive with respect to the effect of urea on toxin production because no cultures produced detectible levels of toxin under any nutrient conditions. Increased cell growth rate was observed prior to nutrient depletion in limited cultures with urea in place of nitrate, but to determine whether these observations were significant it would have been useful to include a urea treatment that was

not limited by silicate or phosphate and to compare this treatment to the cultures growing in replete L1 media. Therefore, the effect of urea on cell growth in this experiment was inconclusive.

While, the effect of urea on cell growth was inconclusive, nutrient data did show evidence for preference of urea as a nitrogen source over nitrate (Figure 15A). Nitrate concentrations decreased steadily for all cultures in the Treatment 1A (L1 replete) and 1B (L1 –P, –Si), but the nitrate present in Treatment 1C (L1 –P, –Si, +urea) remained relatively constant throughout the experiment (Figure 15A). This nitrate was likely carried over from the initial inoculum, which had been growing in L1 media. The lack of depletion of nitrate over the course of the experiment suggests that urea may have acted as the preferred nitrogen source in this treatment. In agricultural regions like Maine, urea is a useful source of relatively inexpensive nitrogen, particularly for use on blueberry barrens. It is possible that runoff from agriculture in nearby regions contributes to nitrogen loading in Gulf of Maine coastal waters, fueling phytoplankton growth.

### Phosphate uptake

The nutrient data show that no cultures were phosphate limited by the end of the experiment. In Treatment 1A, phosphate concentrations decreased slowly but steadily during the exponential growth phase, which continued through the end of the experiment. In Treatments 1B and 1C, phosphate decreased for the first three days, while the cultures were still growing, until the cultures became silicate-limited after day 3 (Figure 15C). At this point, phosphate uptake halted at a concentration of 2.21 μM in treatment 1B and at a concentration of 2.20 μM in treatment 1C (Figure 15B).

Any phosphate present in Treatments 1B and 1C is the result of unintended carryover from the inoculum culture. In subsequent experiments transfer methods were modified in an attempt to reduce this nutrient carryover.

### Silicate uptake

*Pseudo-nitzschia* are diatoms and use silica to form their cell frustule, so they require high concentrations of silica compared to other macronutrients to sustain cell growth (Bates & Trainer, 2006). While there was some carryover of silicate from the inoculum, it did not preclude limitation on cell growth, as the excess silicate was rapidly depleted under all treatments (Figure 15C).

#### *6.3.A.iii Toxin production*

No toxin production was detected under any treatment in this experiment. This lack of toxin production is likely due to the low-toxin-producing nature of *Pseudo-nitzschia pungens* (Casteleyn et al., 2008). At the time of experimentation *P. pungens* was the only species available in culture. *P. pungens* it is not representative of the species and toxin producing potential of the species present in the fall 2016 bloom population in Downeast Maine. Future experimentation on the effects of nutrient limitation on *Pseudo-nitzschia* found in the Gulf of Maine should use cells isolated from the site of a toxic bloom, rather than those found at an unaffected location.

### *6.3.B EXPERIMENT 2*

#### 6.3.B.i Phosphate uptake

To ensure that phosphate drawdown was not halted as a result of silicate limitation, silicate was not limited in this experiment and began at 106 µM (L1 concentrations). A smaller inoculum was also used during the initial culture transfer into experimental bottles to reduce nutrient carryover. Despite these efforts, phosphate never reached below a concentration of 0.6 µM. Instead, it began at a concentration of 2.364 µM and increased marginally at each time point, reaching a final concentration of 3.020 µM at the end of the experiment. The lack of cell growth in Treatment 2B suggests that the cells were, in fact, nutrient limited at these phosphate concentrations, but it also meant that there were not enough cells present in the medium to deplete the phosphate further. Future experiments should begin with a higher initial cell concentration to increase nutrient drawdown efficiency.

#### 6.3.B.ii Toxin production

In Experiment 2, DA was not detected under any treatment. In this iteration, due to time constraints and materials available, a Scotia Rapid Test for Amnesic Shellfish Poisoning was used to test for the present of DA. The detection limit for this method is 50 ng $ml^{-1}$, which is much higher than that of the LC-QQQ-MS or ELISA methods. It would not have been sensitive enough to detect DA levels at the most toxic sites of the 2016 bloom, the highest of which was 37.5 ng $ml^{-1}$. The Scotia Rapid Test was not an appropriate method for toxin analysis for this experiment, but it is likely that there still was not DA present in any treatment. As in Experiment 1, the species used in the experiment was *P. pungens,* a low-toxin-producing species (Casteleyn et al., 2008).

### *6.3.C EXPERIMENT 3*

#### 6.3.C.i Silicate uptake

Average starting silica concentrations for Treatment 3B were lower but not significantly different from those of Treatments 1B and 1C ($t = 2.2263$, $p = 0.1344$; one-sided Welch two-sample t-test). Additional methods development trials are needed to determine whether the alternative transfer method, filtering cells over mesh, is effective in reducing nutrient carryover, compared to transferring a small volume using a disposable transfer pipette.

#### 6.3.C.ii Toxin production

Neither strain produced toxin over the duration of the experiment, reaffirming the low toxin-producing nature of *P. pungens,* regardless of geographic origin (Casteleyn et al., 2008).

# 7. CONCLUSION

This study worked to better understand the biological and chemical factors that affected the fall 2016 toxic bloom event in the Gulf of Maine. In this study, *Pseudo-nitzschia australis* was identified for the first time in the region and was likely responsible for the majority of toxin production during the bloom. A method was developed for estimating *Pseudo-nitzschia* cell concentrations from copy number

given by qPCR analysis, using a conversion factor of 79.4 ± 10 gene copies per cell of *Pseudo-nitzschia pungens.* This method can be expanded to determine the number of gene copies for other species of *Pseudo-nitzschia* or for other microorganisms whose genomes are not available in the literature.

This study also found that, when examining the role of macronutrient limitation in toxic bloom events, it is more salient to look at nutrient stoichiometry, in relation to the Redfield ratio or similar theoretical frameworks, than it is to look at the relationship between individual nutrients and toxin concentrations. Doing so with the data collected as part of the NOAA funded rapid response effort during the bloom offered evidence that silica limitation acted as a primary driver of bloom toxicity. Finally, this study reaffirmed the low-toxin-producing nature of *P. pungens*, through the conduction of three nutrient limitation experiments, none of which induced toxin production in *P. pungens* cultures.

Prior to 2016, the Gulf of Maine had never before experience a toxic bloom of *Pseudo-nitzschia*. In the time since, the region has experience two more toxic blooms with DA concentrations high enough to force closures on shellfish harvesting. The Maine Department of Marine Resources has taken action indicating that they are taking the threat of this new species seriously, such as changing the shellfish harvesting closure DA limit to >0 ppm in shellfish tissue (McGuire, 2017). In addition to changes in management practices, a proactive response to these toxic events should include regular sampling of previously affected harvest areas to understand how changing environmental conditions play a role in *Pseudo-nitzschia* bloom formation and toxin production.

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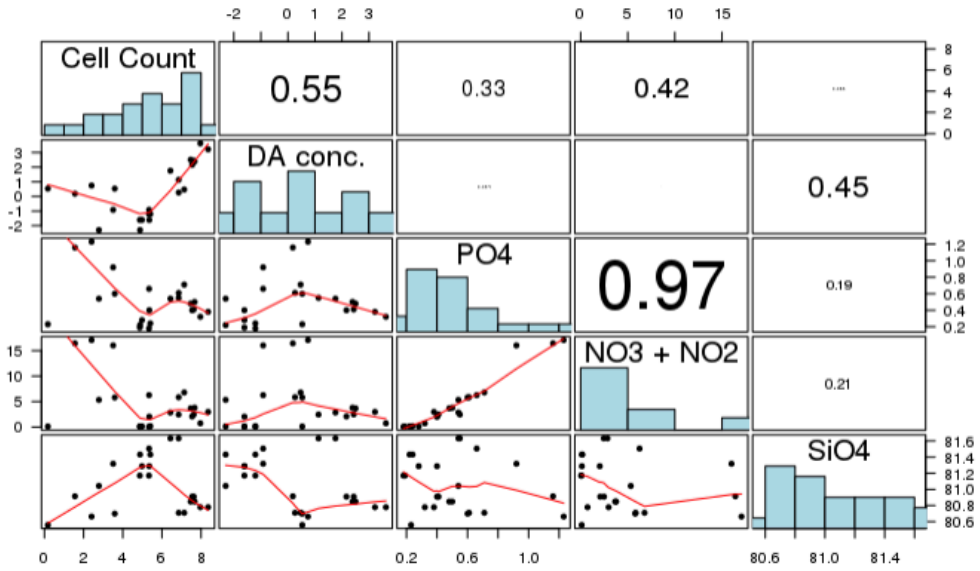
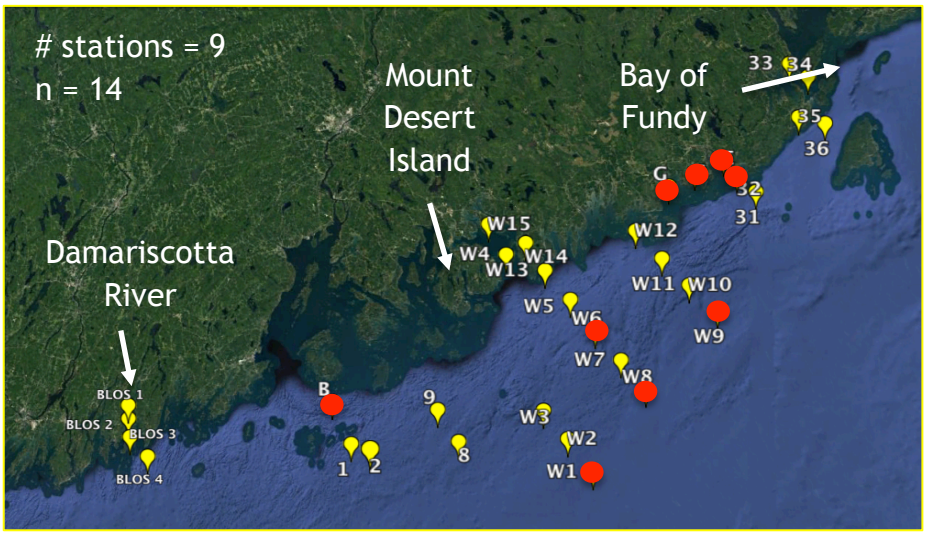
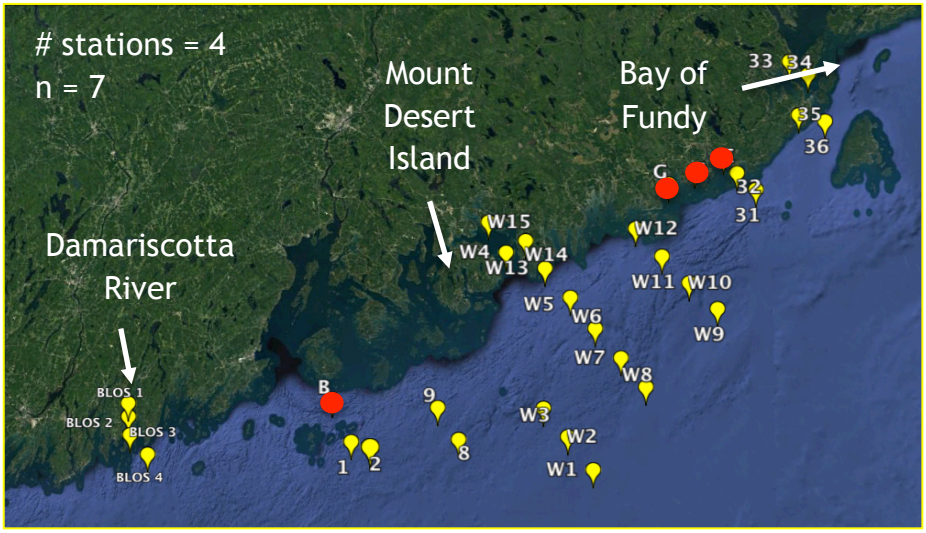
# 9. APPENDIX



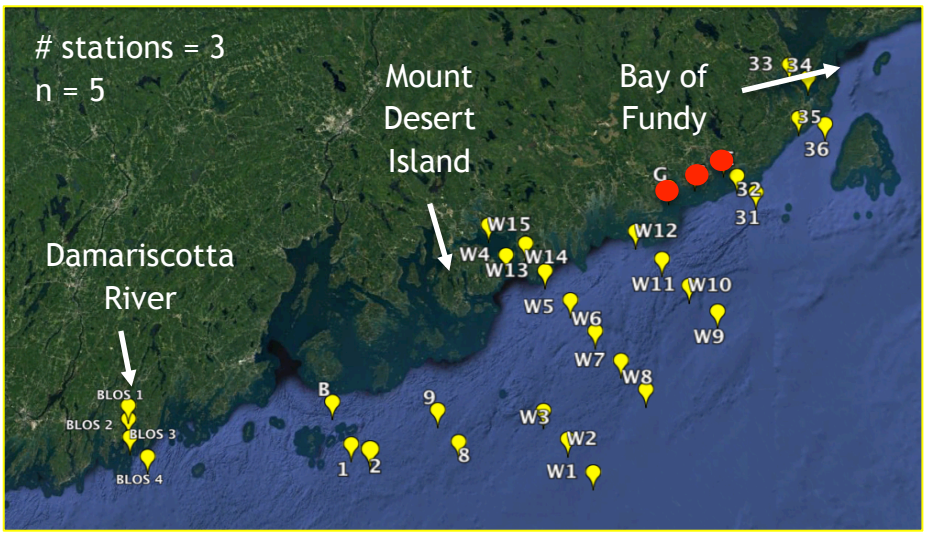

**APPENDIX A – A MATRIX OF FIGURES SHOWING PEARSON PRODUCT-MOMENT CORRELATIONS BETWEEN CELL DENSITY, DA CONCENTRATION, PHOSPHATE, NITRATE + NITRITE AND SILICATE FOR ALL BLOOM SAMPLES WITH DA CONCENTRATIONS GREATER THAN 0 NG ML$^{-1}$.**



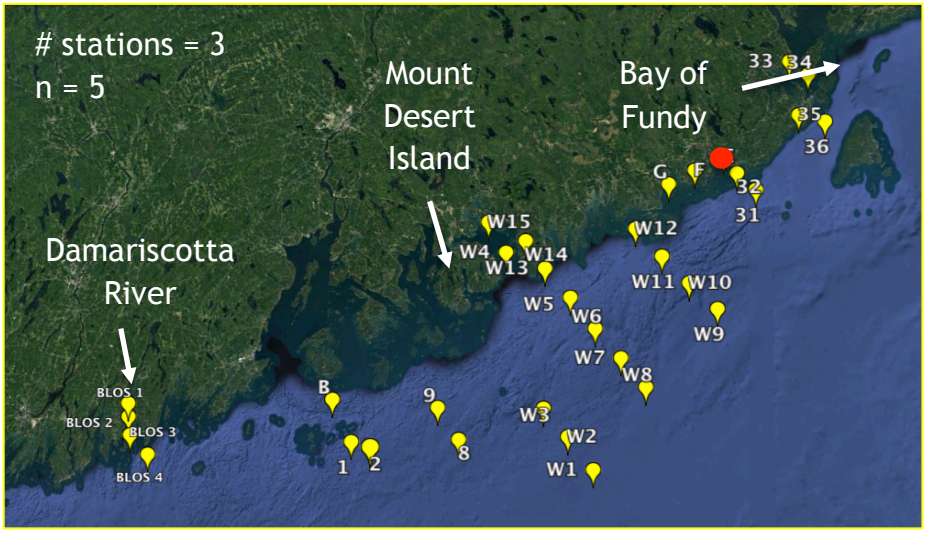

**APPENDIX B – A MAP SHOWING THE NUMBER OF STATIONS (RED DOTS) AND OBSERVATIONS ("N") INCLUDED 'IN-BLOOM' WITH A PARTICULATE DOMOIC ACID (DA) THRESHOLD CONCENTRATION OF 1 NG ML$^{-1}$. EACH STATION CONSISTS OF OBSERVATIONS FROM ONE OR TWO DEPTHS.**



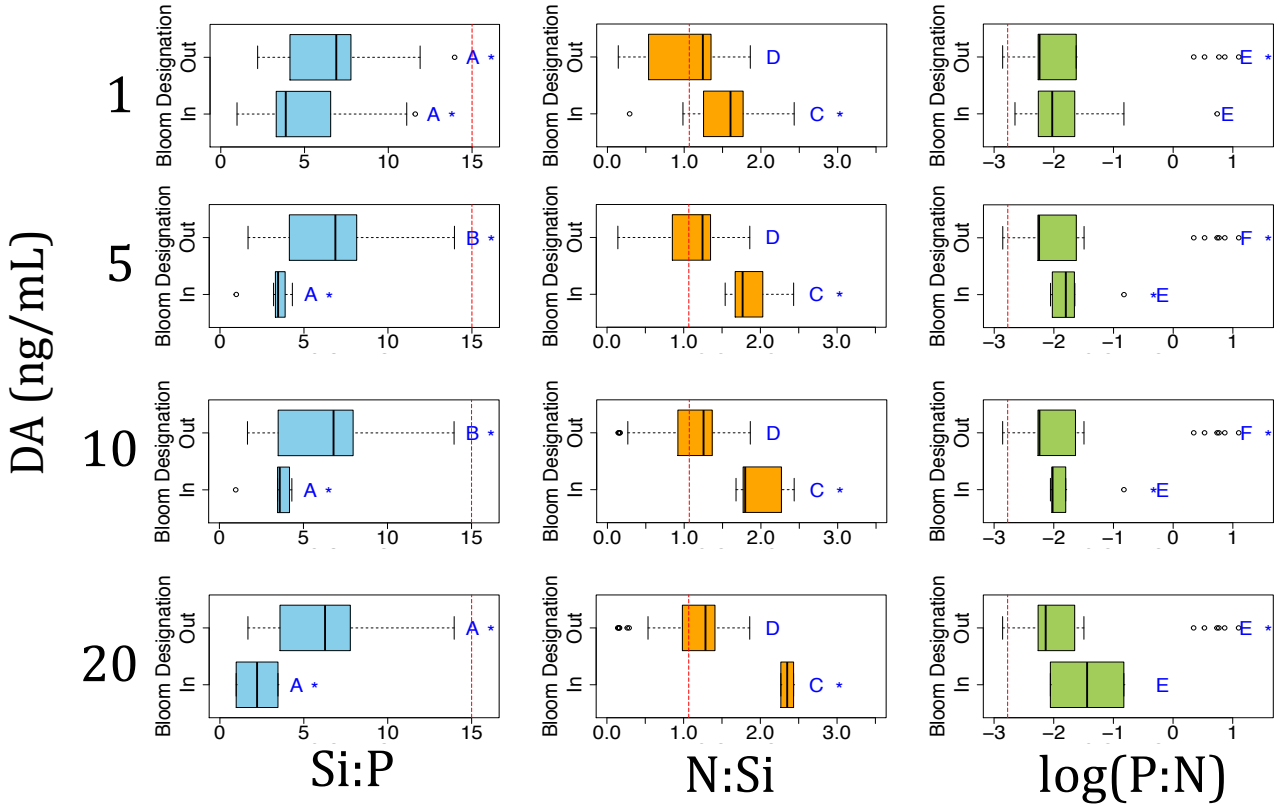

**APPENDIX C – A MAP SHOWING THE NUMBER OF STATIONS (RED DOTS) AND OBSERVATIONS ("N") INCLUDED 'IN-BLOOM' WITH A PARTICULATE DOMOIC ACID (DA) THRESHOLD CONCENTRATION OF 5 NG ML$^{-1}$. EACH STATION CONSISTS OF OBSERVATIONS FROM ONE OR TWO DEPTHS.**




**APPENDIX D – A MAP SHOWING THE NUMBER OF STATIONS (RED DOTS) AND OBSERVATIONS ("N") INCLUDED 'IN-BLOOM' WITH A PARTICULATE DOMOIC ACID (DA) THRESHOLD CONCENTRATION OF 10 NG ML$^{-1}$. EACH STATION CONSISTS OF OBSERVATIONS FROM ONE OR TWO DEPTHS.**

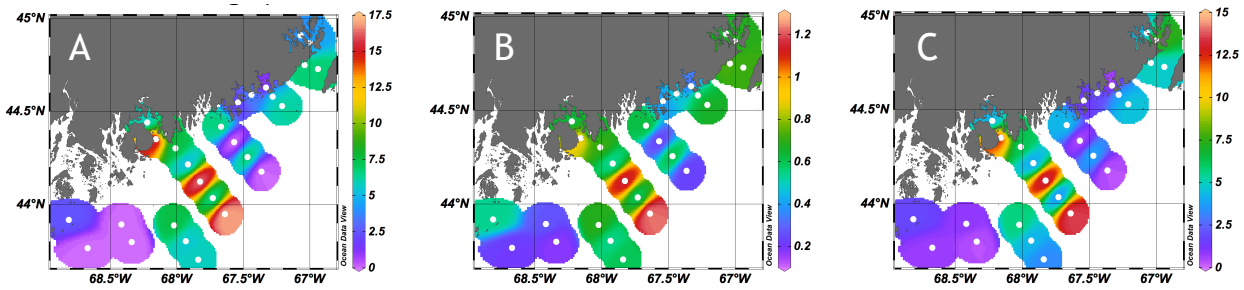



**APPENDIX E – A MAP SHOWING THE NUMBER OF STATIONS (RED DOTS) AND OBSERVATIONS ("N") INCLUDED 'IN-BLOOM' WITH A PARTICULATE DOMOIC ACID (DA) THRESHOLD CONCENTRATION OF 20 NG ML$^{-1}$. EACH STATION CONSISTS OF OBSERVATIONS FROM ONE OR TWO DEPTHS.**




**APPENDIX F – A MATRIX OF BOXPLOTS SHOWING THE DISTRIBUTION OF NUTRIENT RATIOS AT ALL OBSERVATIONS, INCLUDING OBSERVATIONS FROM UP TO TWO DEPTHS AT EACH STATION. EACH ROW DESIGNATES A DIFFERENT HYPOTHETICAL 'IN-BLOOM' THRESHOLD (PARTICULATE DA CONCENTRATION ≥ 1, 5, 10, OR 20 NG ML$^{-1}$). EACH COLUMN DESIGNATES A DIFFERENT NUTRIENT RATIO: SI:P (BLUE, LEFT), N:SI (ORANGE, MIDDLE) AND LOG(P:N) (GREEN, RIGHT). WITHIN EACH BOXPLOT, "OUT" INDICATES THE DISTRIBUTION OF OBSERVATIONS DESIGNATED 'OUT-OF-BLOOM' FOR THE GIVEN THRESHOLD; "IN" INDICATES THE DISTRIBUTION OF OBSERVATIONS**

**DESIGNATED 'IN-BLOOM' FOR THE GIVEN THRESHOLD; THE X-AXIS VALUES INDICATE THE NUTRIENT RATIO VALUE. EACH BOXPLOT ALSO INCLUDES THE HYPOTHETICAL VALUE FOR THE GIVEN NUTRIENT RATIO, ACCORDING TO REDFIELD (DASHED RED LINE; REDFIELD, 1958; BRZEZINSKI, 1982), WHETHER EACH GROUP OF OBSERVATIONS IS SIGNIFICANTLY DIFFERENT FROM THE VALUE DEFINED BY REDFIELD (ASTERISK) AND WHETHER 'IN-BLOOM' AND 'OUT-OF-BLOOM' GROUPS ARE SIGNIFICANTLY DIFFERENT FROM EACH OTHER (LETTERS). A THRESHOLD OF 5 NG ML$^{-1}$ WAS CHOSEN FOR FURTHER NUTRIENT STOICHIOMETRY ANALYSIS BECAUSE IT SHOWED RELATIVELY LOW VARIATION FOR ALL 'IN-BLOOM' GROUPS AND HAD A HIGHER SAMPLE SIZE THAN THRESHOLDS OF 10 OR 20 NG ML$^{-1}$.**




**APPENDIX G MAPS SHOWING A) NITROGEN (NITRATE + NITRITE), B) PHOSPHATE AND C) SILICATE CONCENTRATIONS (µM) FOR ALL STATIONS IN THE BLOOM REGION.**