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Interactive Effects Of Domoic Acid Allelopathy, Salinity, And Eutrophication On Estuarine Phytoplankton Community Structure

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INTERACTIVE EFFECTS OF DOMOIC ACID ALLELOPATHY, SALINITY, AND
EUTROPHICATION ON ESTUARINE PHYTOPLANKTON COMMUNITY
STRUCTURE

by

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DEDICATION

To my Mum and Dad, always here for me, no matter the distance.

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ABSTRACT

Coastal regions and estuaries are particularly sensitive to the increase in nutrient loading and river runoff, threatening the ecosystems with possible spreads in harmful algal blooms (HABs). As an example, blooms of toxic *Pseudo-nitzschia* species can release acute concentrations of the neurotoxin domoic acid (DA) in the water column. When ingested at concentrations higher than 20 ppm, DA can cause the death of marine birds, marine mammals and even humans. The main objectives of my research were to determine the drivers of *Pseudo-nitzschia* abundance and toxicity and to assess how these drivers influenced the phytoplankton community structure and DA allelopathy. Through multiple bioassays using water collected from two sites, North Inlet (high salinity, nutrient deplete) and Winyah Bay (low salinity, nutrient replete), links between the environmental factors and *Pseudo-nitzschia* abundances and toxicity were investigated. Subsequent experiments determined the influence of salinity on DA allelopathy. Finally, the impact of moderate loadings of nitrate and phosphate on *Pseudo-nitzschia* abundance, DA allelopathy and on the entire phytoplankton community composition were examined. The major conclusions of this research are:

1. In North Inlet, increases in *Pseudo-nitzschia* abundance and toxicity were driven by low temperatures and high salinity. In Winyah Bay, *Pseudo-nitzschia* cells were present but had difficulties acclimating to low salinities.

2. DA allelopathy was more effective on salinity-stressed cells (i.e. high salinity in North Inlet, low salinity in Winyah Bay).
3. Although the addition of nutrients enhanced *Pseudo-nitzschia* abundance, it decreased the effect of DA on natural phytoplankton communities and mitigated the influence of salinity on DA allelopathy.
4. Significant shifts in the phytoplankton community composition with the addition of nutrients were observed although the threshold of 40 $\mu\text{g chl } a \text{ l}^{-1}$ wasn't reached. But no shift towards a particular group was established.

My findings highlight how environmental factors such as salinity and nutrients can play important roles on *Pseudo-nitzschia* abundance as well as on DA allelopathy by alleviating or exacerbating its effect. Furthermore, with a possible increase in HABs due to eutrophication, my results demonstrate the ecological risk of using a subjective chl *a* concentration based on total phytoplankton biomass for water quality criteria. Relative abundances of algal groups and species should be included to be able to identify harmful species.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Harmful algal blooms

Responsible for roughly 50% of the total annual production of the Earth's oxygen, microalgae are one of the most important marine microscopic organisms. Although the majority of phytoplankton species is harmless to the environment and the human health, 2% of the described phytoplankton species are toxigenic and can form harmful algal blooms (HABs) (Granéli and Turner, 2006). HABs were first observed in 1770; Captain James Cook observed a "brown scum" on the sea along the Australian coast. In 1832, Charles Darwin described the water of Chile as "a pale reddish tint and, examined under a microscope, was seen to swarm with minute animalculae darting about and often exploding. Their shape is oval and contracted in the middle by a ring of vibrating curved ciliae." (The Voyage of the Beagle, Galtsoff, 1949; Granéli and Turner, 2006).

HAB events are commonly associated with the proliferation of microalgae discoloring the water and releasing phycotoxins in the water column (e.g. the dinoflagellate *Karenia brevis*, the cyanobacterium *Microcystis aeruginosa*) with huge impacts on human economy and health (Granéli and Turner, 2006). Phycotoxins can cause illnesses and/or the death of humans and aquatic organisms (e.g. marine birds, marine mammals) through the ingestion of contaminated fish and shellfish (Van Dolah,

2000). The development of new tools (e.g. PCR, flow cytometry) enables the characterization of new phycotoxins and new HAB species as well as a better control and prediction of dense HABs (Seltenrich, 2014).

Pseudo-nitzschia

HAB species thrive in both benthic and pelagic environments, marine and freshwater, and range in size from cyanobacteria (<2 μm) to dinoflagellates and diatoms (up to 200 μm). Particularly, a cosmopolitan diatom genus, *Pseudo-nitzschia*, can cause severe problems in the environment. Species of *Pseudo-nitzschia* (20-100 μm) are found worldwide at wide ranges of temperature and salinity. Some species have been identified in the North Pacific and Antarctic waters, but most have been characterized in coastal regions (Marchetti et al., 2006; Trainer et al., 2012). In the United States, toxic *Pseudo-nitzschia* blooms predominantly occur along the west coast, such as the Santa Barbara Basin (Sekula-Wood et al., 2011), but some have also been reported on the east coast, especially in the Gulf of Maine (Fernandes et al., 2014) and in the Chesapeake Bay (Anderson et al., 2010). Although mostly non-toxic, blooms of *Pseudo-nitzschia* have also occurred along the coasts of North and South Carolina (Shuler et al., 2012).

Domoic acid characterization, degradation and production

To date, 23 out of a total of 48 identified *Pseudo-nitzschia* species can release the neurotoxin domoic acid (DA) (Teng et al., 2016). DA ($\text{C}_{15}\text{H}_{21}\text{NO}_6$) is a water soluble tricarboxylic amino acid with four dissociation constants (pK_a 's = 2.1, 3.72, 4.97, and 9.82) and eight different isomers (Wright et al., 1989; Falk et al., 1991). The structure of DA is similar to the neurotransmitter glutamic acid, but has a stronger receptor affinity than glutamic acid. Thus, it causes the depolarization of neurons and their death (Todd,

1993). With a lower receptor affinity, DA isomers have a significant lower toxicity compared to DA (Sawant et al., 2007; 2010).

DA is able to intoxicate marine birds and marine mammals through bioaccumulation in fish, shellfish and crustaceans (Perl et al., 1990; Mos, 2001; Trainer et al., 2012). In humans, it causes amnesic shellfish poisoning (ASP) with acute symptoms such as dizziness, short-term memory loss and seizures (Todd, 1993; Jeffery et al., 2004). A regulatory limit of 20 $\mu\text{g DA g}^{-1}$ of tissue was applied after 153 people presented symptoms of strong intoxication in 1987 (Wright et al., 1989; Thessen and Stoecker, 2008). The accumulation of DA in filter feeders can be through the direct consumption of *Pseudo-nitzschia* as well as through the ingestion of DA present in the water column (Liu et al., 2008; Visciano et al., 2016). During a bloom of toxigenic *Pseudo-nitzschia*, the concentration of dissolved DA (dDA) ranges from 1 to 30 ng ml^{-1} in the water column (Bargu et al., 2011) but can reach up to 980 ng ml^{-1} during extraordinary bloom such as the one that occurred along the west coast of the U.S. in 2015 (McCabe et al., 2016; Kudela, 2017).

Once in the water column, photodegradation is thought to be the major sink of dDA (Bouillon et al., 2006). In filtered seawater and deionized water, 75% of dissolved DA were degraded after 22 hours of exposure to full spectrum light (Bates et al., 2003; Bouillon et al., 2006). However, light intensity exponentially decreases in the water column and becomes inconsequential with depth. Furthermore, with an absorption wavelength of $\lambda = 242 \text{ nm}$, DA degradation occurs under UV light which barely penetrates the ocean surface (Bouillon et al., 2006). This was confirmed by studies in which DA whether in dissolved or particulate forms, was measured in deep waters, in

sediment traps as well as in marine snow (Sekula-Wood et al., 2011; Schnetzer et al., 2017). Stewart et al. (2008) suggested a possible bacterial degradation of DA in *Pseudo-nitzschia multiseriis* cultures. In cultures of toxigenic *Pseudo-nitzschia fraudulata*, the transplantation with the bacterial community of the non-toxic species *Pseudo-nitzschia pungens* stopped the production of DA. This was attributed to a possible consumption of DA by the bacterial consortium of *P. pungens* (Sison-Mangus et al., 2014).

In *Pseudo-nitzschia* cultures, the introduction of a foreign bacterial community into the medium can also trigger the production of DA (Bates et al., 1995; Sison-Mangus et al., 2014). Changes in nutrient concentrations and/or pH also induce stress to *Pseudo-nitzschia* cells, which can suddenly stop or trigger the production of DA. Stress can occur in depleted nutrient conditions (nitrate, ammonium, phosphate, iron and silicate) as well as in overconcentrated nutrient conditions (nitrate and ammonium) (reviewed in Lelong et al., 2012). Nutrients are not the only factors driving *Pseudo-nitzschia* toxicity. A change in temperature, light intensity and/or salinity can also enhance or mitigate *Pseudo-nitzschia* abundance and the production of DA (reviewed in Lelong et al., 2012).

For example, an increase in *Pseudo-nitzschia* abundance with salinity was reported in upwelling regions (Trainer et al., 2012), in the Chesapeake Bay (Thessen and Stoecker, 2008), in the Gulf of Mexico (MacIntyre et al., 2011; Liefer et al., 2013; Bargu et al., 2016) and in the Sea of Marmara in Turkey (Tas et al., 2016). An increase in abundances with salinity was also observed with *P. fryxelliana* and *P. fraudulenta* in Puget Sound (Hubbard et al., 2014), *P. calliantha* and *P. pungens* in the Sea of Marmara in Turkey (Tas et al., 2016), *P. brasiliensis* in the Bizerte Lagoon in Tunisia (Sahraoui et al., 2012), *P. multistriata* along the Uruguayan coast (Méndez et al., 2012) and *P.*

delicatissima in the English Channel (Caroppo et al., 2005; Downes-Tettmart et al., 2013) and in Puget Sound (Hubbard et al., 2014). Positive correlations between the measured particulate DA concentrations and salinity were also reported for few of the systems mentioned above (Anderson et al., 2009; MacIntyre et al., 2011; Sahraoui et al., 2012; Bargu et al., 2016; Tas et al., 2016).

Toxic *Pseudo-nitzschia* cells were also observed in lower salinity systems such as the Juan de Fuca region, the San Juan Islands and the Zrmanja River Estuary (Eastern Adriatic Sea) where salinity can be as low as 8 (Rines et al., 2002; Burić et al., 2008; Trainer et al., 2009). Although the production of DA is often reduced in low salinity water, relatively high concentrations of DA were measured in fresher water (Thessen et al., 2005; Hickey et al., 2013).

The ecological reasons for the production of DA are still unknown. DA production was primarily thought to serve as a way to release excess of photosynthetic energy produced during terminal growth stage (Mos, 2001). DA production is also hypothesized to compensate iron depletion as DA would bind with particulate iron Fe(III) in the water and by oxidation would release soluble Fe(II) bioavailable for *Pseudo-nitzschia* (Rue and Bruland, 2001; Wells et al., 2005). A factor often left behind though important for its role in regulating phytoplankton community structure, is the possible use of DA as an allelochemical.

Domoic acid and allelopathy

Allelopathy is the release of one or more chemical substances by individuals of a population that have either an inhibitory or a stimulatory effect on the growth, survival, and reproduction of individuals of another population (Rice, 1984). Among most studies,

it is defined as an inhibitory effect. In the water column, phytoplankton cells compete for light and nutrients and allelochemicals produced by some phytoplankton enable them to overcome their competitors (e.g. other phytoplankton species, bacteria, grazers).

The first study on microalgal allelopathy was done by Keating (1977) who observed a correlation between a shift in microalgal community in a pond (Linsley Pond, Connecticut) and phycotoxin concentration released by cyanobacteria. Afterward, many studies focused on phytoplankton allelopathy mostly through culture experiments and the importance of allelopathy in marine environments was revealed. Allelopathy can regulate phytoplankton diversity and without it, the coexistence of two phytoplankton species on one limited resource may not be possible (Roy, 2015). The mode of action of allelochemicals differed from species to species; toxins can alter the shape or lyse the target cells or inhibit the competitor's photosynthesis by acting on the photosystem II (reviewed in Legrand, 2003).

DA allelopathy is a controversial subject. Subba Rao et al. (1995) obtained an allelopathic interaction between *Pseudo-nitzschia pungens* and the diatom *Rhizosolenia alata*. Windust (1992) and Lundholm et al. (2005) studied the effect of direct addition of dDA on several phytoplankton species but did not find a significant impact. However, Prince et al. (2013) identified a negative allelopathic effect of DA on the growth of the diatom *Skeletonema marinoi* growth in iron depleted medium. More recently, studies have established an allelopathic effect of *Pseudo-nitzschia* on species of dinoflagellates, raphidophytes, haptophytes and cryptophytes (Xu et al., 2015) as well as on the diatom *Bacillaria* spp. (Sobrinho et al., 2017). Most research on allelopathy is based on mono or co-cultures of phytoplankton in ideal conditions (i.e. replete nutrients). But microcosm

incubations with natural phytoplankton communities are essential to understand the role played by DA in interspecies competition.

Establishing allelopathic interactions on natural phytoplankton communities is a real challenge. While some phytoplankton species can resist the attacks of allelopathic compounds in some conditions, altering these conditions may weaken the phytoplankton strategies and make them more vulnerable to allelopathy (Prince et al., 2008).

Inconstancies in the phytoplankton response to allelochemicals in natural environmental are common with allelopathy being mitigated by environmental factors as well as by competitors (Prince et al., 2008; Poulston-Ellestad et al., 2014). Abiotic and biotic factors can influence allelopathy with nutrient concentrations in the water column one of the most important (Granéli et al., 2008). Studies, whether field observations or culture experiments, have demonstrated a higher allelopathic activity in nutrient depleted conditions (reviewed in Granéli et al., 2008). Even anthropogenic nitrogen and phosphorus inputs can lead to unbalanced N:P ratios, with whether N or P as the limited source for phytoplankton growth. In this case, phytoplankton species releasing phycotoxins have an advantage to thrive by eliminating competitors (Granéli et al., 2008; Peñuelas et al., 2013).

***Pseudo-nitzschia* abundance and toxicity with nutrients**

Nutrient loading can lead to the spread of toxic blooms of *Pseudo-nitzschia*. This was confirmed by sediment records demonstrating a positive correlation between the abundance of *Pseudo-nitzschia* and nitrogen fluxes (Parsons et al., 2002). Furthermore, along the west coast of the United States an unprecedented *Pseudo-nitzschia* bloom appeared during a particularly warm upwelling season in 2015 (McCabe et al., 2016; Du

et al., 2016). Along the coast of Washington, dense blooms of *Pseudo-nitzschia* were also triggered by the addition of ammonium through the sewage system (Trainer et al., 2007).

Furthermore, most *Pseudo-nitzschia* cells have the ability to both photosynthesize and assimilate external organic nutrients (i.e. mixotrophy). It allows them to proliferate in low light environments concentrated in dissolved organic matter (Cochlan et al., 2008; Burkholder et al, 2008; Flynn and Mitra, 2009; Loureiro et al., 2009). Therefore, eutrophication through the pulses of combined phosphorus and nitrogen, whether organic or inorganic, can be a driver of the increase in the occurrence of *Pseudo-nitzschia* as well as other HAB species (Anderson et al. 2002; Heisler et al., 2008; Granéli et al., 2008).

Eutrophication

The primary symptom of eutrophication is an increase in chlorophyll *a* (chl *a*) concentrations (Whitall et al. 2007) and many agencies select an arbitrary chl *a* concentration as an indicator of eutrophic conditions, commonly 40 µg chl *a* l⁻¹ (<https://www.epa.gov>). However, this concentration limit may not be an ecologically sound regulation parameter as it only takes into account the total phytoplankton biomass, not the state of the phytoplankton community structure (Ferreira et al., 2011; Glibert 2017). Several studies have demonstrated that the addition of nutrients resulted in shifts in phytoplankton communities towards cyanobacteria, dinoflagellates and mixotrophic species (Anderson et al. 2002; Heisler et al. 2008; Reed et al. 2016; Caron et al. 2017). But few of them have determined if significant shifts in the phytoplankton community composition occur at chl *a* concentrations lower than the common regulatory limit.

1.2 THESIS OBJECTIVES

In the second chapter, I examined the effect of salinity in the *Pseudo-nitzschia* abundance and toxicity from a nutrient depleted site (North Inlet, SC). I also investigated the possible allelopathic effect of DA on the phytoplankton community composition through multiple bioassays with direct additions of dDA. The influence of several environmental factors and particularly salinity in the allelopathic effect of DA was established. In the third chapter, I determined the role of river and nutrient inputs on the *Pseudo-nitzschia* abundance and toxicity from two sites through multiple bioassays with direct additions of dDA. The first site was a low salinity site (Winyah Bay, SC) and the second one was a high salinity site in which I added nutrients simultaneously with DA (North Inlet, SC). The influence of salinity on DA allelopathy was also examined for both sites. In the final chapter, I investigated the effect of nutrient loading on the phytoplankton community composition as well as on the phytoplankton size at a total chl *a* concentration lower than the regulatory limit $40 \mu\text{g l}^{-1}$.

CHAPTER 2

THE INFLUENCE OF SALINITY IN THE DOMOIC ACID EFFECT ON ESTUARINE PHYTOPLANKTON COMMUNITIES¹

2.1 ABSTRACT

Toxic species of the diatom genus *Pseudo-nitzschia*, observed worldwide from coastal waters to the open ocean, produce the neurotoxin domoic acid (DA). DA is an important environmental and economic hazard due to shellfish contamination with subsequent effects on higher trophic levels. Previous research has demonstrated that, among other environmental factors, salinity influences the abundance and toxicity of *Pseudo-nitzschia*. In this study, the environmental factors driving the growth of *Pseudo-nitzschia* and the production of dissolved DA (dDA) in North Inlet estuary were examined. The effect of salinity on the growth inhibition of phytoplankton induced by the initial presence as well as by an addition of dDA was also assessed. Initially, the diatom abundance was negatively correlated with the abundance of *Pseudo-nitzschia* and with the concentration of dDA. With the addition of a concentrated solution of dDA, the percent inhibition of cryptophytes and diatoms was significantly correlated with salinity and suggested a higher sensitivity to dDA at extreme salinities. These results emphasize the importance of salinity in assessing the properties of DA and potentially of other

¹ Van Meerssche, E. and J.L. Pinckney. 2017. *Harmful Algae*. 69: 65-74.
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phycotoxins on phytoplankton.

2.2 INTRODUCTION

Domoic acid (DA), produced by toxic species of the diatom genus *Pseudo-nitzschia*, is a water-soluble tricarboxylic amino acid with a structure similar to that of the neurotransmitter glutamate (Wright et al., 1989). Because of its stronger receptor affinity, DA activates glutamate receptors and causes the depolarization and death of neurons (Bejarano et al., 2008). Thus, through consumption of contaminated fish and shellfish at concentrations higher than $20 \mu\text{g g}^{-1}$ of tissue, DA can cause severe amnesic shellfish poisoning (ASP) symptoms and can lead to the death of marine mammals, marine birds and even humans (Wright et al., 1989; Todd, 1993; Pulido, 2008). Due to its major environmental and health impacts, the influence of multiple factors (nutritional, physiological, physical and biological) on the development of *Pseudo-nitzschia* blooms and their associated toxicity have been thoroughly studied (reviewed in Lelong et al., 2012).

Pseudo-nitzschia is a ubiquitous genus with species capable of growing at salinities ranging from 1 to over 35 and at temperatures between -1.5°C and 30°C (Bates et al., 1998; Thessen et al., 2005). Although most species were identified in coastal regions, some species have been isolated from the North Pacific and Antarctic waters with cells capable of heterotrophy by assimilating organic nitrogen (Marchetti et al., 2006; Loureiro et al., 2009; Trainer et al., 2012; Lelong et al., 2012). Therefore, different combinations of multiple environmental factors can result in blooms of toxic *Pseudo-nitzschia*. However, differing species and strains within the same *Pseudo-nitzschia* bloom with different physiological, physical and chemical preferences such as temperature,

light, and macronutrient concentrations make it difficult to predict *Pseudo-nitzschia* blooms and toxicity (Lundholm et al., 1997; Lelong et al., 2012; Kim et al., 2015).

For example, an increase in the abundance of *Pseudo-nitzschia* with salinity was reported in upwelling regions (Trainer et al., 2012), in the Chesapeake Bay (Thessen and Stoecker, 2008), in the Gulf of Mexico (MacIntyre et al., 2011; Liefer et al., 2013; Bargu et al., 2016) and in the Sea of Marmara in Turkey (Tas et al., 2016). Although *Pseudo-nitzschia multiseriata* and *P. pungens* abundances presented different outcomes in relation to salinity depending on the region (Méndez et al., 2012; Downes-Tettmar et al., 2013; Hubbard et al., 2014; Tas et al., 2016), an increase in abundances with salinity was observed with *P. fryxelliana* and *P. fraudulenta* in Puget Sound (Hubbard et al., 2014), *P. calliantha* and *P. pungens* in the Sea of Marmara in Turkey (Tas et al., 2016), *P. brasiliensis* in the Bizerte Lagoon in Tunisia (Sahraoui et al., 2012), *P. multistriata* along the Uruguayan coast (Méndez et al., 2012) and *P. delicatissima* in the English Channel (Caroppo et al., 2005; Downes-Tettmar et al., 2013) and in Puget Sound (Hubbard et al., 2014). These species may be osmoadapted and tolerate hyperosmotic conditions (Kirst et al., 1989). However, phytoplankton species are not only regulated by salinity but also by temperature, light and nutrients, therefore other environmental factors may also influence the abundance of *Pseudo-nitzschia* in these regions.

In upwelling regions, the Chesapeake Bay and the Gulf of Mexico, blooms were also stimulated by low temperature and high nutrient concentrations particularly silica and nitrogen, which was also observed in Puget Sound where the abundance of *P. fryxelliana*, *P. delicatissima* and *P. fraudulenta* was negatively correlated with temperature (Thessen and Stoecker, 2008; MacIntyre et al., 2011; Trainer et al., 2012;

Liefer et al., 2013; Hubbard et al., 2014; Bargu et al., 2016). However, a positive correlation between the abundance of *Pseudo-nitzschia* spp. with temperature (Méndez et al., 2012; Downes-Tettmart et al., 2013; Tas et al., 2016) as well as a negative correlation with nutrient concentrations (Sahraoui et al., 2012; Tas et al., 2016) were observed in other regions. Only a few locations and studies noticed the same combination of environmental factors to explain the growth of *Pseudo-nitzschia*. This is also true with the measurement of DA concentrations, mostly particulate DA.

Positive correlations between the measured particulate DA concentrations and salinity were reported for many systems (Anderson, 2009; MacIntyre et al., 2011; Sahraoui et al., 2012; Bargu et al., 2016; Tas et al., 2016). In the Gulf of Mexico, a positive correlation with dissolved inorganic carbon (DIC) and silicate concentrations as well as a negative correlation with temperature were also observed (MacIntyre et al., 2011; Bargu et al., 2016). On the opposite, Tas et al. (2016) established a positive correlation with temperature and a negative correlation between particulate DA with nutrient concentrations in the Sea of Marmara in Turkey. However, the ecological reason for DA production remains unknown.

DA has been hypothesized to have allelochemical properties (i.e. the positive and negative effects of a toxin produced by a phytoplankton species on other phytoplankton species) allowing *Pseudo-nitzschia* to dominate the water column and overcome the other microalgal competitors. This has been the focus of culture-based studies that resulted in an absence of allelopathic effect by a direct addition of DA in different monocultures of phytoplankton groups including a few species of diatoms, prymnesiophytes, euglenophytes, dinoflagellates, raphidophytes, prasinophytes, and cryptophytes (Windust,

1992; Lundholm et al., 2005). An allelopathic effect was suggested in co-cultures of *P. multiseriis* and *Chrysochromulina ericina* but the growth inhibition of the prymnesiophyte was demonstrated to be due to a slight change in pH and not to the released DA (Lundholm et al., 2005). More recently, Xu et al. (2015) established an allelopathic effect of *P. multiseriis* and *P. pungens* on species of dinoflagellates, raphidophytes, haptophytes and cryptophytes but also concluded that it was not caused by DA. These experiments were performed with mono- or co-cultures under optimal conditions, conditions that rarely occur in the ocean (Windust, 1992; Subba Rao et al., 1995; Lundholm et al., 2005; Xu et al., 2015). Prince et al. (2013) partially modified the culture medium to have iron concentrations similar to those encountered in the field and observed a slight but significant growth inhibition of the monocultured *Skeletonema marinoi* induced by a DA addition in iron depleted medium. However, when co-cultured with *P. delicatissima* in iron-replete conditions, the addition of DA increased the abundance of *P. delicatissima* at the expense of the abundance of *Skeletonema marinoi* suggesting a bond between DA and iron, which thus became unavailable for *Skeletonema marinoi* (Rue and Bruland, 2001; Maldonado et al., 2002; Prince et al., 2013). Therefore, other physical and/or chemical factors could also influence the effect of DA on phytoplankton cells.

In this study, the influence of different environmental factors on the initial abundance and toxicity of *Pseudo-nitzschia* was examined. Their role on the inhibitory and stimulatory effects of the initial dissolved DA (dDA) in the water column and of the addition of a highly-concentrated solution of dDA on an estuarine phytoplankton community (North Inlet, South Carolina) was also assessed. This study was aimed to

better anticipate toxic blooms of *Pseudo-nitzschia* and DA levels, and to predict their associated effects on the phytoplankton community. Particularly, DA was hypothesized to be used as an osmolyte by *Pseudo-nitzschia* cells, allowing them to outcompete the other phytoplankton species at high salinity (Doucette et al., 2008). Therefore, the other phytoplankton cells, stressed at high salinity, might be more sensitive to dDA and be inhibited by an addition of a concentrated dDA solution.

2.3 MATERIALS AND METHODS

Study sites

North Inlet, located in a National Estuarine Research Reserve near Georgetown, South Carolina (Fig 2.1), is a pristine bar-built estuary characterized by low freshwater input and high flushing rates. The estuary is subjected to strong tidal forces bringing salt water into the inlet, with salinity ranging from 15 to 35 (Dame et al., 1991). Because of the influence of a temperate subtropical climate, the water temperature varies from 8°C to 30°C and precipitation averages 130 cm yr⁻¹ with maximum rainfall reached during summer (Dame et al., 1991; Sea Grant Consortium, 1992; Allen et al., 2014). The concentrations of orthophosphate, nitrate + nitrite and ammonium average 0.03 µmol l⁻¹, 0.55 µmol l⁻¹ and 1.73 µmol l⁻¹ respectively (Wolaver et al., 1984; Sea Grant Consortium, 1992). The concentration of silicate is usually over 25 µmol l⁻¹, therefore silicate is not considered as a limited nutrient in North Inlet (White et al., 2004; Wetz et al., 2006). Diatoms are the dominant phytoplankton group in the estuary with seasonally variable contributions of cryptophytes, cyanobacteria, chlorophytes, euglenophytes and dinoflagellates (Lawrenz et al., 2013; Allen et al., 2014).

Bioassays

Water samples from North Inlet (Clambank Landing; 33.3500°N, 79.1902°W) were collected monthly in a 10-liter carboy from May to November 2016 during high tide (Fig 2.1). Volumes of 250 ml of water were transferred into 3 control and 3 treatment square Nalgene polycarbonate clear bottles. Ten ml of an initial 10- $\mu\text{g DA ml}^{-1}$ solution, obtained by dissolving 5 mg of DA (Sigma-Aldrich cat. no. D6152) in 500 ml of high purity de-ionized water, were added to the treatment bottles to reach a final concentration of 400 ng DA ml^{-1} . This dissolved DA (dDA) concentration approximates the calculated maximum concentration possible during a toxic *Pseudo-nitzschia* bloom in the field (i.e., 300 ng DA ml^{-1}) (Lundholm et al., 2005).

Samples were incubated in outside water tables covered by two layers of fiberglass screen to simulate *in situ* temperature and irradiance for 2 days, an incubation time adjusted from Gordon et al. (2015) and Pinckney et al. (2017). Salinity (portable refractometer) and temperature were measured at the beginning of each incubation, and pH (Orion, model 250A) was monitored at the beginning and at the end of each bioassay.

Analyses

Monthly cumulative rainfall and photosynthetically active radiations (PAR) were recorded at the Winyah Bay-North Inlet weather station and data were retrieved from <http://cdmo.baruch.sc.edu>.

To measure the initial nutrient concentration, 50 ml of water from the 10-l carboy were collected and to measure the final concentrations, 15 ml from each triplicate were combined. Water was filtered through a 25-mm dia. Whatman GF/F glass fiber filter and stored in polyethylene Nalgene bottles at -20°C until nutrient analysis. Ammonium,

nitrate + nitrite and orthophosphate concentrations were determined with a Lachat Quikchem 8000T autoanalyzer according to photometric methods (Johnson and Petty 1983; Grasshoff et al. 1999). Dissolved inorganic nitrogen (DIN) was calculated as the sum of nitrate, nitrite and ammonium concentrations, and dissolved inorganic phosphorus (DIP) was considered equal to the orthophosphate concentration. The ratios of each macronutrient and pH between the treatment and the control bottles were calculated following the equation:

$$X \text{ ratio} = \frac{X_{\text{treatment}}}{X_{\text{control}}}$$

where X is the final macronutrient concentration or the final pH value.

To measure the initial photopigment concentrations, triplicates of 100 ml of water from the 10-l carboy were collected and to measure the final concentrations, 100 ml from each triplicate were collected. Water was filtered through a 25-mm dia. Whatman GF/F glass fiber filter under gentle vacuum. The filters were transferred into 2-ml microcentrifuge tubes and stored at -80°C until photopigment analysis with high performance liquid chromatography (HPLC) following the method from Pinckney et al. (1996). The photopigment concentrations were examined with the CHEMTAX program (v. 1.95) (Mackey et al., 1996) to determine the absolute abundance of major phytoplankton groups (in $\mu\text{g chl } a^{-1}$) using the convergence procedure outlined by Latasa (2007) to minimize errors in algal group biomass. The initial pigment ratio matrix was derived from two coastal phytoplankton matrices (Schlüter et al., 2000; Lewitus et al., 2005). Photopigments from each experiment were analyzed separately and provided the abundance of cyanobacteria, euglenophytes, chlorophytes, prasinophytes, dinoflagellates,

cryptophytes, and diatoms. Because dinoflagellates and prasinophytes were detected in very low abundances, they were excluded from further analysis.

In each sample, the percent inhibition of the different phytoplankton groups was calculated with the following equation:

$$\% \text{ inhibition} = 100 \left(1 - \frac{\text{abundance}_{\text{treatment}}}{\text{averaged abundance}_{\text{control}}} \right)$$

To identify the initial phytoplankton community, 40 ml of water from the 10-l carboy were collected, and to identify the community at the end of each experiment 10 ml from each triplicate were combined. After settling each sample preserved with Lugol's iodine for a minimum of 6 h in a 10-ml chamber, a minimum of 400 cells were counted, using an inverted light microscope at x 200 and x 400 magnifications (Nikon Eclipse TS100) (Lund et al., 1958). The genera *Chaetoceros*, *Cylindrotheca*, *Skeletonema*, *Rhizosolenia*, *Pseudo-nitzschia* and unknown centric diatoms were the most abundant. Other diatoms included the genera *Thalassosira*, *Thalassionema*, *Leptocylindrus*, *Asterionellopsis*, *Guinardia* and *Pleurosigma*, with each genus representing less than 10% of the total diatom abundance in all samples. Although the identified species were the same month to month, the contribution of each species to the total community was different (Table 2.1). The abundances of *Pseudo-nitzschia* measured at the beginning and at the end of each experiment (control and dDA additions) were summarized in table 2.7.

At the beginning and at the end of each experiment, triplicates of 150 µl were transferred to a 96 microwell plate for the total cell abundance analysis (Guava flow cytometer, Millipore). The percent growth inhibition was calculated with the following equations:

$$\text{growth rate (per day)} = \frac{\ln(\text{abundance}_{\text{tfinal}} - \text{abundance}_{\text{t0}})}{2}$$

$$\% \text{ growth inhibition} = 100 \left(1 - \frac{\text{growth rate}_{\text{treatment}}}{\text{averaged growth rate}_{\text{control}}} \right)$$

Concentrations of dDA were measured before any addition of dDA, right after the addition of dDA and at the end of each experiment. To measure the initial dDA concentration, 10 ml of water from the 10-l carboy were collected, and to measure the concentration just after the addition of dDA and at the end of each experiment, 2 ml from each triplicate were combined. Samples were filtered through a 25-mm dia. Whatman GF/F glass fiber filter and stored at -20°C in amber polyethylene Nalgene bottles until dDA analysis. Only dissolved DA was analyzed; no particulate DA was measured. The samples were prepared following the Biosense protocol and analyzed with enzyme-linked immunosorbent assay (ASP cELISA) kits from Biosense (Kleivdal et al., 2007). The remaining dDA after the 2-day incubations was calculated following the equation:

$$dDA \text{ remaining} = \frac{\text{final dDA concentration}}{\text{initial dDA concentration}}$$

The released DA (rDA) by *Pseudo-nitzschia* cells (in ng DA cell⁻¹) was calculated using the equation:

$$rDA = \frac{\text{final dDA concentration}}{\text{final Pseudo nitzschia abundance}}$$

All homogenous and normally distributed data were analyzed using parametric tests (single and multifactor ANOVAs). All statistical analyses and Pearson's correlation tests were completed using SPSS Software 24.0 (IBM Corporation, Armonk, NY) and principal component analyses (PCA) were performed using Origin (OriginLab, Northampton, MA).

2.4 RESULTS

Initial environmental factors and *Pseudo-nitzschia* abundances

The *Pseudo-nitzschia* abundance was maximum in May with 1032 cell ml⁻¹ corresponding to the highest dDA concentration recorded with 1.21 ng ml⁻¹. In May, salinity was relatively high (37), the phosphate concentration and the pH were maximum with 3.94 μmol l⁻¹ and 7.98 respectively and the ammonium concentration was the lowest with 3.64 μmol l⁻¹. May was also a month of drought with no precipitation (Table 2.2). The lowest *Pseudo-nitzschia* abundance was observed in August with an abundance of 34 cell ml⁻¹ and a dDA concentration of 0.12 ng ml⁻¹. In August, pH was the lowest and the water temperature and the ammonium concentration were maximum with 32.1°C and 4.64 μmol l⁻¹ respectively.

The principal component analysis (PCA) with the first two components representing 80.1% of variability indicated that *Pseudo-nitzschia* abundances were associated with salinity, pH and phosphate concentrations, and were negatively related to temperature and ammonium concentrations (Fig 2.2A). These observations were confirmed by the Pearson's correlation tests indicating a strong positive and significant correlation between the initial *Pseudo-nitzschia* abundances with salinity ($r = 0.796$, $N = 10$, $p < 0.05$), with phosphate concentrations ($r = 0.874$, $N = 10$, $p < 0.05$) and with pH ($r = 0.959$, $N = 10$, $p < 0.05$) (Table 2.3). It also indicated a strong negative and significant correlation with temperature ($r = -0.860$, $N = 10$, $p < 0.05$), with ammonium concentrations ($r = -0.881$, $N = 10$, $p < 0.05$), with the ratio DIN : DIP ($r = -0.976$, $N = 10$, $p < 0.05$) and with the initial PAR ($r = -0.923$, $N = 10$, $p < 0.05$).

The initial dDA concentration, strongly correlated with the abundance of *Pseudo-nitzschia*, was not significantly correlated with salinity but was positively correlated with phosphate concentrations and pH ($r = 0.760$ and $r = 0.761$, $N = 10$, $p < 0.05$). However, the released DA by *Pseudo-nitzschia* cells (rDA) was negatively correlated with salinity ($r = -0.792$, $N = 10$, $p < 0.01$) and positively correlated with the initial nitrate + nitrite and ammonium concentrations ($r = 0.779$ and $r = 0.657$, $N = 10$, $p < 0.05$) (Table 2.3).

The PCA also indicated that the initial diatom, chlorophyte and cryptophyte abundances were negatively related to *Pseudo-nitzschia* abundances and dDA concentrations (Fig 2.2A). Some of these observations were validated by the Pearson's correlation tests indicating a strong negative and significant correlation between the abundance of cyanobacteria and dDA ($r = -0.665$, $N = 10$, $p < 0.05$). The diatom abundance was negatively correlated not only with dDA ($r = -0.723$, $N = 10$, $p < 0.05$) but also with the *Pseudo-nitzschia* abundance ($r = -0.912$, $N = 10$, $p < 0.05$) (Table 2.3). Interestingly, the *Rhizosolenia* abundance was negatively correlated with dDA ($r = -0.717$, $N = 10$, $p < 0.05$) and the unidentified centric diatom abundance was negatively correlated with both dDA ($r = -0.868$, $N = 10$, $p < 0.05$) and the abundance of *Pseudo-nitzschia* ($r = -0.924$, $N = 10$, $p < 0.05$). However, the other diatom genera including *Thalassosira*, *Thalassionema*, *Leptocylindrus*, *Asterionellopsis*, *Guinardia* and *Pleurosigma* were positively correlated with the abundance of *Pseudo-nitzschia* ($r = 0.674$, $N = 10$, $p < 0.05$) (Table 2.4).

Effect of dDA additions on the abundances of the different phytoplankton groups

The response to the addition of dDA was neither consistent nor significant each month. Over the seven different incubations, most groups responded significantly only

once to the addition of dDA and this significant response was mainly an increase in abundance (Fig 2.3). For instance, the abundances of cyanobacteria and chlorophytes were significantly stimulated by the addition of dDA in May (ANOVA, $p < 0.01$), the abundance of diatoms was significantly enhanced in June (ANOVA, $p < 0.01$) and the abundance of euglenophytes increased significantly in July (ANOVA, $p < 0.05$) (Fig 2.3A, B, C and E). Only the significant responses of cryptophytes differed from month to month with a growth inhibition in May but a growth stimulation in June, July and November (ANOVA, $p < 0.05$) (Fig 2.3D). Overall the total cell abundance was not significantly altered by the addition of dDA with only a significant increase in September and a significant inhibition in October (ANOVA, $p < 0.05$) (Fig 2.3F).

Influence of environmental factors on the percent inhibition of phytoplankton

The second PCA (Fig 2.2B) with the first two components representing 66.9% of variability indicated that the percent inhibition of diatoms and the total percent growth inhibition were associated with the remaining dDA and salinity. It also suggested that the percent inhibition of cryptophytes was positively related with salinity and the ammonium and nitrate + nitrite ratios, and that the percent inhibition of chlorophytes and cyanobacteria was associated with the phosphate and pH ratios but was negatively related to the nitrate + nitrite ratio. These observations were partially confirmed with the Pearson's correlation tests with a strong and negative correlation between the percent inhibition of cyanobacteria and chlorophytes with the nitrate + nitrite ratio ($r = -0.941$, $N = 5$, $p < 0.01$ and $r = -0.942$, $N = 5$, $p < 0.05$) and with the ratio DIN : DIP ($r = -0.940$, $N = 7$, $p < 0.01$ and $r = -0.941$, $N = 5$, $p < 0.05$). The percent inhibition of cryptophytes was not only correlated with the ammonium ratio ($r = 0.929$, $N = 5$, $p < 0.05$) but also with

salinity ($r = 0.728$, $N = 21$, $p < 0.01$). A positive correlation with salinity was also observed with the percent inhibition of diatoms ($r = 0.622$, $N = 21$, $p < 0.01$) and the total percent growth inhibition ($r = 0.603$, $N = 21$, $p < 0.01$) (Table 2.5). Their percent inhibition increased with salinity starting with a growth stimulation from salinities 33 to 35 and reaching a maximum percent inhibition of cryptophytes ($74.9 \pm 10.6\%$) at salinity 37, of diatoms ($38.4 \pm 15.2\%$) and of the total growth inhibition ($88.6 \pm 53.6\%$) at salinity 38 (Fig 2.4A, B and C).

Although positively but not significantly correlated with the percent inhibition of euglenophytes, cryptophytes and diatoms, the remaining dDA was significantly correlated with the total percent growth inhibition ($r = 0.649$, $N = 21$, $p < 0.01$) (Table 5) and with salinity in the bottles with the addition of dDA ($r = 0.686$, $N = 10$, $p < 0.05$) (Table 2.6). The averaged lowest ratio (0.63) was obtained at salinities 33 and 34. The ratio intensified with salinity and exceeded 1 at salinities higher than 35 with the maximum ratio (1.66) obtained at salinity 35 (Fig 2.4D). The calculated released DA by *Pseudo-nitzschia* was negatively correlated with salinity in both the initial conditions ($r = -0.792$, $N = 10$, $p < 0.05$) and the control bottles ($r = -0.710$, $N = 10$, $p < 0.05$), implying that *Pseudo-nitzschia* cells released less dDA as salinity increased (Table 2.6).

2.5 DISCUSSION

In this study, the abundance of *Pseudo-nitzschia* was significantly and positively correlated with salinity and negatively correlated with temperature and PAR, which is in accordance with many blooms of *Pseudo-nitzschia* observed in upwelling regions (Schnetzer et al., 2007; Trainer et al., 2012) as well as with the abundance of *Pseudo-nitzschia calliantha* in the Adriatic Sea and the Chesapeake Bay (Caroppo et al., 2005;

Thessen and Stoecker, 2008), of *P. delicatissima*, *P. fryxelliana* and *P. fraudulenta* in Puget Sound (Hubbard et al., 2014) and of *P. delicatissima* in the Mediterranean Sea (Sahraoui et al., 2012).

In North Inlet, the abundance of *Pseudo-nitzschia* was also negatively correlated with the ammonium concentration and positively correlated with the phosphate concentration and pH (Table 2.3). Although a negative correlation with nitrogen is regularly observed (Schnetzer et al., 2007; Downes-Tettmar et al., 2013; Tas et al., 2016), a positive correlation with phosphate concentrations is less frequent. It was observed in the Yangtze River but was accompanied with a positive correlation with nitrate concentrations (Zou et al., 1993) and also noticed in the Bay of Fundy where *P. pungens* was positively correlated with phosphate concentrations and negatively correlated with nitrate concentrations as observed in this study. However, *P. pungens* was negatively correlated with salinity unlike *Pseudo-nitzschia* spp. in North Inlet (Kaczmarska et al., 2007).

Although the positive relationship between the *Pseudo-nitzschia* abundance with pH established in this study is in contradiction with Lundholm et al. (2004) observations, in which the lowest growth rate of *Pseudo-nitzschia multiseriis* in cultures was detected at pH 9.0, a positive correlation with pH was also observed in the Sea of Marmara in Turkey (Tas et al., 2016). However, no study reported the same combination of environmental factors as in North Inlet to explain the bloom of *Pseudo-nitzschia* in May, suggesting that the optimal conditions are not only species and strains specific but also depend on the location. The same conclusion was reached for the DA concentration.

In North Inlet, the initial dDA concentration was positively correlated with the phosphate concentration and negatively correlated with DIN : DIP ratio. Typically, the concentration of particulate and dissolved DA is negatively correlated with phosphate or positively correlated with the ratio DIN : DIP since it has been demonstrated that *Pseudo-nitzschia* produced more DA when phosphorus-stressed (Pan et al., 1998; Felhing et al., 2004; Sun et al., 2011; Liefer et al., 2013; Hubbard et al., 2014; Bargu et al., 2016; Tas et al., 2016). However, the released dDA by *Pseudo-nitzschia* positively and significantly correlated with the ammonium and nitrate + nitrite concentrations, which is consistent with the decrease in DA production in nitrogen limited conditions as nitrogen is necessary to produce DA (Bates et al., 1991).

Although *Pseudo-nitzschia* abundances and dDA concentrations were positively correlated with salinity in the control bottles, the correlation with the released DA, whether initially in the water column or in the control bottles, was negative (Table 2.6). One possible explanation is the reduction in size and volume of *Pseudo-nitzschia* cells at high salinity, thus producing less DA (Roubeix and Lancelot, 2008). As *Pseudo-nitzschia* was more abundant at high salinity, a higher dDA concentration was measured in the water column. It is also possible that the production of DA increased with salinity but *Pseudo-nitzschia*, instead of releasing dDA in the water column, stored DA inside the cell for osmotic reasons (Doucette et al., 2008; MacIntyre et al., 2011). Higher plants increase their amino acids content in response to high salinity, and this modulates their membrane permeability and ion uptake (reviewed in Rai, 2002). An increase in the amino acid content was also observed in two *Pseudo-nitzschia* species (Jackson et al., 1992). Therefore, the production of DA could help protect enzymes against salinity and avoid

enzyme denaturation as in higher plants. Another hypothesis is a reduction of the released dDA due to the decrease in carbon dioxide and bicarbonate ions, thus due to the increase in pH in the water column (MacIntyre et al., 2011), which is in accordance with the negative but not significant correlation between rDA and pH in this study.

This increase in *Pseudo-nitzschia* abundance and dDA concentrations at high salinity in North Inlet could impact the abundance of other phytoplankton groups and species which were negatively correlated with salinity, indicating a sensitivity to high salinity (Tables 2.3 and 2.4). The initial diatom abundance and particularly the abundances of *Rhizosolenia* and unidentified centric diatoms were negatively and significantly correlated with the initial dDA concentration suggesting a possible effect of dDA on the growth of phytoplankton particularly the diatoms (Table 2.3). However, the results of the seven incubations with the addition of a highly-concentrated solution of dDA demonstrated no direct effect of dDA on any phytoplankton group with inconsistencies between positive and negative responses from month to month and significant responses occurring only 1 out of the 7 times for most groups except for the cryptophytes (Fig 2.3). This is in accordance with the findings by Lundholm et al. (2005) where no allelopathic effect of the addition of $23 \mu\text{g DA ml}^{-1}$ was observed on nine different monocultures of phytoplankton species from many different groups and by Windust et al. (1992) where 50 and 50,000 ng DA ml^{-1} had no effect on the growth of two diatom species *Skeletonema costatum* and *Chaetoceros gracilis*.

In the field, environmental factors can play an indirect role in the effect of a toxin on phytoplankton as observed in studies on the allelopathic effect of phycotoxins produced by cyanobacteria (Brutemark et al., 2015; Śliwińska-Wilczewska et al., 2016).

In this study, while the percent inhibition of cyanobacteria and chlorophytes between the controls and the bottles with dDA added were negatively correlated with the nitrate + nitrite ratio, the percent of the total growth inhibition and the percent inhibition of diatoms and cryptophytes were positively correlated with salinity (Table 2.5). This could be explained by an increase in sensitivity to dDA by the phytoplankton and particularly by the cryptophytes and the diatoms at extreme salinities. However, only the percent of the total growth inhibition was also positively correlated with the remaining dDA (Table 2.5).

The remaining dDA in the treatment bottles, positively correlated with salinity, was observed to be the highest at salinities 35 and 37 and to be the lowest and below 1 at salinities 33 and 34, suggesting an intensification of the degradation of dDA as salinity declined (Fig 2.4D). Bates et al. (2003) also obtained a slightly lower percent degradation of DA in deionized water with a ratio of 0.36 compared to seawater where a ratio of 0.41 was obtained. But because the difference was not significant, the salinity factor was not considered in other studies on DA degradation (Fisher et al., 2006; Hagström et al., 2007; Sison-Mangus et al., 2014).

As demonstrated in this study, salinity affected the growth of most phytoplankton groups providing *Pseudo-nitzschia* with an advantage to thrive possibly due to the production of DA as an osmolyte. Although releasing less dDA at high salinity, the higher concentration in the water column corresponding to a higher abundance of *Pseudo-nitzschia* reduced the abundance of the diatoms and the cryptophytes more importantly as salinity increased. This was accompanied by a decrease in the degradation of dDA at high salinity. Therefore, not only may salinity play a key role in the inhibition

of different phytoplankton groups exposed to dDA, it could also be important in the degradation of the toxin, and, consequently, its residence time in the water column. As an indirect effect of climate change, salinity alterations have already been observed and are predicted to intensify worldwide (IPCC, 2007), which, as a result, could impact the abundance of toxic species of *Pseudo-nitzschia*, the production of DA, the toxin residence time in the water column and the effect of DA on phytoplankton communities particularly on diatoms and cryptophytes.

Table 2.1 Averaged initial phytoplankton abundances (in cell ml⁻¹) from May to September 2016, acquired by microscopy (N = 2). Other diatoms included the genera *Thalassosira*, *Thalassionema*, *Leptocylindrus*, *Asterionellopsis*, *Guinardia* and *Pleurosigma*, with each genus representing less than 10% of the total diatom abundance in all samples. No samples were collected in October and November

	May	Jun	Jul	Aug	Sep
Flagellates	722	386	403	663	568
<i>Skeletonema</i>	292	128	217	1119	1262
<i>Chaetoceros</i>	236	39	13	618	316
<i>Rhizosolenia</i>	17	156	566	352	53
<i>Cylindrotheca</i>	108	10	92	127	491
<i>Pseudo-nitzschia</i>	1032	84	112	34	100
Unidentified centric diatoms	169	861	862	955	608
Other diatoms	621	371	447	553	324

Table 2.2 Initial parameters at collection site from May to November 2016

	May	Jun	Jul	Aug	Sep	Oct	Nov
Temperature (°C)	22.1	26.9	27.6	32.1	29	24.3	15.7
Salinity	37	35	34	35	33	38	35
Nitrate + nitrite ($\mu\text{mol l}^{-1}$)	2.05	2.10	2.02	2.09	2.14	-	-
Ammonium ($\mu\text{mol l}^{-1}$)	3.64	4.25	4.20	4.64	4.61	-	-
Phosphate ($\mu\text{mol l}^{-1}$)	3.94	3.45	3.55	3.62	3.68	-	-
pH	7.98	7.03	6.85	6.70	7.08	7.90	7.80
dDA (ng ml^{-1})	1.21	0.28	0.32	0.12	1.11	-	-
PAR at sampling time ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	96	1182	1094	1713	1589	614	580
Monthly cumulative precipitation (mm)	0	166	120	158	349	145	27
<i>Pseudo-nitzschia</i> abundance (cell ml^{-1})	1032	84	112	34	100	-	-
Total cell abundance (cell ml^{-1})	3260	7560	11057	23253	24837	23580	4819

Table 2.3 Pearson's correlation factors from initial parameters and initial phytoplankton abundances obtained from CHEMTAX. Bold numbers represent significant correlations ($p < 0.05$)

	Salinity	Temperature	Nitrate + nitrite	Ammonium	Phosphate	DIN : DIP	dDA	pH	PAR	<i>Pseudo-nitzschia</i>
Cyanobacteria	-0.239	0.488	-0.580	0.269	-0.354	0.280	-0.665	-0.624	0.320	-0.421
Euglenophytes	-0.296	0.209	-0.690	0.057	-0.379	0.154	-0.504	-0.473	0.120	-0.337
Chlorophytes	-0.375	0.867	0.276	0.766	-0.169	0.560	-0.407	-0.652	0.743	-0.551
Cryptophytes	-0.121	0.811	0.366	0.693	-0.177	0.534	-0.486	-0.565	0.676	-0.459
Diatoms	-0.690	0.610	0.098	0.616	-0.947	0.830	-0.723	-0.859	0.689	-0.912
<i>Pseudo-nitzschia</i> abundance	0.796	-0.860	-0.373	-0.881	0.874	-0.976	0.656	0.959	-0.923	
Total cell abundance	-0.118	0.881	0.603	0.914	-0.207	0.674	-0.167	-0.386	0.663	-0.636
dDA	0.154	-0.590	0.183	-0.420	0.760	-0.609		0.761	-0.503	
rDA	-0.792	0.427	0.779	0.657	-0.123	0.506		-0.282	0.599	

Table 2.4 Pearson's correlation factors from initial parameters and initial phytoplankton abundances obtained from microscopy. Bold numbers represent significant correlations ($p < 0.05$)

	Salinity	Temperature	Nitrate + nitrite	Ammonium	Phosphate	DIN : DIP	dDA	pH	PAR	<i>Pseudo-nitzschia</i>
Flagellates	0.306	-0.082	0.056	-0.116	0.513	-0.311	0.477	0.309	-0.168	0.600
<i>Skeletonema</i>	-0.410	0.569	0.572	0.627	0.102	0.368	0.138	-0.285	0.562	-0.346
<i>Chaetoceros</i>	0.032	0.354	0.254	0.304	0.187	0.114	-0.033	-0.112	0.263	-0.076
<i>Rhizosolenia</i>	-0.286	0.435	-0.552	0.249	-0.505	0.344	-0.717	-0.647	0.317	-0.511
<i>Cylindrotheca</i>	-0.551	0.229	0.564	0.411	0.211	0.180	0.552	-0.029	0.335	-0.153
Unidentified centric diatoms	-0.335	0.497	0.066	0.448	-0.547	0.537	-0.868	-0.585	0.489	-0.924
Other diatoms	0.417	-0.166	-0.284	-0.277	0.325	-0.342	0.063	0.236	-0.285	0.674

Table 2.5 Pearson's correlation coefficients from ratios calculated between the treatment and the control bottles and the percent inhibition of each phytoplankton group. Bold numbers represent significant correlations ($p < 0.05$)

	Salinity	Nitrate + nitrite ratio	Ammonium ratio	Phosphate ratio	DIN : DIP ratio	dDA remaining	pH ratio
% inhibition cyanobacteria	-0.504	-0.941	-0.810	0.054	-0.940	-0.338	0.296
% inhibition euglenophytes	0.316	0.423	0.610	-0.746	0.598	0.475	-0.764
% inhibition chlorophytes	-0.494	-0.942	-0.844	-0.197	-0.941	-0.340	0.301
% inhibition cryptophytes	0.728	0.799	0.929	-0.059	0.859	0.428	-0.353
% inhibition diatoms	0.622	0.212	0.691	-0.201	0.316	0.518	0.064
Total % growth inhibition	0.603	-0.064	0.471	0.242	0.056	0.649	0.634

Table 2.6 Pearson's correlation coefficients between the salinity with the dDA concentrations, the remaining dDA and the released dDA from the initial conditions and from the final conditions in the control and the treatment bottles. Bold numbers represent significant correlations ($p < 0.05$)

Treatment	dDA concentration (ng DA ml ⁻¹)	Remaining dDA (ng DA ml ⁻¹)	rDA (ng DA cell ⁻¹)
Initial	0.154	-	-0.792
Final control	0.741	0.209	-0.710
Final dDA addition	0.497	0.686	-0.145

Table 2.7 Initial and final *Pseudo-nitzschia* abundances and final abundances collected from each control and the bottles with added dDA (cell ml⁻¹)

	May	Jun	Jul	Aug	Sep	Oct	Nov
Salinity	37	35	34	35	33	38	35
Initial	1032	84	112	34	100	-	-
Final control	3219	226	240	0	31	338	158
Final dDA addition	3688	994	798	300	552	253	234

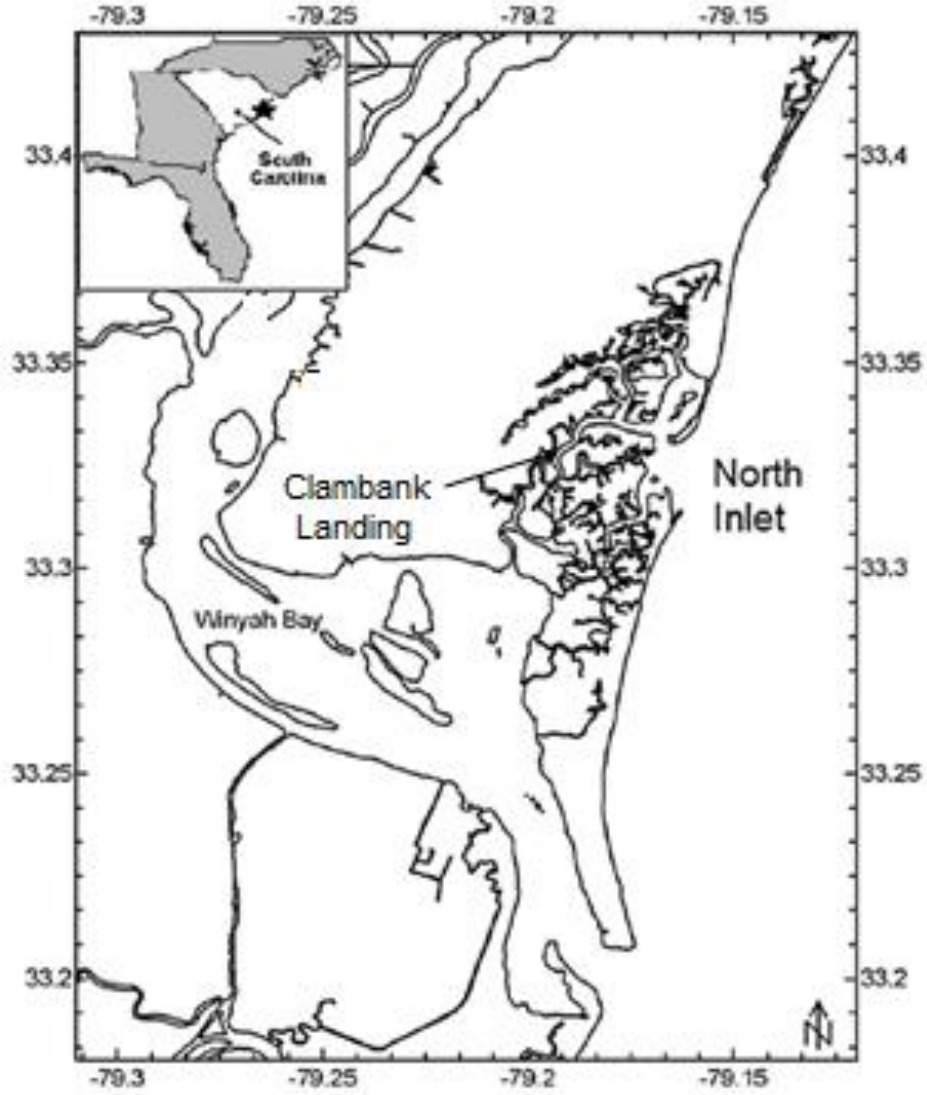


Fig 2.1 Site of water collection in North Inlet (Clambank Landing), South Carolina

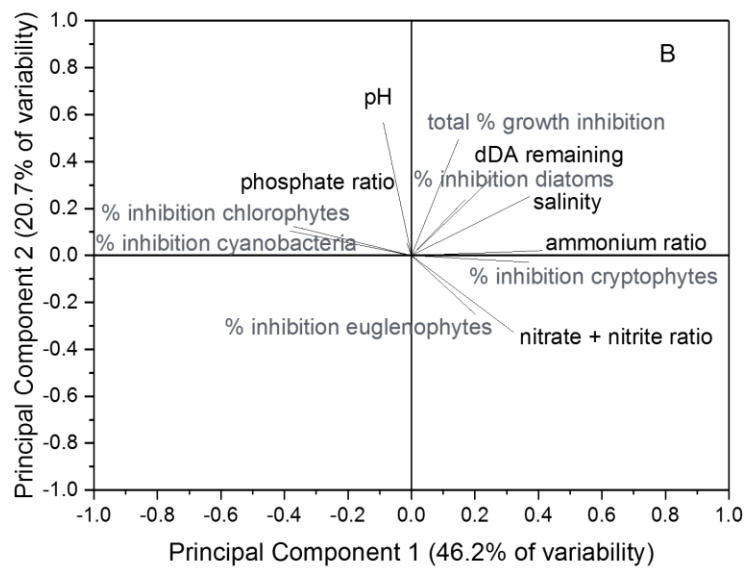
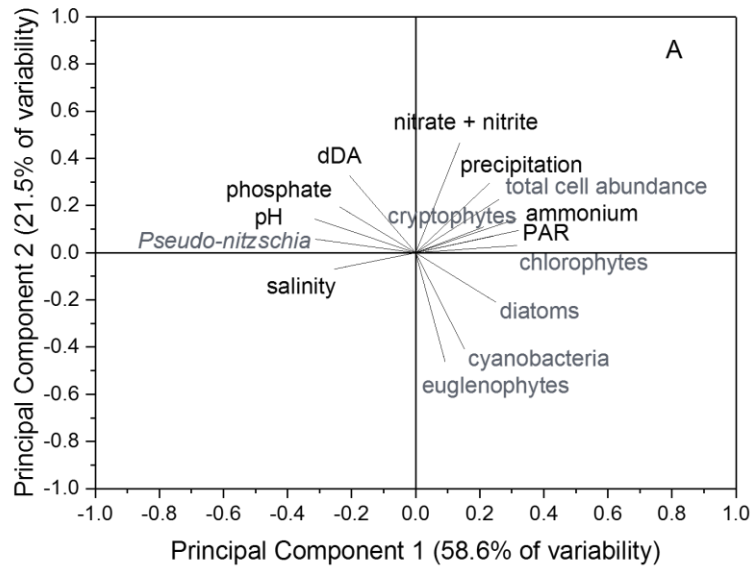


Fig 2.2 Eigenvectors of the initial environmental factors (in black) and of the initial abundances of each phytoplankton group and *Pseudo-nitzschia* (in grey) (A) and eigenvectors of the ratio of environmental factors between the control and the treatment bottles and the percent inhibition of the phytoplankton groups (in grey) (B)

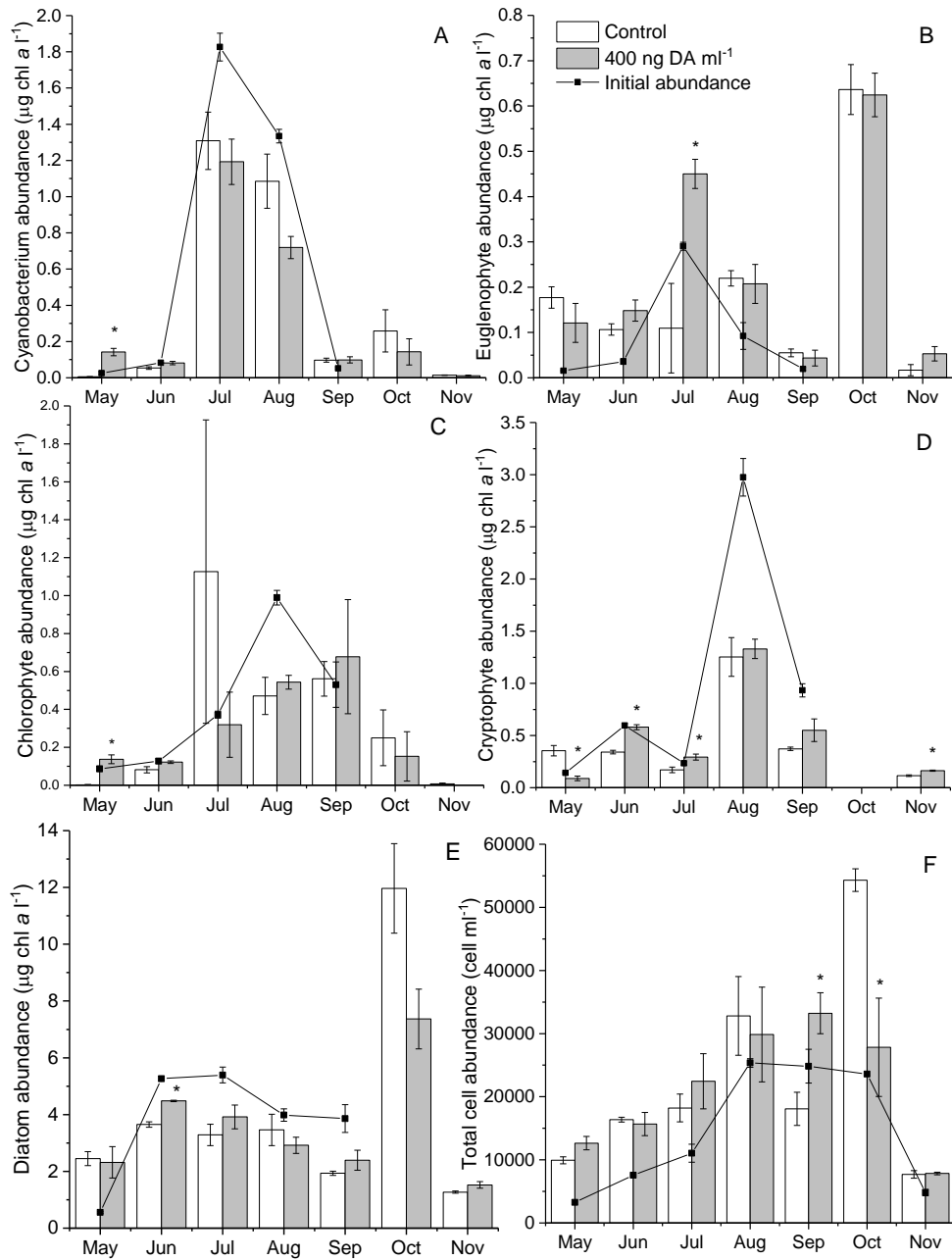


Fig 2.3 Phytoplankton abundances (mean \pm SE) from May to November 2016 collected at the beginning of each experiment (black line) and collected in the control bottles (white) and the treatment bottles (grey) at the end of each experiment with the abundances of cyanobacteria (A), euglenophytes (B), chlorophytes (C), cryptophytes (D), diatoms (E) and the total cell abundances (F). The star represents a significant difference of abundance between the control and the addition of dDA ($p < 0.05$). Note the differences in the y-axis scale between the different phytoplankton groups

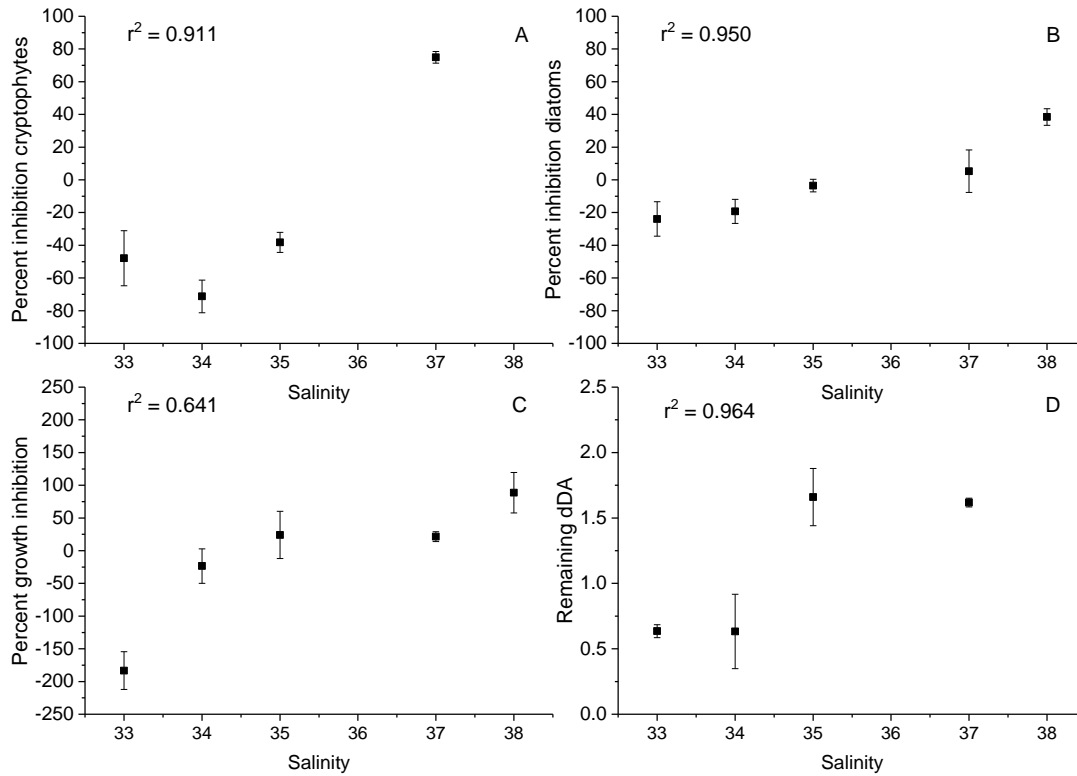


Fig 2.4 Percent inhibition (\pm SE) of cryptophytes (A) and diatoms (B) and the percent growth inhibition of the total cells (C) induced by the addition of dDA at different salinities, and the remaining dDA (\pm SE) from the treatment bottles at different salinities (D). Note the differences in the y-axis scale

CHAPTER 3
COASTAL EUTROPHICATION AND FRESHENING: IMPACTS ON
PSEUDO-NITZSCHIA ABUNDANCE AND DOMOIC ACID
ALLELOPATHY²

3.1 ABSTRACT

During phytoplankton bloom events, the diatom genus *Pseudo-nitzschia* may produce chronic and/or acute concentrations of the neurotoxin domoic acid (DA), which we hypothesized to be an allelochemical to outcompete the other phytoplankton species. Previous research in a nutrient depleted ecosystem demonstrated an increase in the allelopathic effect of dissolved DA (dDA) on cryptophytes and diatoms with salinity. But the potential for DA to be allelopathic in nutrient replete environments across a salinity gradient was not considered. In this study, we examined how salinity reductions and nutrient loading affected the abundance of *Pseudo-nitzschia*. We also investigated how river and nutrient inputs influenced the effect of dDA on phytoplankton community compositions as well as the influence of salinity on DA allelopathy. Through bioassays, we measured the phytoplankton responses to 400 ng DA ml⁻¹ at a low salinity site we detected an increase in *Pseudo-nitzschia* abundance with the addition of dDA and nutrients. DA allelopathy was observed to be more efficient at lower salinity in fresher

² Van Meerssche, E., D.I. Greenfield and J.L. Pinckney. 2017. Submitted to *Estuarine, Coastal and Shelf Science*, 12/15/2017

water. However, the effect of nutrient loading alone neutralized the influence of salinity on DA allelopathy. This study highlights how environmental factors such as salinity and nutrients can play important roles on a toxin allelopathy by alleviating or exacerbating its effect.

3.1 INTRODUCTION

Distributed worldwide, *Pseudo-nitzschia* is a cosmopolitan genus composed of 48 species identified to date and is able to grow at wide ranges of temperature (-1.5 – 30°C) and salinity (6 – 40) (Bates et al., 1998; Thessen et al., 2005; Teng et al., 2016). To date, 23 species of *Pseudo-nitzschia* have been recognized as producers of the neurotoxin domoic acid (DA) (Teng et al., 2016). The consumption of DA through contaminated shellfish, fish and crustaceans can cause amnesic shellfish poisoning (ASP), a syndrome affecting birds, marine mammals and even humans (Wright et al., 1989; Thessen and Stoecker, 2008). Although species have been isolated from the North Pacific and Antarctic waters, most species have been detected in coastal regions causing a threat to wildlife and human health (Marchetti et al., 2006; Lelong et al., 2012; Trainer et al., 2012).

During the past few years, toxic *Pseudo-nitzschia* blooms have increased in frequency and magnitude. As a recent example, an unprecedented *Pseudo-nitzschia* bloom was observed along the west coast of the U. S. in 2015 (Du et al., 2016; McCabe et al., 2016). During this event, the abundance of *Pseudo-nitzschia* reached almost 2,500 cells ml⁻¹ and the concentration of dissolved DA (dDA) reached up to 980 ng ml⁻¹ in the water column (McCabe et al., 2016; Kudela et al., 2017). In addition to the influence of warmer temperatures, this dense bloom appeared during the upwelling season in April,

favoring the flourishing of the toxigenic species *Pseudo-nitzschia australis* (McCabe et al., 2016). Increases in nutrients, particularly nitrogen, often stimulates blooms of *Pseudo-nitzschia* spp. in upwelling regions, the Chesapeake Bay, the Gulf of Mexico, and Los Angeles Harbor (Schnetzer et al., 2007; Thessen and Stoecker, 2008; Anderson et al., 2009; Trainer et al., 2012; Bargu et al., 2016). This suggests that pulses of nutrients can lead to the spread of toxic blooms of *Pseudo-nitzschia* intensifying the production of DA and increasing the concentration of dissolved DA (dDA) in the water column.

Salinity also influences the abundance of *Pseudo-nitzschia* and its toxicity. In several coastal systems such as the upwelling regions, the Chesapeake Bay and the Gulf of Mexico, the abundance of *Pseudo-nitzschia* was positively correlated with salinity (Thessen and Stoecker, 2008; MacIntyre et al., 2011; Trainer et al., 2012; Liefer et al., 2013; Bargu et al., 2016). However, this relationship depends on the regions as well as on the species and strains. For example, *Pseudo-nitzschia multiseriata* and *P. pungens* abundances increased with salinity along the Uruguayan coast and in the Sea of Marmara in Turkey but decreased with salinity in the Puget Sound, the Bay of Fundy, and the English Channel (Kaczmarek et al., 2007; Méndez et al., 2012; Downes-Tettmar et al., 2013; Hubbard et al., 2014; Tas et al., 2016). DA production is also often associated with high salinity (Anderson et al., 2009; MacIntyre et al., 2011; Sahraoui et al., 2012; Bargu et al., 2016; Tas et al., 2016).

Toxic *Pseudo-nitzschia* cells have also been observed in lower salinity systems such as the Juan de Fuca region, in the San Juan Islands and the Zrmanja River Estuary (Eastern Adriatic Sea), where salinity can be as low as 8 (Rines et al., 2002; Burić et al., 2008; Trainer et al., 2009). Freshwater discharge is also an important factor for *Pseudo-*

nitzschia blooms in the Chesapeake Bay (Anderson et al., 2010). Another example is the Columbia River plume, that enables toxic *Pseudo-nitzschia* to acclimate to lower salinities as it transports cells along the Washington coast (Trainer et al., 2009; Hickey et al., 2013, PICES report, 2017). Although the production of DA is often reduced at low salinity, relatively high concentrations of particulate DA were measured in fresher water (Thessen et al., 2005; Hickey et al., 2013). Among other hypotheses to explain the ecological reason for its production, DA may have allelopathic properties.

In the ocean, phytoplankton cells compete for light and nutrients, and the production of allelochemicals (i.e. compounds excreted by phytoplankton species that inhibit or stimulate the growth of other phytoplankton species) provides an ecological mechanism to outcompete other phytoplankton species. It can restrain a competitor's growth through a variety of processes such as cell lysis, cell shape alterations, and photosynthesis inhibition (reviewed in Legrand et al., 2003). Often neglected as a factor in the succession of phytoplankton species, allelopathy may play a major role in the regulation of phytoplankton diversity and in the expansion of harmful algal blooms (HABs) (Legrand et al., 2003; Granéli et al., 2008; Roy, 2015).

The first monoculture-based studies on DA allelopathy resulted in an absence of effect of dDA on different phytoplankton groups including species of diatoms, prymnesiophytes, euglenophytes, dinoflagellates, raphidophytes, prasinophytes, and cryptophytes (Windust, 1992; Lundholm et al., 2005). But more recently, studies have established an allelopathic effect of *Pseudo-nitzschia* in co-cultures with species of dinoflagellates, raphidophytes, haptophytes and cryptophytes (Xu et al., 2015) as well as on the diatom *Bacillaria* spp. in iron depleted medium (Sobrinho et al., 2017). Prince et

al. (2013) also identified a growth inhibition of the diatom species *Skeletonema marinoi* Sarno & Zingone induced by the direct addition of dDA in iron depleted medium.

This rekindled an interest in the study of DA allelopathy including on natural phytoplankton communities, which is necessary to understand the role of DA in interspecific competition. A previous study on DA allelopathy using microcosm experiments conducted in a high salinity and nutrient depleted ecosystem demonstrated an increase in the inhibition of cryptophytes and diatoms as salinity increased (Van Meerssche and Pinckney, 2017). However, DA allelopathy in nutrient replete environments, whether in saltwater or freshwater systems was not considered. This is important as studies reported a decrease in allelopathy when target cells were in nutrient excess conditions (Granéli and Johansson, 2003; Fistarol et al., 2005).

In our study, the influence of river and nutrient inputs on *Pseudo-nitzschia* abundance was first established. Then, through bioassays with phytoplankton exposed to an acute dDA concentration, we determined the effect of dDA on the phytoplankton community composition from a low salinity and nutrient replete site. We hypothesized that in environments with fresher water the allelopathic effect of dDA will be minimal as it was previously found to decrease with salinity (Van Meerssche and Pinckney, 2017). Finally, we established the effect of dDA on the phytoplankton community from a high salinity site through bioassays with the addition of an acute dose of dDA simultaneously with the addition of nitrate and phosphate. We suggested that DA allelopathy will be lower in ecosystems with excess nutrients as demonstrated in a few studies (Granéli and Johansson, 2003; Fistarol et al., 2005). This research highlights the complexity of establishing the allelopathic effect of a toxin in natural environments. Multiple factors

such as salinity and nutrients can influence concurrently, sometimes in contradictory ways, a toxin allelopathy.

3.3 MATERIALS AND METHODS

Study sites

Winyah Bay, near Georgetown, South Carolina, is a 65 km² temperate-subtropical coastal plain estuary, partially mixed with semi-diurnal tides (Fig. 3.1). This estuary receives freshwater and materials from four rivers (Pee Dee, Waccamaw, Black, and Sampit) with the Pee Dee River contributing ca. 90% of the total discharge into the bay (Sea Grant Consortium, 1992; Patchineelam et al., 1999). Winyah Bay is surrounded by industrial, agricultural, forested, natural and managed wetlands and has a total drainage of 47,000 km² across Virginia, North and South Carolina. The salinity ranges from 0.6 to 8.4 during tidal cycles (Sea Grant Consortium, 1992). The phytoplankton community is mostly composed of diatoms and cryptophytes with variable contributions of cyanobacteria, chlorophytes, euglenophytes and dinoflagellates (Lewitus et al., 1998; Lawrenz et al., 2010; Reed et al., 2016). Winyah Bay is a nutrient replete estuary with concentrations of orthophosphate, ammonium and nitrate respectively two, eight and thirty times more elevated than in North Inlet (Sea Grant Consortium, 1992; Reed et al., 2016).

North Inlet, located roughly 10 km northeast of Winyah Bay, is a tidally dominated bar-built estuary (Fig 3.1). Surrounded by only a few residential communities, North Inlet is a relatively undisturbed salt marsh with low freshwater input. This estuary is characterized by numerous shallow tidal creeks, mudflats, sand flats, *Crassostrea virginica* oyster reefs, and bordered by the cordgrass *Spartina alterniflora* (Sea Grant

Consortium, 1992; Allen et al., 2014). Salinity is higher than in Winyah Bay and ranges from 15 to 35 (Dame et al., 1991). The phytoplankton community is mostly composed of diatoms with variable contributions of cryptophytes, cyanobacteria, chlorophytes, euglenophytes and dinoflagellates (Lawrenz et al., 2013). The concentrations of orthophosphate, nitrate + nitrite and ammonium average $0.30 \mu\text{mol l}^{-1}$, $0.55 \mu\text{mol l}^{-1}$ and $2.50 \mu\text{mol l}^{-1}$ respectively (Wolaver et al., 1984; Sea Grant Consortium, 1992; Allen et al., 2014). With concentrations of silicate usually over $25 \mu\text{mol l}^{-1}$, silicate is not considered as a limiting nutrient in North Inlet (White et al., 2004; Wetz et al., 2006).

Phytoplankton bioassays

Water samples from Winyah Bay (the Georgetown Marina; 33.3652° N , 79.2663° W) and North Inlet (Clambank Landing; 33.3341° N , 79.1929° W) were collected monthly during high tide in 10 liter carboys from May to October 2016 in Winyah Bay and from May to September 2016 in North Inlet (Fig. 1). From each site, 250 ml of water were transferred into 6 Nalgene square polycarbonate clear bottles (250 ml) with 3 bottles for the controls and 3 for the addition of dDA. Simultaneously to the addition of dDA in North Inlet, volumes of an initial 10 mmol l^{-1} of nitrate (NaNO_3) solution and of an initial 5 mmol l^{-1} of phosphate (NaH_2PO_4) solution were added into all bottles to reach a final concentration of $20 \mu\text{mol l}^{-1}$ of nitrate and $10 \mu\text{mol l}^{-1}$ of phosphate. Volumes of an initial $10 \mu\text{g DA ml}^{-1}$ solution, obtained by dissolving 5 mg of DA (Sigma-Aldrich cat. no. D6152) in 500 ml of high purity de-ionized water, were added into the triplicate treatment bottles to reach a final concentration of $400 \text{ ng DA ml}^{-1}$. Although not the highest concentration observed in the field, 400 ng ml^{-1} is a relatively high concentration (Lundholm et al., 2005; Kudela, 2017).

Winyah Bay water was incubated in the lab for 2 days. Light for incubations was supplied using a 91 cm, 4 × 39 W Ocean Light T5 hood (10,000K 39W – TRU fluorescent lamps) to achieve an irradiance of ca. 130 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Light was cycled to correspond to times of sunrise and sunset on the dates the water samples were collected. Temperature was a constant 22°C. North Inlet water was incubated outside the Belle W. Baruch Institute for 2 days in water tables covered with two layers of fiberglass screen to simulate *in situ* temperature and irradiance (50% of full irradiance).

Analyses

Salinity was measured at the beginning of each experiment using a refractometer. The initial and final photopigment concentrations were measured by collecting 100 ml of water from the 10-l carboy at time 0 and from each triplicate at the end of the incubations. Water was filtered through a 25-mm dia. Whatman GF/F glass fiber filter under gentle vacuum. The filters were transferred into 2-ml microcentrifuge tubes and stored at -80°C until photopigment analysis by high performance liquid chromatography (HPLC). For the analysis, filters were lyophilized for 18 - 24 hours at -50°C then extracted for 12 - 20 hours at -20°C by adding 750 μl of 90% acetone. A volume of 50 μl of the synthetic carotenoid β -apo-8'carotenal, used as an internal standard, was also added to the solution. Filtered extracts (250 μl) were injected into a Shimadzu HPLC with a monomeric column (Rainin Microsorb-MV, 0.46 x 10 cm, 3 μm) and a polymeric (Vydac 201TP54, 0.46 x 25 cm, 5 μm) reverse phase C18 column. The mobile phase was composed of two solvents; solvent A, 80% methanol : 20% 0.5 M ammonium acetate and solvent B, 80% methanol : 20% acetone (Pinckney et al., 1996).

Pigment peaks were identified by comparing retention times and absorption spectra with pigment standards (DHI, Denmark). The photopigment concentrations were analyzed with the program CHEMTAX (v. 1.95) to determine the absolute abundance of major phytoplankton groups (in $\mu\text{g chl } a^{-1}$) (Mackey et al., 1996; Higgins et al., 2011). The initial pigment ratio matrix was derived from two coastal phytoplankton matrices (Schlüter et al., 2000; Lewitus et al., 2005). The convergence procedure outlined by Latasa (2007) was used to minimize errors in algal group biomass due to inaccurate pigment ratio seed values. Photopigments from each experiment were analyzed separately and provided the abundance of 7 algal groups (cyanobacteria, euglenophytes, chlorophytes, prasinophytes, dinoflagellates, cryptophytes, and diatoms). However, dinoflagellates were detected in very low abundance in both sampling sites, therefore, they were excluded from further analysis.

In each sample, the percent inhibition of the different phytoplankton groups was calculated with the following equation:

$$\% \text{ inhibition} = 100 \left(1 - \frac{\text{abundance}_{\text{treatment}}}{\text{averaged abundance}_{\text{control}}} \right)$$

Concentrations of dDA were measured at time 0, right after the addition of dDA and at the end of each experiment. To measure the initial dDA concentration, 10 ml of water from the 10-l carboy were collected, and to measure the concentration just after the addition of dDA and at the end of each experiment, 2 ml from each triplicate were combined. Samples were filtered through a 25-mm dia. Whatman GF/F glass fiber filter and stored at -20°C in amber polyethylene Nalgene bottles until dDA analysis. Only dissolved DA was analyzed; no particulate DA was measured. The samples were

prepared following the Biosense protocol and analyzed with enzyme-linked immunosorbent assay (ASP cELISA) kits from Biosense (Kleivdal et al., 2007).

The initial and final abundances of *Pseudo-nitzschia* were determined by collecting 40 ml of water from the 10-l carboy at time 0, and by collecting and combining 10 ml from each triplicate at the end of the incubations. Samples were preserved with Lugol's iodine. After settling each sample for a minimum of 6 hours in a 10-ml chamber, a minimum of a total of 400 cells were counted. *Pseudo-nitzschia* cells were then identified and counted using an inverted light microscope at x 200 and x 400 magnifications (Nikon Eclipse TS100) (Lund et al., 1958).

The initial nutrient concentrations were measured in North Inlet and data can be retrieved from Van Meerssche and Pinckney (2017). In Winyah Bay nitrate, ammonium, phosphate and silicate are not considered as limiting in this system, therefore, their concentrations were not measured.

All homogenous and normally distributed data were analyzed using parametric tests (multifactor and multivariate ANOVA). However, when transformed data were neither normally distributed nor their variances were homogenous, non-parametric Kruskal-Wallis tests were performed. All statistical analyses including ANOVA and Pearson's correlation tests were completed using SPSS Software 24.0 (IBM Corporation, Armonk, New York).

3.4 RESULTS

***Pseudo-nitzschia* abundances and domoic concentrations at both sites**

The initial dDA concentrations reported in Winyah Bay, the low salinity site, were low and ranged from 0.04 to 0.59 ng ml⁻¹ with a *Pseudo-nitzschia* abundance

between 10 and 53 cells ml⁻¹ (Table 3.1). In North Inlet, the high salinity site, initial dDA concentrations ranged from 0.12 to 1.21 ng ml⁻¹ with *Pseudo-nitzschia* abundance between 34 and 1032 cells ml⁻¹ (Table 3.1). At high salinity, the addition of nitrate and phosphate increased the abundance of *Pseudo-nitzschia* in May, July and September 2016 suggesting a positive response to nutrient loading by *Pseudo-nitzschia* (Table 3.1). Interestingly, at both sites the addition of dDA increased the abundance of *Pseudo-nitzschia* (Tables 3.1).

Phytoplankton response to the addition of dDA in low salinity water

The addition of dDA significantly stimulated the abundance of cyanobacteria and cryptophytes but only in May and July respectively (Fig 3.1A and D). Euglenophytes and diatoms demonstrated different significant responses to dDA over the different months. The abundance of euglenophytes was significantly stimulated in May but inhibited in June (Fig 3.1B). The abundance of diatoms was significantly inhibited in June and in September but was stimulated in July (Fig 3.1E). No significant difference in the abundance of chlorophytes and prasinophytes between the control and the treatment bottles was observed (Fig 3.1C and F).

Phytoplankton response to the addition of dDA in high salinity water enriched with nutrients

Only three groups responded to the addition of dDA. It significantly stimulated the abundance of euglenophytes but only in June (Fig 3.2B). It also significantly increased the abundance of cryptophytes in May but significantly decreased it in June (Fig 3.2D). It also inhibited the abundance of prasinophytes in June (Fig 3.2F). The other

groups including cyanobacteria, chlorophytes and diatoms demonstrated no significant response to dDA compared to the controls (Fig 3.2A, C and E).

Effect of salinity on the domoic acid allelopathy at both sites

As previously demonstrated, salinity can play an important role in DA allelopathy on certain phytoplankton groups initially stressed by an elevation in salinity (Van Meerssche and Pinckney, 2017). Therefore, a first Pearson's correlation test between the salinity and the initial phytoplankton abundances was performed for both sites (Table 3.2). At the low salinity site, the initial abundance of most phytoplankton groups was positively correlated with salinity with only a significant correlation with the initial abundance of cryptophytes. At the high salinity, the opposite was observed with the initial abundance of most phytoplankton groups negatively correlated with salinity. Only a significant correlation with the initial abundance of diatoms was observed (Table 3.2). A second Pearson's correlation test was completed between the salinity and the percent inhibition of each phytoplankton group (Table 3.3). Strong and negative correlations between salinity and the percent inhibition of cryptophytes ($r = -0.894$, $p < 0.05$) and of diatoms ($r = -0.812$, $p < 0.05$) were observed (Table 3.3). A linear fit demonstrated strong and negative linear relationships between the salinity and the percent inhibition of cryptophytes ($r^2 = 0.800$) and diatoms ($r^2 = 0.659$) (Fig 3.3). For both phytoplankton groups, the highest percent inhibition was obtained at salinity 0 with an average of $17.2 \pm 3.5\%$ for cryptophytes and $32.6 \pm 5.1\%$ for diatoms.

At the high salinity site enriched with nutrients, no significant correlation was observed between salinity with the percent inhibition of cryptophytes and diatoms. Only a strong negative correlation was observed with the percent inhibition of prasinophytes (r

= -0.602, $p < 0.05$) (Table 3.3). However, the negative linear relationship between the percent inhibition of prasinophytes with salinity was weak ($r^2 = 0.362$) (Fig 3.4).

3.5 DISCUSSION

At the low salinity site, measurements of dDA suggest the presence of toxic *Pseudo-nitzschia* species in relatively fresh water (salinity 0 – 6). However, *Pseudo-nitzschia* abundance was low (Table 3.1) and appeared only as single cells rather than in chains (pers. obs.). Furthermore, comparing the initial and the final abundances in the controls, *Pseudo-nitzschia* abundance mostly decreased (Table 3.1). It indicates a possible transport of *Pseudo-nitzschia* cells from the mouth to the bay during tidal cycles but a difficult acclimation to low salinity as previously shown by Thessen et al. (2008). From the cell morphology observed with a light inverted microscope, *Pseudo-nitzschia pungens* seemed to be the dominant species. It is usually a non-toxic species regularly detected in South Carolina and able to grow at a salinity between 6 and 30 (Jackson et al., 1992; Shuler et al., 2012). The absence of correlation between the initial dDA concentrations and *Pseudo-nitzschia* abundances also suggests the presence of non-toxic species (data not shown).

River inputs not only freshen the water column but also bring nutrients (e.g. nitrogen, phosphorus, organic matter) into the system and can lead to the spread of toxic blooms of *Pseudo-nitzschia*. At the low salinity site, the effect of river inputs with the concurrent impact of freshening and nutrient loading on DA allelopathy could be observed. The addition of nutrients in a nutrient depleted ecosystem with low river runoff allowed us to observe the effect of nutrient loading alone on *Pseudo-nitzschia* abundance. The addition of nutrients mainly increased the abundance of *Pseudo-nitzschia* (Table

3.1). This was also observed along the coast of Washington where dense blooms of *Pseudo-nitzschia* were triggered by the addition of ammonium through the sewage system (Trainer et al., 2007). Sediment records also demonstrated a positive correlation between the abundance of *Pseudo-nitzschia* and nitrogen fluxes (Parsons et al., 2002).

Interestingly, the addition of dDA increased the abundance of *Pseudo-nitzschia* whether at low salinity or at high salinity and whether in nutrient replete environment (Table 3.1) or in nutrient depleted conditions (Van Meerssche and Pinckney, 2017). The same phenomenon was observed in monocultures of *Pseudo-nitzschia delicatissima* (Cleve) Heiden when exposed to 20 ng DA ml⁻¹ and in a microcosm study where the abundance of unidentified species of *Pseudo-nitzschia* increased by 20% when the phytoplankton community was exposed to 7.8 ng DA ml⁻¹ (Trick et al., 2010; Prince et al., 2013). It implies a beneficial role played by dDA in the growth of *Pseudo-nitzschia*.

DA has been hypothesized to bind with particulate iron Fe(III) in the water and by oxidation releases soluble Fe(II) bioavailable for *Pseudo-nitzschia* (Rue and Bruland, 2001; Wells et al., 2005; Lelong et al., 2013). Salt marshes like North Inlet and industrialized estuaries like Winyah Bay can be depleted in bioavailable iron, weakening the phytoplankton community (Miller and Kester, 1994; Kawaguchi et al., 1994; Lewitus et al., 2004). Therefore, the addition of dDA may have helped *Pseudo-nitzschia* to overcome the reduction in iron concentrations. Moreover, iron depletion has been demonstrated to play an important role in DA allelopathy by exacerbating its effect (Prince et al., 2013; Sobrinho et al., 2017).

It has also been previously observed that the allelopathic effect of dDA was influenced by salinity. The percent inhibition of cryptophytes and diatoms increased with

salinity in a nutrient depleted ecosystem (Van Meerssche and Pinckney, 2017). These two groups were negatively correlated with salinity, therefore, we suggested a possible stress by an elevation in salinity. With an increase in DA allelopathy with salinity, we hypothesized that DA was more effective at higher salinities as the target cells were more salinity-stressed at higher salinities. Although with an opposite trend, the same phenomenon was observed at the low salinity site replete by nutrients. At this site, diatom and cryptophyte abundances were positively correlated with salinity (Table 3.3), therefore they were suggested to be more salinity-stressed at lower salinities. Like at the high salinity site, we observed that DA allelopathy was more efficient when target cells were salinity-stressed (Table 3.3).

At the high salinity site with limited river runoff and enriched with nutrients, the influence of salinity on the allelopathic effect of dDA previously observed in Van Meerssche and Pinckney study (2017) was neutralized (Table 3.3). Sjöqvist and Kremp (2016) demonstrated that in salinity-stressed environments the increase in clonal richness allowed phytoplankton to maintain their primary production at the same rate as in the controls. Therefore, the increase in species richness induced by the addition of nutrients (Larson and Belovsky, 2013) may have helped reducing the effect of dDA on phytoplankton when cells were salinity-stressed. This is also in accordance with studies, whether field observations or culture experiments, that demonstrated a higher allelopathic activity in nutrient depleted environment (reviewed in Granéli et al., 2008).

While some phytoplankton species can resist the attacks of allelopathic compounds in some conditions, altering these conditions may weaken the phytoplankton strategies and make them more vulnerable to allelopathy (Prince et al., 2008).

Inconstancies in the phytoplankton response to allelochemicals are common in natural environments. Allelopathy can be mitigated by environmental factors as well as by competitors (Prince et al., 2008; Poulston-Ellestad et al., 2014). In this study, we demonstrated that nutrient loading alone could reduce DA allelopathy in estuaries with low river inputs as predicted from previous studies (Granéli et al., 2008). However, river inputs bringing fresh water in addition to nutrients may stress phytoplankton cells by lowering the salinity. This will possibly emphasize the effect of dDA on phytoplankton particularly on cryptophytes and diatoms. With the increase in *Pseudo-nitzschia* abundance with the addition of nutrients as well as its possible survival in freshwater, the increase in river inputs in some regions (IPCC, 2001) present a threat to the environment. This is particularly important for estuaries and bays with strong river runoff

Table 3.1 Initial salinity, domoic acid concentration and *Pseudo-nitzschia* (PN) abundance as well as the final *Pseudo-nitzschia* abundances from the control bottles and the bottles with added dDA (cell ml⁻¹) from both Winyah Bay and North Inlet. Initial data from North Inlet are from Van Meerssche and Pinckney (2017)

Site	Parameter	May	Jun	Jul	Aug	Sep	Oct
Winyah Bay	Salinity	1	0	6	1	0	0
	Initial dDA (ng ml ⁻¹)	0.59 ± 0.08	0.23 ± 0.08	0.19 ± 0.02	0.18 ± 0.03	0.04 ± 0.00	-
	PN Initial	53	19	19	10	49	-
	PN Final (control)	0	32	0	0	29	50
	PN Final (dDA addition)	71	83	124	0	36	90
North Inlet replete with nutrients	Salinity	37	35	34	35	33	-
	Initial dDA (ng ml ⁻¹)	1.21 ± 0.40	0.28 ± 0.11	0.32 ± 0.09	0.12 ± 0.15	1.11 ± 0.17	-
	PN Initial	1032	84	112	34	100	-
	PN Final (control)	2288	74	365	0	175	-
	PN Final (dDA addition)	3352	916	571	346	116	-

Table 3.2 Pearson's correlation test results between salinity with the initial total phytoplankton abundance as well as with the initial abundances of the different phytoplankton groups at both Winyah Bay and North Inlet. Bold numbers represent significant correlations ($p < 0.05$). North Inlet data are from Van Meerssche and Pinckney (2017)

	Winyah Bay	North Inlet
Cyanobacteria	-0.054	-0.239
Euglenophytes	0.098	-0.296
Chlorophytes	0.205	-0.375
Cryptophytes	0.968	-0.121
Diatoms	0.061	-0.690
Prasinophytes	0.319	-

Table 3.3 Pearson's correlation test results between salinity and the total percent growth inhibition and the percent inhibition of the different phytoplankton groups in Winyah Bay and North Inlet replete with nutrients. Nutrient depleted North Inlet data are from Van Meerssche and Pinckney (2017)

	Winyah Bay	North Inlet (nutrient replete)	North Inlet (nutrient depleted)
% inhibition cyanobacteria	-0.116	0.328	-0.504
% inhibition euglenophytes	0.019	-0.051	0.316
% inhibition chlorophytes	-0.375	0.508	-0.494
% inhibition cryptophytes	-0.894	-0.275	0.728
% inhibition diatoms	-0.812	-0.375	0.622
% inhibition prasinophytes	-0.153	-0.602	-

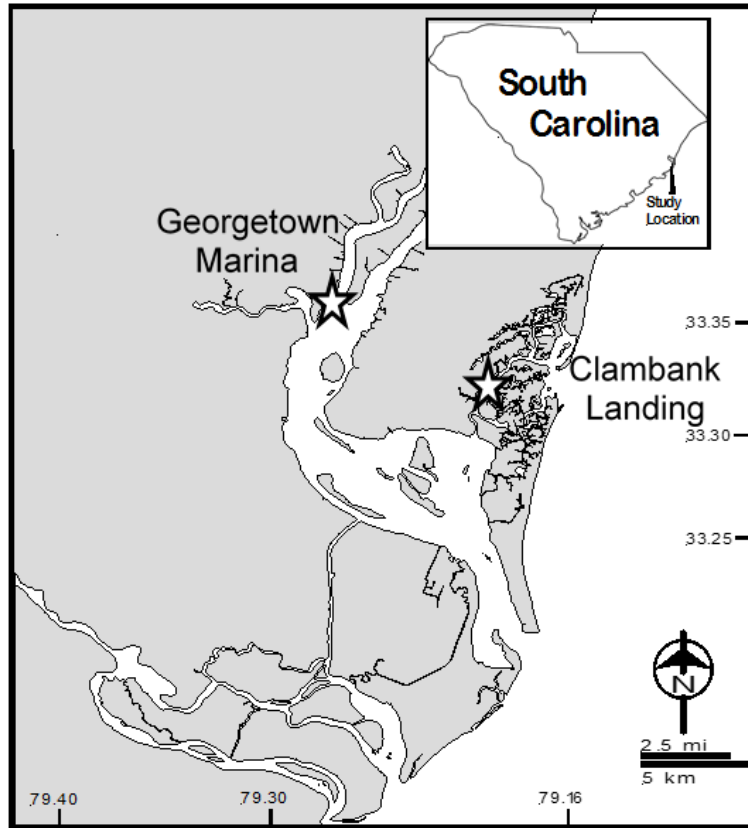


Fig 3.1 Map of the two collection sites Winyah Bay (the Georgetown marina) and North Inlet (Clambank Landing)

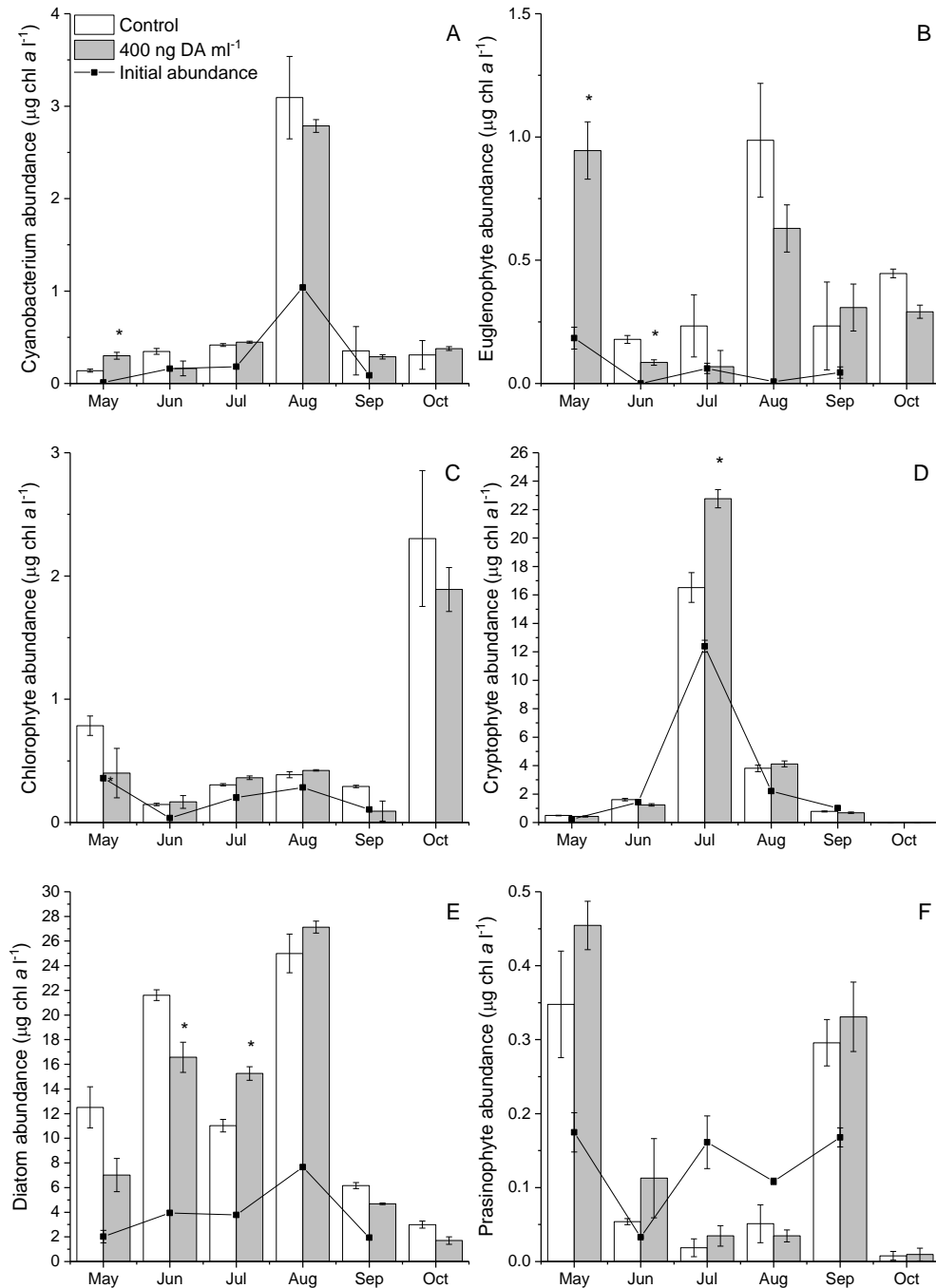


Fig 3.2 Abundances of cyanobacteria (A), euglenophytes (B), chlorophytes (C), cryptophytes (D), diatoms (E) and prasinophytes (F) collected in Winyah Bay from May to October 2016 at the beginning of each experiment (black line) and collected at the end of each experiment in the control bottles (white) and the treatment bottles (grey). The star represents a significant difference of abundance between the control and the addition of dDA ($p < 0.05$). Note the differences in the y-axis scale between the different phytoplankton groups

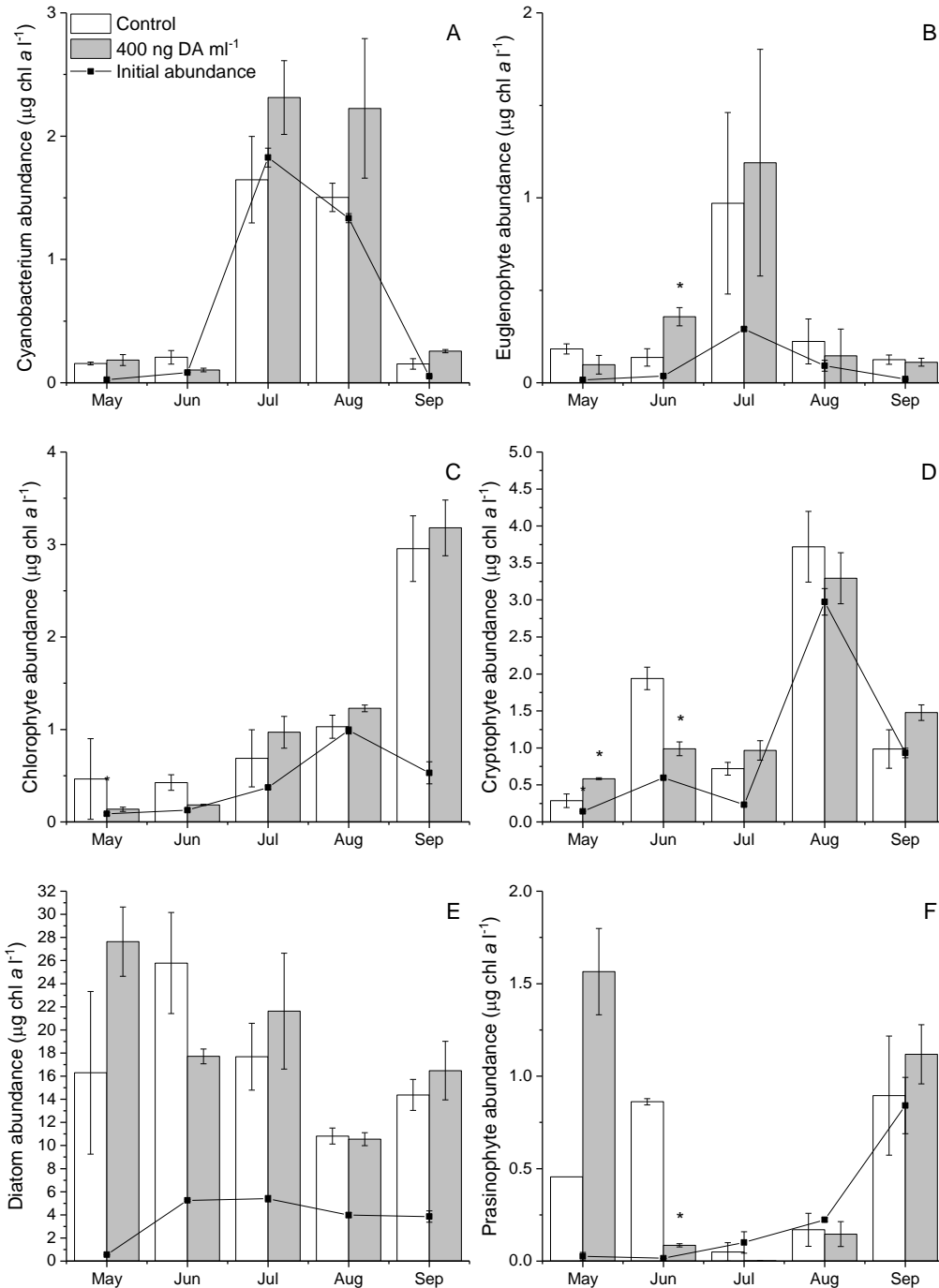


Fig 3.3 Abundances of cyanobacteria (A), euglenophytes (B), chlorophytes (C), cryptophytes (D), diatoms (E) and prasinophytes (F) collected in North Inlet replete with nutrient from May to September 2016 at the beginning of each experiment (black line) and collected at the end of each experiment in the control bottles (white) and the treatment bottles (grey). The star represents a significant difference of abundance between the control and the addition of dDA ($p < 0.05$). Note the differences in the y-axis scale between the different phytoplankton groups

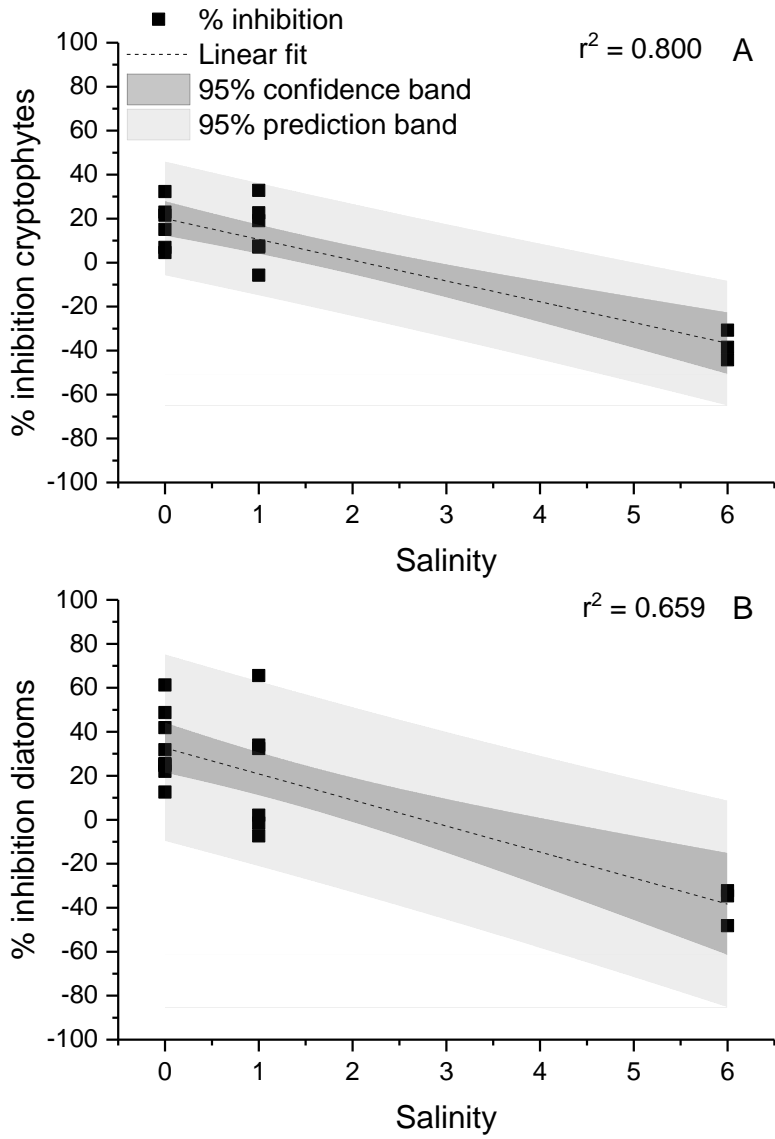


Fig 3.4 Percent inhibition of cryptophytes (A) and diatoms (B) induced by the addition of dDA at different salinities in Winyah Bay

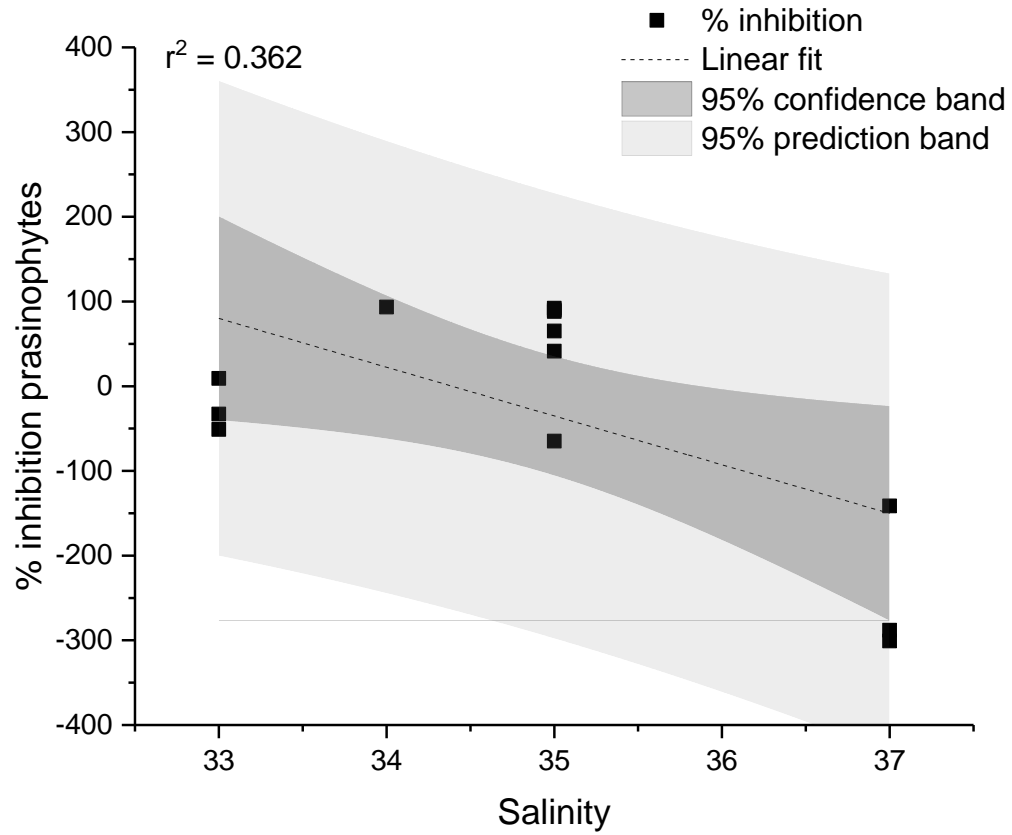


Fig 3.5 Percent inhibition of prasinophytes induced by the addition of dDA at different salinities in North Inlet replete with nutrients

CHAPTER 4
REGULATORY CRITICAL VALUES FOR PHYTOPLANKTON
BIOMASS NEGLECT SIGNIFICANT ALTERATIONS IN COMMUNITY
STRUCTURE³

4.1 ABSTRACT

With about 40% of the world's population living along the coastline, the acceleration of eutrophication endangers the economic and recreational use of estuarine ecosystems. Strategies have been developed to reduce nutrient loading in estuaries to limit the total concentration of chlorophyll *a* (chl *a*) to an arbitrary maximum concentration (e.g. 40 µg chl *a* l⁻¹). But the potential alterations in the phytoplankton community composition below this limit are not generally considered. In this study, the effects of moderate loadings of nitrate and phosphate on an unimpacted phytoplankton community was investigated through multiple bioassays from 2014 to 2016. Using photopigment biomarkers, we observed significant shifts in the phytoplankton community composition. However, the response from each phytoplankton group was unpredictable and differed depending on the season as well as the initial community composition. This research highlights the ecological risk of using a subjective chl *a*

³ Van Meerssche, E. and J.L. Pinckney. 2018. Submitted to *Estuaries and Coasts*, 01/29/2018

concentration based on total phytoplankton biomass for water quality criteria. Although more expensive and time consuming to measure compared to fluorescence, phytoplankton-based water quality criteria should ideally be based on the relative abundances of algal groups in addition to total biomass (as chl *a*).

4.2 INTRODUCTION

Eutrophication, defined as “an increase in the rate of supply of organic matter to an ecosystem” (Nixon 1995) is a process affecting coastlines worldwide. Although occurring naturally (e.g. river runoff, remineralization, upwelling), human activities have greatly accelerated its rate through anthropogenic activities (e.g. agriculture, industry, urbanization) resulting in high concentrations of nutrients in the water. With these pulses of combined nitrogen and phosphorus, dense phytoplankton blooms form and reduce light penetration. This limits the growth of macroalgae and submerged aquatic vegetation, diminishing habitats, nurseries, and refugia for multiple organisms (Paerl 2006; Chislock et al. 2013). Eutrophication also reduces phytoplankton species diversity and promotes uni-algal blooms, with a possible shift towards harmful species (Anderson et al. 2002; Bužančić et al. 2016; Heisler et al. 2008; Lim et al. 2017). Furthermore, microbial degradation of these phytoplankton blooms drastically decreases dissolved oxygen concentrations creating hypoxic or even anoxic conditions causing fish kills (Pinckney et al. 2001; Rabalais et al. 2009). The reduction of the overall water quality caused by eutrophication has disastrous consequences on human health (e.g. through drinking water) as well as the international economy (e.g. fisheries and tourism) (Pinckney et al. 2001; Smith and Schlinder 2009; Chislock et al. 2013). In the U.S. alone, 65% of estuaries present signs of medium to high eutrophication (Bricker et al. 1999;

Pinckney et al. 2001) and the cost of the damages has been estimated at \$2.2 billion every year (Dodds et al. 2009; Chislock et al. 2013).

To monitor eutrophication and assess water quality, governmental agencies measure the chlorophyll *a* (chl *a*) concentration, an easy, quick and relatively inexpensive parameter to evaluate the total phytoplankton biomass. An arbitrary chl *a* concentration is usually used as an indicator of eutrophic conditions based on the trophic state index, with a common regulatory limit of 40 $\mu\text{g chl } a \text{ l}^{-1}$ for estuaries (Bricker et al. 2003; <https://www.epa.gov>). However, this concentration limit may not be an ecologically sound regulation parameter as it only takes into account the total phytoplankton biomass, not the state of the phytoplankton community structure (Glibert 2017). Several studies have demonstrated that the addition of nutrients results in shifts in the phytoplankton community composition but few have specifically determined if significant shifts in composition occur at chl *a* concentrations lower than the common regulatory limit (Anderson et al. 2002; Heisler et al. 2008; Reed et al. 2016; Caron et al. 2017).

The goal of this study was to investigate the effect of nitrate and phosphate additions on an unimpacted, relatively pristine estuarine phytoplankton community through multiple bioassays from 2014 to 2016. For each bioassay, the effect of nutrient additions on phytoplankton community composition was determined through changes in the different photopigment concentrations used as specific biomarkers for different phytoplankton groups. We hypothesized that nutrient loading, although enhancing chl *a* concentrations below the 40 $\mu\text{g l}^{-1}$ threshold, would result in a significant alteration in the phytoplankton community composition. This may have cascading impacts on the biota and on processes essential for ecosystem services such as biogeochemical cycling,

trophodynamics and primary productivity (Sarmiento et al. 2004; Beardall and Stojkovic 2006; Finkel et al. 2010).

4.3 MATERIALS AND METHODS

Study site

North Inlet, a tidally dominated bar-built estuary near Georgetown, South Carolina, USA is a relatively undisturbed salt marsh with low freshwater input. This estuary is characterized by numerous shallow tidal creeks, mudflats, sand flats, *Crassostrea virginica* oyster reefs, and bordered by the cordgrass *Spartina alterniflora* (Sea Grant Consortium, 1992; Allen et al., 2014). Over an annual cycle, water temperature ranges from 8°C to 30°C and the salinity from 15 to 35 (Dame et al., 1991). The concentrations of phosphate, nitrate + nitrite and ammonium average 0.30 $\mu\text{mol l}^{-1}$, 0.55 $\mu\text{mol l}^{-1}$ and 2.80 $\mu\text{mol l}^{-1}$ respectively (Allen et al., 2014). The concentration of silicate is usually over 25 $\mu\text{mol l}^{-1}$ and is not considered as a limiting nutrient in North Inlet (White et al., 2004; Wetz et al., 2006). The microalgal community is mostly composed of diatoms with seasonally variable contributions of cryptophytes, cyanobacteria, chlorophytes, euglenophytes and dinoflagellates (Lewitus et al., 1998; 2005; Lawrenz et al., 2013; Allen et al., 2014).

Microalgal bioassays

Water samples from North Inlet (Clambank Landing, 33.3500°N, 79.1902°W) were collected in 10-liter carboys during high tide in May, June and November 2014, in May, June, July, September and October 2015 and in February, April, May, June, July and September 2016. A volume of 250 ml of water was transferred into 6 Nalgene square, clear polycarbonate bottles with three bottles for control. In three other bottles,

volumes of an initial 10 mmol l⁻¹ of nitrate solution (NaNO₃) and of an initial 5 mmol l⁻¹ of phosphate solution (NaH₂PO₄) were added to reach a final concentration of 20 μmol l⁻¹ of nitrate and 10 μmol l⁻¹ of phosphate. All bottles were incubated outside the Belle W. Baruch Institute for 2 days in water tables covered with two layers of fiberglass screen to simulate *in situ* temperature and irradiance (50% of ambient sunlight).

Analyses

The initial nutrient concentrations were measured by collecting 50 ml of water from the 10-l carboy. The sample was filtered through a 25-mm dia. Whatman GF/F glass fiber filter and stored in acid-washed Nalgene bottles at -20°C for nutrient analysis.

Ammonium, nitrate + nitrite and phosphate concentrations were determined by using a Lachat Quickchem 8000T autoanalyzer according to wet chemical methods (Johnson and Petty 1983; Grasshoff et al. 1999). The initial temperature, salinity and nutrient concentrations for each bioassay are presented in Table 4.1.

Triplicates of 100 ml of water were collected at time 0 and after the 2-day incubation to measure the photopigment concentrations. Water was filtered through a 25-mm dia. Whatman GF/F glass fiber filter under gentle vacuum. A subset of incubation dates was randomly selected for size fraction analyses. On those dates, triplicates of 100 ml of water were collected and filtered through a 20 μm mesh before filtration to obtain the 0-20 μm size-fraction at time 0 and after a 2-day incubation time. All filters were transferred into 2-ml microcentrifuge tubes and stored at -80°C until photopigment analysis by high performance liquid chromatography (HPLC). For analysis, filters were lyophilized for 18 - 24 hours at -50°C then extracted for 12 - 20 hours at -20°C by adding 750 μl of 90% acetone. A volume of 50 μl of the synthetic carotenoid β-apo-8' carotenal,

used as an internal standard, was also added to the solution. Filtered extracts (250 µl) were injected into a Shimadzu HPLC with a monomeric column (Rainin Microsorb-MV, 0.46 x 10 cm, 3 µm) and a polymeric (Vydac 201TP54, 0.46 x 25 cm, 5 µm) reverse phase C18 column. The mobile phase was composed of two solvents; solvent A, 80% methanol : 20% 0.5 M ammonium acetate and solvent B, 80% methanol : 20% acetone (Pinckney et al., 1996). Pigment peaks were identified by comparing retention times and absorption spectra with pigment standards (DHI, Denmark). Photopigments were used as specific biomarkers for different phytoplankton groups (Higgins et al., 2011) In our study, fucoxanthin (fuco) was considered as the biomarker for diatoms as diatoms dominate the other phytoplankton groups such as haptophytes, dinoflagellates, pelagophytes and chrysophytes in North Inlet. The other pigments of interest prasinoxanthin (prx), alloxanthin (alx), zeaxanthin (zeax), chlorophyll *b* (chl *b*) were considered biomarkers of prasinophytes, cryptophytes, cyanobacteria and chlorophytes respectively (Table 4.2). For each bioassay, the response of photopigments relative to the respective controls was calculated with the following equation:

$$\text{Response relative to control} = \left(\frac{\text{pigment concentration}_{\text{treatment}}}{\text{averaged pigment concentration}_{\text{control}}} \right)$$

A value lower than 1 referred to a decrease in concentration relative to the controls and a value higher than 1 referred to an increase in concentration relative to the controls.

For each size-fraction experiment, the contribution of the fraction with cells larger than 20 µm (> 20 µm) was calculated with the following equation:

$$[\text{pigment}]_{>20 \mu\text{m}} = [\text{pigment}]_{\text{whole water}} - [\text{pigment}]_{<20 \mu\text{m}}$$

With [pigment] the pigment concentration.

All homogeneous and normally distributed data were analyzed using parametric tests (multifactor and multivariate ANOVAs). However, when transformed data were neither normally distributed nor their variances were homogeneous, non-parametric Kruskal-Wallis tests were performed. All ANOVA, Kruskal-Wallis and discriminant analyses were completed using SPSS software 24.0 (IBM Corporation, Armonk, New York).

4.4 RESULTS

For each bioassay, the addition of nitrate and phosphate enhanced the total chlorophyll *a* (chl *a*) concentration with a significant increase in all nutrient bottles except in February and May 2016. However, this increase in chl *a* only exceeded the common regulatory limit of 40 $\mu\text{g l}^{-1}$ in October 2015 (Fig. 4.1). All bioassays combined, the pigments of interest all responded positively (≥ 1) to the addition of nitrate and phosphate with chl *a*, chl *b* and fuco demonstrating the highest response (Table 4.3). The overall pigment composition was quantitatively different between the control and the treatment bottles (MANOVA and discriminant analysis, $p < 0.001$) with the concentrations of the pigments of interest (fuco, alx, zeax, chl *a* and *b*) quantitatively different as well (Kruskal-Wallis, $p < 0.05$) (Tables 4.4 and 4.5). All statistical analyses indicated a shift in the phytoplankton community composition induced by the addition of nutrients but the scattered points observed in the discriminant analysis suggest a variable phytoplankton response (Fig. 4.2).

Each season analyzed separately, all pigments of interest had the lowest response in fall/winter. Fuco, zeax and chl *a* responded more importantly in spring whereas the other pigments (alx, prx and chl *b*) demonstrated the highest response in summer (Table

4.3). In spring (April, May), summer (June, July and August) and fall/winter (September, October, November and February) a quantitative difference between the control and the treatment bottles was observed (MANOVA, $p < 0.001$). All the pigments of interest were quantitatively different in spring and in summer except for prx (Kruskal-Wallis, $p < 0.05$) but in fall/winter statistical analyses indicated a quantitative difference only for zeax and fuco (Kruskal-Wallis, $p < 0.05$) (Table 4.4). Although the discriminant analyses indicated the formation of two distinct groups each season, a variability in the phytoplankton response to the addition of nutrients was suggested by the scattered data points (Fig. 4.3).

Pigment compositions of both size-fractions ($> 20 \mu\text{m}$ and $< 20 \mu\text{m}$) demonstrated a quantitative difference between the control and the treatment bottles (MANOVA, $p < 0.01$) (Table 4.5). All the pigments of interest were quantitatively different for the small size-fraction (Kruskal-Wallis, $p < 0.01$). However, only fuco and chl *a* demonstrated a significant difference for the large size-fraction (Kruskal-Wallis, $p < 0.01$) (Table 4.5). The discriminant analysis also demonstrated a quantitatively different response in photopigments between the control and the nutrient bottles for each size-fraction ($p < 0.01$) (Fig. 4.4; Table 4.6).

4.5 DISCUSSION

In our study, programs like CHEMTAX were not applied because of the few number of algal groups present in the samples (e.g. mostly diatoms) in addition to the uncertainty associated with partitioning into algal groups (Higgins et al. 2011). Only unique diagnostic pigments were used for the identification of the different phytoplankton groups and chl *a* for the phytoplankton biomass.

The increase in chl *a* concentration induced by the addition of nutrients rarely exceeded the common regulatory limit of 40 µg l⁻¹ (Fig. 4.1). However, based on the changes in the pigment concentrations, MANOVA and discriminant analyses demonstrated an alteration in the phytoplankton community composition (Tables 4.4 and 4.5; Fig. 4.2). Fuco and zeax, biomarkers of diatoms and cyanobacteria respectively, were the two photopigments which concentrations increased quantitatively each season (Table 4.3). In Reed et al. (2016) study, the abundance of cyanobacteria and diatoms also significantly increased with the addition of nitrogen (i.e. urea). Cyanobacteria can uptake a variety of nitrogen sources whether organic or inorganic including nitrate (Flores and Herrero 2005). The increase in diatoms could be explained by their ability to store nutrients, particularly nitrate, thus they often dominate nitrate-rich waters (Clark et al. 2002; Kamp et al. 2015).

However, in our study, discriminant analyses demonstrated an unpredictable phytoplankton response to the addition of nutrients and no clear shift towards a particular phytoplankton group (Fig. 4.2 and 4.3). Initial community composition as well as initial environmental factors may also play an important role in the phytoplankton response. For example, nitrate assimilation by phytoplankton is a temperature and light dependent process occurring at a light intense enough to satisfy their energetic requirements (Domingues et al. 2011; Kamp et al. 2015).

The variation in temperature is also an important factor regulating phytoplankton size (Paerl and Huisman 2008; Daufresne et al. 2009). While several studies in different estuaries have established an increase in the abundance of larger cells with the addition of nutrients (reviewed in Marañón 2015), others observed a high abundance of

cyanobacteria and phytoflagellates in coastal regions overloaded with nutrients (Bodeanu and Ruta 1998; Clarke et al. 2008). In our study, although both size-fractions responded significantly to the addition of nutrients, the smaller size-fraction exhibited a higher response (Fig. 4.4; Table 4.6). This could be explained by Cloern (2017) suggested that a 15°C threshold should be adjusted; in coastal regions with temperatures higher than 15°C, eutrophication would alter the large diatom communities towards a smaller-sized community. A shift towards cyanobacteria, not effectively grazed, could result in a limitation of carbon cycling and a decrease in the dissolved oxygen in the water column (Paerl et al. 2003). However, our results from the size-fractions showed no shift towards one particular group, as evidenced in the whole water.

Although the phytoplankton response was variable, major alterations in the phytoplankton community structure were observed at relatively low levels of nutrient loading and with chl *a* concentrations well below the 40- $\mu\text{g l}^{-1}$ threshold. If the primary goal of water quality criteria is to maintain ecosystem integrity and function, using an arbitrary chl *a* concentration as a metric may not be an ecologically sound approach. As observed, changes in the community composition occur below the selected threshold. Furthermore, at a similar chl *a* concentration, a bloom of centric diatoms would probably be more desirable than a bloom of toxic cyanobacteria. But the measurement of chl *a* alone doesn't allow the differentiation between both. Like Feirrer et al. (2011) and Glibert (2017), we suggest that criteria based on phytoplankton abundance should include consideration of community composition.

One potential solution would be to use the ratios of the abundances of the different algal groups in combination with total chl *a* concentrations. For example, the

ratio of each diagnostic pigment relative to total chl *a* could be determined for “normal nutrient” concentrations and used for comparison with ratios at nutrient replete conditions. These ratios could be derived experimentally (using dilution bioassays) or empirically, using historical values from comparable undisturbed natural systems. However, pigment analyses by HPLC are costly and time-consuming. Measurements of chl *a* by fluorescence is the most practical and affordable way to obtain the total phytoplankton biomass. Therefore, a good compromise would be the analyze of photopigments with a spectrophotometer. Although they cannot separate carotenoids accurately (e.g. fuco, zeax), new methods are being developed to improve spectrophotometer resolutions to have an inexpensive alternative to HPLC accessible by governmental agencies (Thrane et al. 2015).

Table 4.1 Averaged initial conditions at the collection site

	Salinity	Temperature (°C)	Nitrate ($\mu\text{mol l}^{-1}$)	Ammonium ($\mu\text{mol l}^{-1}$)	Phosphate ($\mu\text{mol l}^{-1}$)
May-14	35	23	-	-	-
Jun-14	35	26	-	-	-
Nov-14	35	13	-	-	-
May-15	33	25	-	-	-
Jun-15	34	31	-	-	-
Jul-15	33	31	2.80	2.21	3.80
Sep-15	35	28	-	-	-
Oct-15	35	25	-	-	-
Feb-16	24	13	13.70	3.07	0.55
Apr-16	36	24	-	-	-
May-16	37	22	2.05	3.64	3.94
Jun-16	35	27	2.10	4.25	3.45
Jul-16	34	28	2.02	4.20	3.55
Aug-16	35	32	2.09	4.64	3.62
Sep-16	33	29	2.14	4.61	3.68

Table 4.2 List of photopigments and their correspondence to the different phytoplankton groups present in North Inlet (Higgins et al. 2011)

Pigment	Abbreviation	Phytoplankton group correspondence
fucoxanthin	<u>fuco</u>	<u>diatoms</u> , haptophytes (all types), <u>dinoflagellates</u> (types 2 and 3), pelagophytes, chrysophytes
prasinoxanthin	<u>prx</u>	prasinophytes (type 3)
alloxanthin	<u>alx</u>	cryptophytes
zeaxanthin	<u>zeax</u>	cyanobacteria
chlorophyll <i>b</i>	chl <i>b</i>	chlorophytes, prasinophytes
chlorophyll <i>a</i>	chl <i>a</i>	all groups except cyanobacteria (type 4)

Table 4.3 Responses to the relative controls of the pigments of interest for each season with N the number of samples and se the standard error (in grey). A value lower than 1 referred to a decrease in concentration relative to the controls and a value higher than 1 referred to an increase in concentration relative to the controls

	N	Fuco		Prx		Alx		Zeax		Chl <i>b</i>		Chl <i>a</i>	
		mean	se	mean	se	mean	se	mean	se	mean	se	mean	se
All seasons	54	6.5	0.8	4.6	1.0	4.2	1.3	3.1	0.5	6.1	0.9	5.7	0.6
Spring	20	8.9	1.0	5.2	0.7	3.6	0.5	4.8	1.2	7.7	2.0	7.4	0.8
Summer	15	7.7	2.1	9.1	4.1	8.6	5.0	2.4	0.2	8.0	1.5	7.2	1.7
Fall/Winter	19	3.1	0.5	1.0	0.1	1.5	0.2	1.9	0.3	3.0	0.4	2.7	0.4

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Table 4.4 Results of single-factor MANOVA tests for the effect of nitrate and phosphate additions on photopigments as well as the results of Kruskal-Wallis (KS) tests for the effect of nitrate and phosphate additions on each photopigment. Pillai's trace is the statistic for the test, F is the F-value, and p is the significance test

Season	Pillai's trace	F	p	Pigments with significant difference relative to controls (KS, p < 0.05)
All seasons	0.603	9.32	< 0.001	<u>fuco</u> , <u>alx</u> , <u>zeax</u> , chl b, chl a
Spring	0.911	18.66	< 0.001	<u>fuco</u> , <u>alx</u> , <u>prx</u> , <u>zeax</u> , chl b, chl a
Summer	0.973	36.61	< 0.001	<u>fuco</u> , <u>alx</u> , <u>zeax</u> , chl b, chl a
Fall/Winter	0.776	5.09	< 0.001	<u>fuco</u> , <u>zeax</u>

Table 4.5 Results of the discriminant analyses of photopigments with Wilks' λ the statistic, p the p-value for hypothesis testing, % variance explained the variance explained by the first two functions, and % classified correctly the number of samples assigned correctly to each group (control and NP treatment)

Season	Wilks' λ	p	% variance explained	% classified correctly
All seasons	0.361	< 0.001	88.3	74.1
Spring	0.732	< 0.001	96.5	88.2
Summer	0.269	< 0.001	85.7	97.9
Fall/Winter	0.561	< 0.001	80.3	85.5

Table 4.6 Results of single-factor MANOVA tests for the effect of nitrate and phosphate additions on photopigments for each size fraction as well as the results of Kruskal-Wallis (KS) tests for the effect of nitrate and phosphate additions on each photopigment. Pillai's trace is the statistic for the test, F is the F-value, and p is the significance test

Fraction	Pillai's trace	F	p	Pigments with significant difference (KS, $p < 0.05$)
> 20 μm	0.536	2.75	< 0.01	<u>fuco</u> , chl <i>a</i>
< 20 μm	0.811	10.4	< 0.01	<u>fuco</u> , <u>prx</u> , <u>alx</u> , <u>zeax</u> , chl <i>b</i> , chl <i>a</i>

Table 4.7 Results of the discriminant analyses of photopigments for each size fraction and each treatment with Wilks' λ the statistic, p the p-value for hypothesis testing, % variance explained the variance explained by the first two functions, and % classified correctly the number of samples assigned correctly to each group (control and NP treatment)

Size-fraction	Wilks' λ	p	% variance explained	% classified correctly
Combined	0.060	< 0.001	77.0	82.9
> 20 μm	0.655	0.004	100	79.3
< 20 μm	0.231	< 0.001	100	94.6

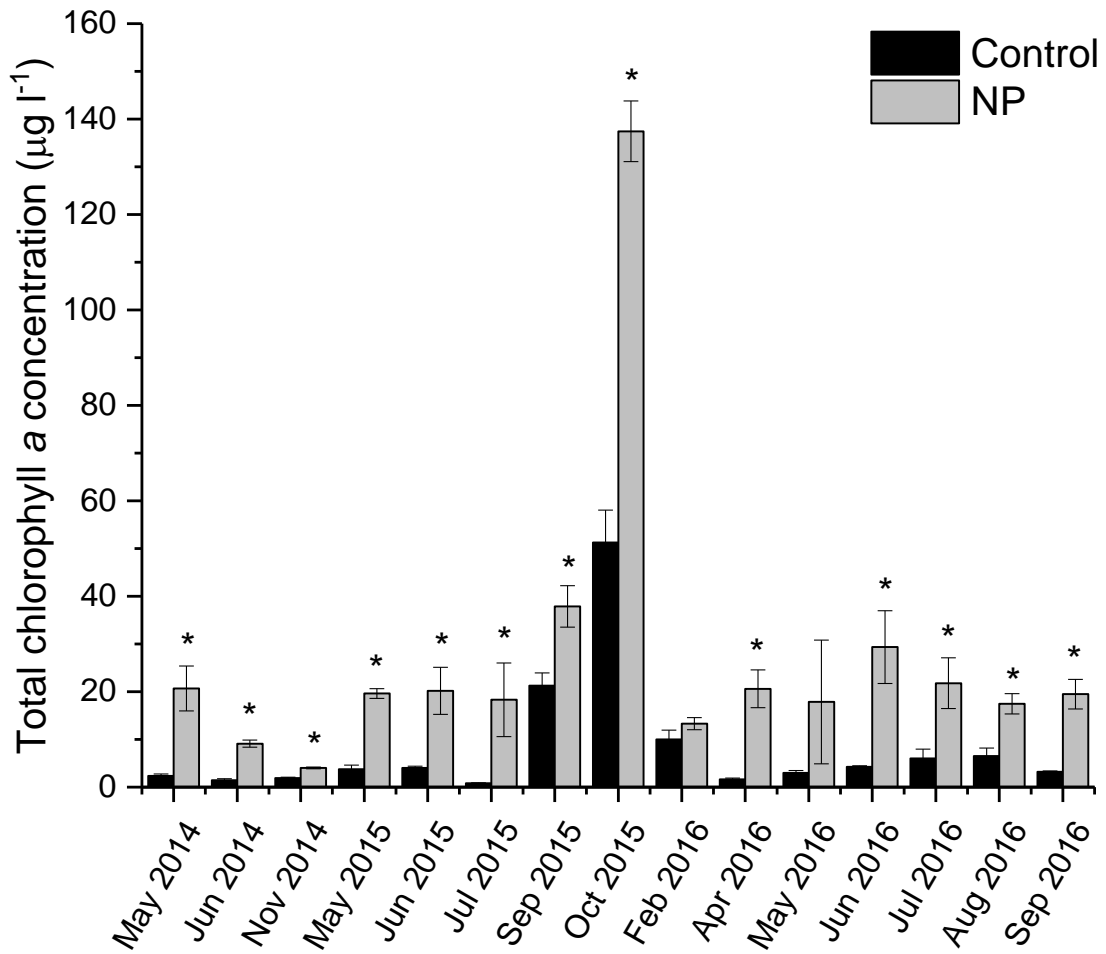


Fig 4.1 Total chl *a* concentrations in the controls (black) and in the treatment bottles with nitrate and phosphate added (NP) (grey) for each bioassay (mean \pm sd). The star represents significant differences between the control and the treatment bottles ($p < 0.05$)

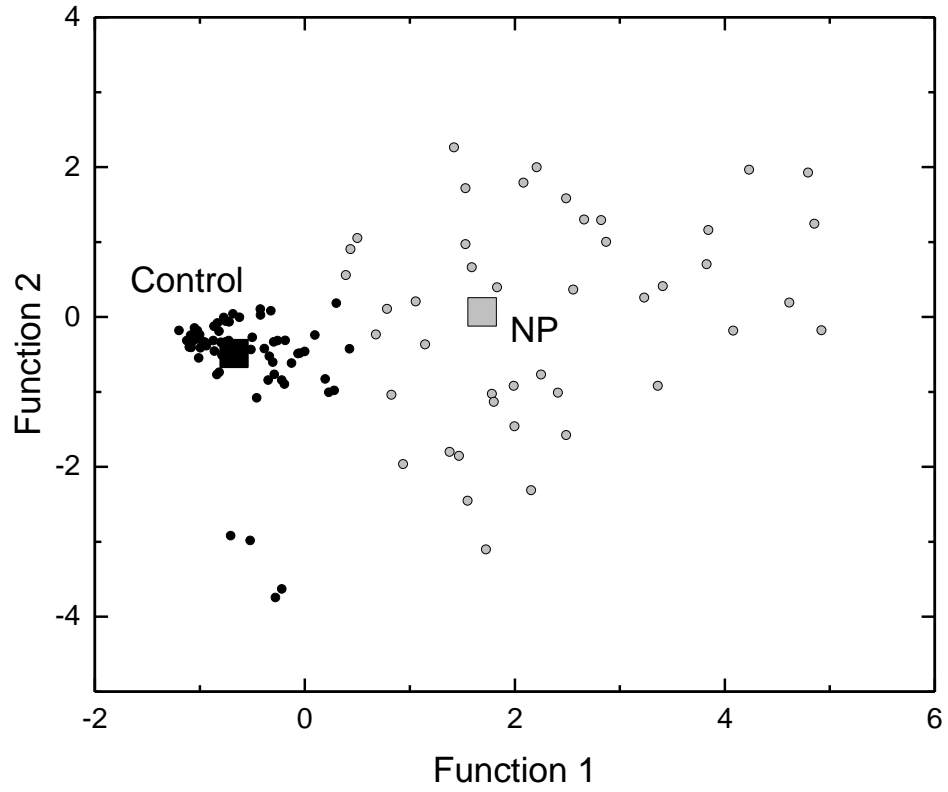


Fig 4.2 Results of the discriminant analysis of all seasons combined with group centroids indicated by the large squares and with data points for the controls in black and the data points for the addition of nitrate and phosphate (NP) in grey

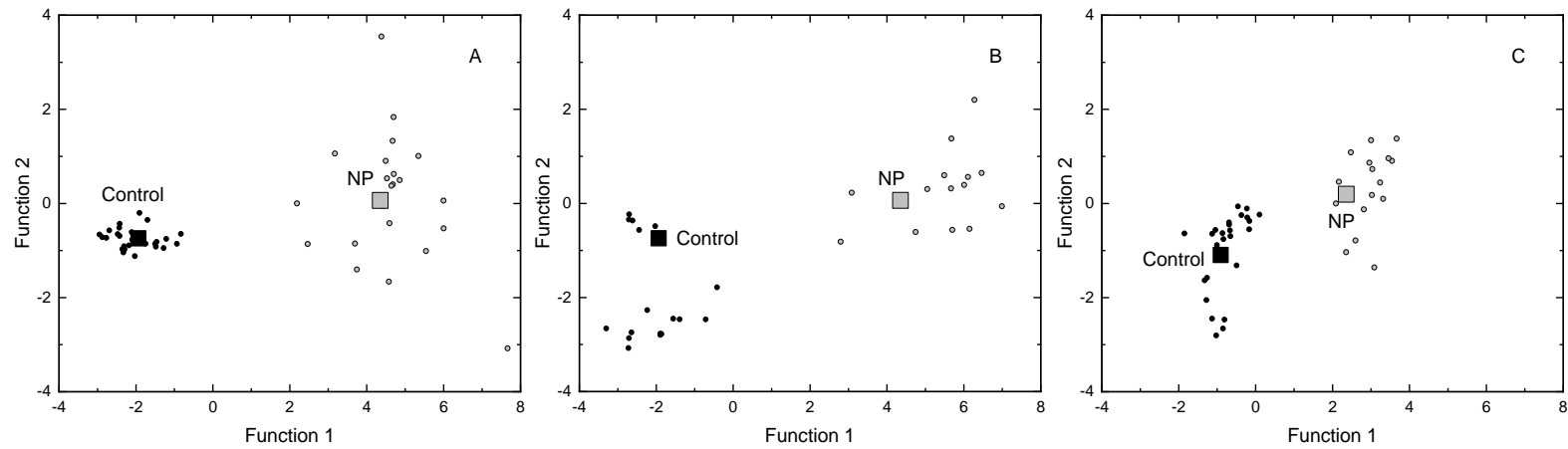


Fig 4.3 Results of the discriminant analysis of spring (A), summer (B) and fall/winter (C) with group centroids indicated by large squares and with data points for the controls in black and the data points for the addition of nitrate and phosphate (NP) in grey

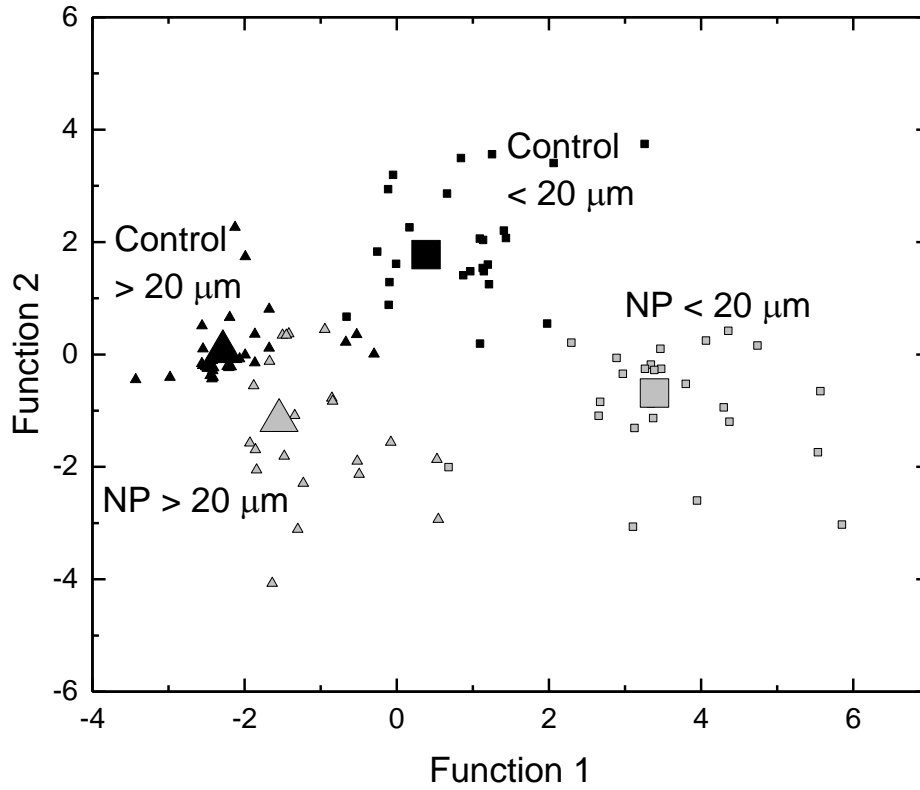


Fig 4.4 Results of the discriminant analysis of the controls with group centroids indicated by the large squares for the > 20 μm fraction with group centroids indicated by the large triangles for the < 20 μm . Data points for the control are in black and the data points for the NP treatment are in grey

CHAPTER 5

CONCLUSION

The spread of HAB species like *Pseudo-nitzschia* spp. depends on multiple environmental factors such as salinity and nutrients (Anderson, 2009). In our study, salinity played an important role on the abundance of *Pseudo-nitzschia* spp. and its toxicity with a correlation with salinity at both sites. We also observed an increase in dDA allelopathy on phytoplankton and particularly on cryptophytes and diatoms at higher salinities in North Inlet (i.e. the high salinity site) and lower salinities in Winyah Bay (i.e. the low salinity site). With these two phytoplankton groups being more sensitive to high salinity in North Inlet and to low salinity in Winyah Bay, we suggested that dDA provided *Pseudo-nitzschia* with a way to outcompete the other phytoplankton at salinities where the target cells were more salinity-stressed.

These results are essential, as salinity alterations have already been observed with higher freshening in some regions (e.g. subpolar waters) and an elevation in salinity in others (e.g. subtropical waters), both predicted to intensify worldwide (IPCC, 2007). An increase in salinity could lead to a spread of toxic *Pseudo-nitzschia* species, which release more dDA at higher salinity and could possibly use it to overcome the other phytoplankton groups (Anderson, 2009; MacIntyre et al., 2011; Sahraoui et al., 2012; Bargu et al., 2016; Tas et al., 2016). In case of a decrease in salinity, *Pseudo-nitzschia* will still be present at lower salinities. Their survival could depend on DA production and allelopathy.

Another parameter also influencing the spread of *Pseudo-nitzschia* and HAB species in general is nutrients. Our results demonstrated not only an increase in the *Pseudo-nitzschia* abundance with the addition of nitrate and phosphate but also an increase in the abundance of all phytoplankton groups. We observed a change in the phytoplankton community composition even though the total concentration of chl *a* was lower than the regulatory limit of 40 µg l⁻¹. No shift towards one particular group was observed but size-fractionation analyses indicated a higher response from small cells compared to large cells. We suggested that the variation in temperatures also played an important role in regulating phytoplankton size in eutrophic systems as small cells prefer higher temperature (Paerl and Huisman 2008; Daufresne et al., 2009; Cloern, 2017).

However, governmental agencies only measure chl *a* as an indicator of eutrophication, which poses the problem of an impossible differentiation between two phytoplankton size-fractions and between blooms of HAB species and non-toxic groups like cryptophytes. With the relatively expensive cost of HPLC analyses, an alternative would be to measure the different specific pigments with a spectrophotometer. As of right now, spectrophotometers cannot separate carotenoids accurately but technology is advancing to make it possible in the near future (Thrane et al., 2015). However, microscopy samples will still be necessary to differentiate blooms of non-toxic (e.g. *Cylindrotheca*) and of toxic diatom species (e.g. *Pseudo-nitzschia*). Knowing that eutrophic conditions can increase drastically HAB biomass, the differentiation between both is crucial (Anderson et al., 2002; Bužančić et al., 2016; Heisler et al., 2008; Lim et al., 2017).

Fortunately, multiple studies, whether field observations or culture experiments, demonstrated a higher allelopathic activity in nutrient depleted environments (reviewed in Granéli et al., 2008). This was confirmed in our study, in which the addition of nutrients increased the abundance of *Pseudo-nitzschia* but neutralized the influence of salinity on dDA allelopathy. We suggested that the increase in species richness induced by the addition of nutrients may have helped reducing the effect of dDA on phytoplankton when cells were salinity-stressed (Larson and Belovsky, 2013; Sjöqvist and Kremp, 2016).

Unfortunately, as far as human health and economy are concerned, eutrophic conditions exacerbate the toxic effects of harmful species with dramatic consequences on drinking water, fisheries and tourism (Pinckney et al. 2001; Smith and Schlinder 2009; Chislock et al. 2013). Monitoring changes in salinity and nutrients is essential to understand their interactions with the biota and to better predict the outcome of these alterations on the phytoplankton community structure, particularly on HABs.

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